1

Engineering Antibodies for Therapy

A. MOUNTAIN AND J.R. ADAIR

Celltech Research, Celltech Ltd, Slough, UK

Introduction

Antibodies have long been viewed as potential agents for targeted drug delivery and other therapeutic interventions, largely with a view to exploiting the combination of high specificity and affinity of the antibody–antigen interaction. Since the development of rodent monoclonal antibody (MAb) technology (Kohler and Milstein, 1975) it has been possible in principle to produce rodent MAbs to virtually any antigen, and a large number of rodent MAbs relevant to human therapy have been generated. MAbs have already been used clinically for the diagnosis and therapy of several human disorders, notably cancer and infectious diseases, and for the modulation of immune responses. The target antigens have been tumour-associated antigens (TAAs, Boyer *et al.*, 1988; Herlyn, Menrad and Koprowski, 1990), specific cell type markers, viruses, bacteria and specific human proteins of physiological

importance (particularly cytokines or their receptors). At the end of 1991 there were 132 biotechnology-based medicines in formal clinical development (Pharmaceutical Manufacturers Association Report, 1992) or awaiting final approval from the US Food and Drugs Administration (FDA). Sixty-six of the 132 were for cancer therapy and 58 of the 132 were MAbs. Three rodent MAbs have so far been approved and launched as products: OKT3 is a naked MAb and has been approved by the FDA for treatment of acute kidney transplant rejection; OncoScint and MyoScint are Mab–isotope conjugates which have been approved outside the US as imaging agents for colorectal cancer and myocardial infarction, respectively. The human MAb 'Centoxin' has also been approved in Europe for treatment of septic shock. The total market for MAbs is presently around $330 million per annum and is estimated to grow approximately ten-fold by 1996. Therapeutic MAbs will account for most of this market.

Although the specificity of MAbs undoubtedly gives them immense potential in medicine, rodent MAbs are certainly not ideal therapeutic agents. The five most important issues and technical challenges in the development of MAb-based therapies are: (1) identifying MAbs of suitable affinity and specificity; (2) overcoming human immune responses against rodent MAbs and against any cell-killing agents attached to them; (3) identifying and harnessing appropriate cell-killing agents; (4) achieving appropriate pharmacokinetics and biodistribution; (5) achieving economic manufacture, which is of particular relevance for highly engineered MAbs and for MAb–cytotoxic agent conjugates (as opposed to naked MAbs).

As the above statistics indicate, a large proportion of the MAb-based agents presently in clinical development are for treatment of cancer and in this review the development of anti-cancer MAbs and MAb conjugates will largely be used to illustrate the approaches being taken to address the five key issues. The review begins with a brief description of the structure of antibodies and antibody genes, followed by a summary of the arguments and evidence relating to the importance of affinity and specificity for MAb-based therapies. We then briefly summarize the available clinical results with naked rodent MAbs. Next we describe the approaches being taken to overcome the immunogenicity in patients of rodent MAbs, which is certainly the most serious and general problem for MAb-based therapies. This section concentrates largely on antibody humanization, which is the most promising solution to the problem. The processes developed for efficient cloning of antibody genes and for production of engineered whole antibodies are then described. This is followed by a summary of the approaches being taken to improve the pharmacokinetics and biodistribution of MAbs, focusing particularly on the development of engineered antibody fragments, and then by a summary of production systems being used for such fragments. The first half of the review is then completed by a summary of the various cell-killing strategies being developed for MAb-based therapies. The second half of the review is largely devoted to a detailed summary of the construction, expression, pre-clinical studies and data on efficacy and immunogenicity for engineered MAbs and MAb conjugates that have been used in clinical studies by the time of writing.

The review finishes with some conclusions and a summary of recent advances in antibody technology which may lead eventually to the successors of humanized rodent MAbs for therapy.

The structure of antibodies and antibody genes

In order to understand the later sections of this review (especially those on antibody gene cloning and humanization) it is necessary to have some knowledge of the structure and organization of antibodies and their genes. We give a brief description here.

Higher mammals have five classes of immunoglobulin, termed IgG, IgM, IgA, IgE and IgD. The structures and functions of these five classes have been very well described by Roitt, Brostoff and Male (1987). Almost all MAbs of therapeutic potential are of the IgG class, and have the basic structure shown in *Figure 1A*. IgG antibodies have a tetrameric structure consisting of two identical 55 kDa glycosylated proteins (termed 'heavy chains') and two identical 25 kDa proteins, which are normally not glycosylated (termed 'light chains'), covalently linked by disulphide bridges. The proteins are organized into discrete folding domains of around 110 amino acids which are encoded in the genome on separate exons (*Figures 1B and 1C*). Each light chain associates with and is covalently linked via a disulphide bridge to a cysteine in the N-terminal region of one heavy chain, and the C-terminal half of the heavy chains associate with each other to form a Y- or T-form structure. The heavy chains are also covalently linked to each other via disulphide bridges in the hinge domain.

Sequence information is now available for hundreds of antibodies of many different species and reveals that the N-terminal domains of each chain are much more variable in sequence than the other domains. The N-terminal domains are therefore termed 'variable domains' and the others 'constant domains'. Three non-contiguous regions within these variable domains are particularly variable and are usually referred to as 'hypervariable loops' or 'complementarity determining regions' (CDRs). This sequence variation is postulated to provide the variability (within these otherwise highly conserved proteins) which enables antibodies to recognize and bind to a very wide range of antigens (Wu and Kabat, 1970; Kabat *et al.*, 1987). The proposal has been confirmed by structural studies, which show that the hypervariable sequences are (in most cases) associated on the surface of the antibody as a set of loops. The loops form a large surface patch and are in contact with antigen in cases for which structural information on the antibody–antigen complex is available (Amit *et al.*, 1986; Boulot *et al.*, 1987; Colman *et al.*, 1987; Sheriff *et al.*, 1987; Davies *et al.*, 1989; Padlan *et al.*, 1989; Tulip *et al.*, 1989, reviewed in Alzari *et al.*, 1987; Bentley *et al.*, 1990; Bhat *et al.*, 1990; Davies, Padlan and Sheriff, 1990 and Poljak, 1991). The variable region residues that are not part of the CDR or loops together constitute the 'framework' of the variable region. It has been shown that the exons for the variable domains are assembled from a number of repeated gene families — V and J for the light chain and V, D and J for the heavy chain — by a series of recombination events during the
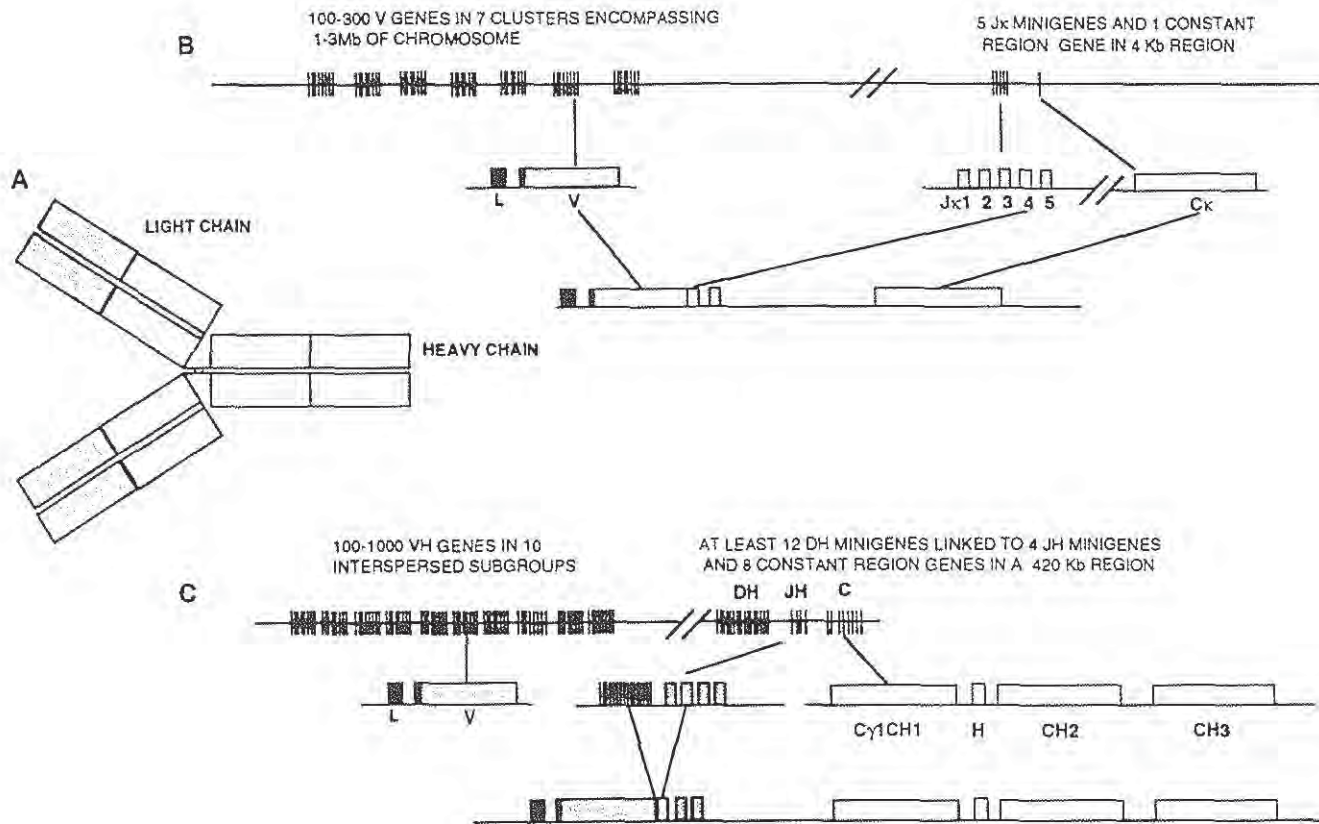
Figure 1. Protein and gene organization of murine IgGκ.

development of the antibody-producing B-cell lineage. The variable region exon along with the signal sequence exon and the promoter/enhancer (involved in transcription) is then juxtaposed with the constant region gene family by further recombination events for subsequent expression (*Figures 1A, 1B*; reviewed by Alt, Blackwell and Yancopolous, 1987). The organization of the Ig loci in mice and humans has been reviewed recently by Lai, Wilson and Hood (1989).

The constant regions tend to be conserved in sequence among antibodies of a given species, and also to a lesser extent between species. Light chains have a single constant domain for which there are two gene loci, Cκ and Cλ. IgGs have three constant domains on the heavy chains, CH1, CH2 and CH3. Between the CH1 and CH2 domains (for IgGs) is a short proline-rich peptide sequence termed the 'hinge' which contains the cysteines that bridge the two heavy chains. IgGs also have a site in the CH2 domain for *N*-linked glycosylation, which is required for structural integrity of the antibody and for some of its effector functions. Sequence motifs within the CH2 and CH3 domains are responsible for the effector functions, such as complement activation and binding to other cells of the immune system. In humans and rodents there are four different types of IgGs, termed 'isotypes', which vary in their spectrum of effector functions as a result of amino acid sequence variation in the constant regions (Burton, 1990). In humans there are a number of immunologically distinct variants of IgG1, 2 and 3, termed allotypes (Gorman and Clark, 1990). These allotypes are racially distributed, for example the G1m(3) marker predominates in Caucasian IgG1 whereas G1m(1,17) predominates in Asian and Japanese individuals.

Until the advent of recombinant DNA technology antibody fragments (*Figure 2*) were generated by proteolytic digestion. Pepsin cleaves IgGs on the C-terminal side of the hinge, liberating an antigen binding fragment referred to as the F(ab')$_2$. Papain cleaves on the N-terminal side of the hinge and liberates two F(ab) fragments and a single Fc fragment. The F(ab) fragments have a single antigen binding site (monovalent), while the F(ab')$_2$ has two (bivalent). The term F(ab') means monovalent but with the hinge sequence also present. The heavy chain of the F(ab) or F(ab') is usually referred to as the Fd or Fd'. The variable domains of the heavy and light chains (V_H and V_L) together comprise a fragment called the Fv. This is the smallest fragment which retains the full antigen binding activity of the monovalent antibody. Although the Fv can be obtained for some antibodies by proteolytic digestion of the IgG the process is very inefficient. Fvs dissociate into V_H and V_L under physiological conditions, and so are not useful for therapy. The single chain Fv (scFv) represents the most successful strategy for stabilizing the Fv. It has V_H and V_L linked by a short peptide linker (between the C-terminus of one domain and the N-terminus of the other) and expressed as a single polypeptide chain. It is possible to make scFv variants for most MAbs which retain most or all of the monovalent antigen binding activity of the MAb. In some cases V_H alone displays significant antigen binding activity, an observation which has led to use of the term 'single domain antibodies' (DAbs; Ward *et al.*, 1989). Molecular biology

procedures now allow the efficient production of the F(ab), F(ab′), F(ab′)$_2$ and Fv, as well as novel engineered variants of these fragments and of course whole IgG.

MAb affinity and specificity

Laboratories developing MAbs for therapy usually choose the highest affinity MAb available because it is widely assumed that high affinity confers a therapeutic advantage. There is, however, very little actual experimental data relevant to the importance of affinity. This is largely because all comparative studies in patients or animal models have concerned MAbs which recognize different epitopes as well as having different affinities. For example comparative studies have been performed in an animal model with rodent MAbs which neutralize human TNF-α as part of a programme to develop a MAb for treatment of septic shock, in which TNF-α is an important mediator. Two mouse MAbs were compared for ability to prevent pyrexia induced in rabbits by human TNF-α (R. Foulkes, personal communication). The 100-fold difference of the MAbs in ability to bind TNF-α *in vitro* was shown to give a seven-fold difference in the doses required for complete neutralization. A similar correlation between affinity and effective dose has also been observed for MAbs neutralizing IL-5 (M. Bodmer, personal communication). These demonstrations of the importance of affinity are not quite conclusive, however, since the antibodies were directed to different epitopes.

Most other relevant data concern MAbs recognizing TAAs, for which the affinity issue is complicated by several factors, including the tendency of such antigens to be shed from the tumour into the circulation, and tumour penetration. Circulating antigen may in some cases interfere with MAb localization to the antigen on the tumour, and MAbs which recognize different epitopes of the antigen on the tumour cells may also be differentially affected by the presence of circulating antigen. It has also been suggested that higher affinity antibodies may show poorer tumour penetration through an increased tendency to bind tightly to the antigen on the tumour cells close to the blood vessel through which the MAb gains access to the tumour. Many studies have been performed on the tumour localization in colorectal cancer patients of MAbs recognizing the TAA's carcinoembryonic antigen (CEA) and polymorphic epithelial mucin (PEM). For both antigens the affinities of the MAbs concerned have been measured and reported to be different. Although marked differences between the MAbs in tumour localization are also reported the data do not allow the effect of affinity differences on localization to be distinguished from differences in circulating half-life (conferred by differences in isotype and immune response), tumour site and size, tumour vascularity and permeability. Perhaps the most direct evidence (Schlom *et al.*, 1992) on the importance of MAb affinity for tumour therapy concerns two MAbs recognizing the TAA TAG72, which is expressed on several human tumour types. MAb B72.3, which binds this antigen, has been administered to over 1000 patients and shown to localize to about 75% of gastrointestinal, ovarian, prostate and breast tumours. B72.3 has gained
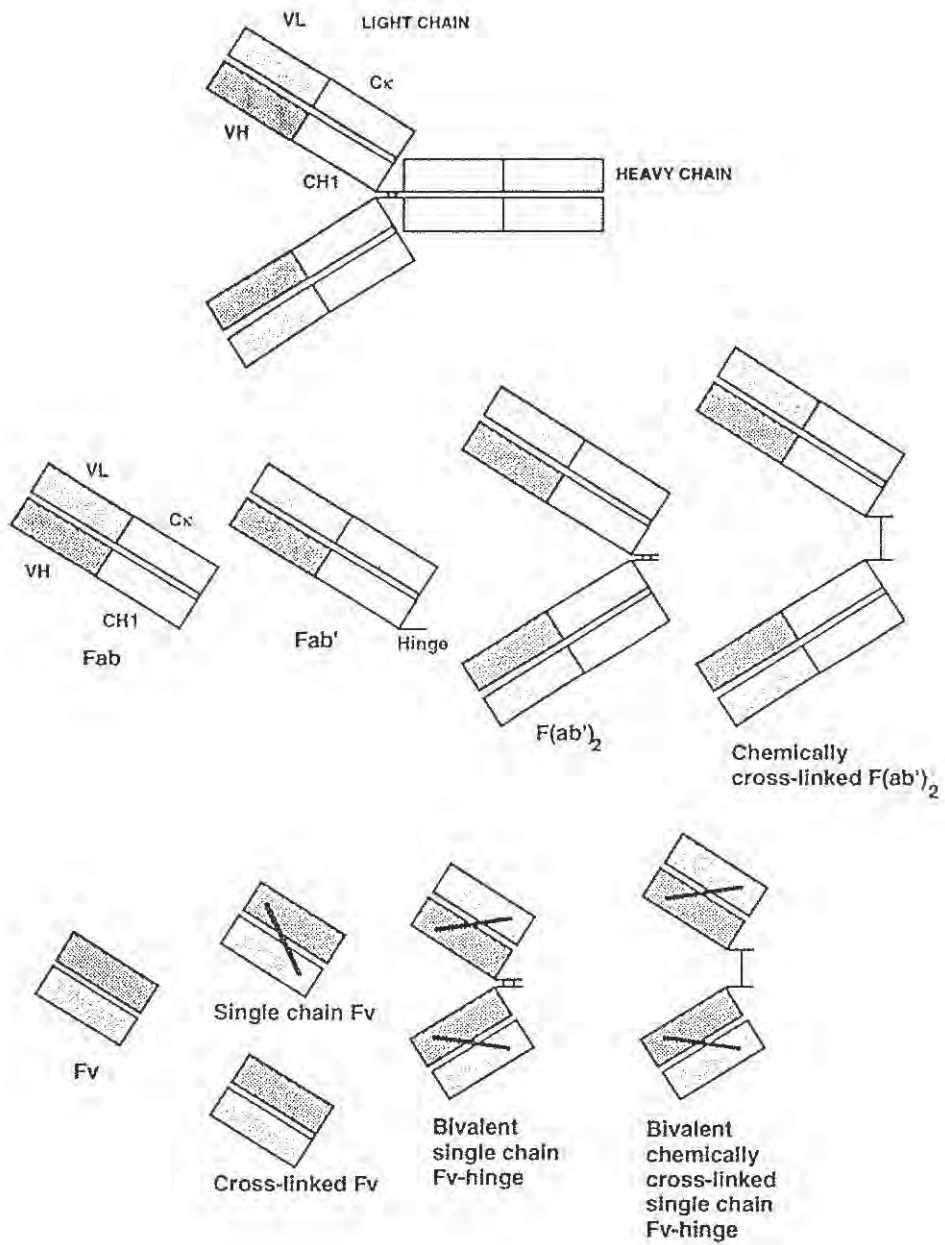
Figure 2. Engineered antibody fragments.

regulatory approval as part of a colorectal cancer imaging agent called 'OncoScint'. Recently a MAb CC49 has been characterized which binds to an overlapping epitope on TAG72 and which has approximately seven-fold greater affinity for this antigen than B72.3 (dissociation constants are 16.2×10^{-9} and 2.5×10^{-9} M, respectively). The two MAbs have the same range of reactivities to normal adult tissues. The higher affinity MAb was clearly shown to have a greater anti-tumour effect (two- to three-fold at the same dose) than B72.3 when both MAbs were conjugated to the therapeutic radioisotope ^{131}I and used in tumour regression experiments in nude mice. Other studies in animal models have concluded that higher affinity MAbs give greater tumour uptake at low doses which do not saturate the antigen, but not at higher doses (Sung et al., 1992).

Other relevant data concern comparisons of MAbs and MAbs conjugated to cytotoxic agents for ability to bind to, and kill, cancer cells in tissue culture. Cell killing by MAbs carrying low molecular weight cytotoxic drugs or protein toxins requires internalization of the conjugates and intracellular release of the active cytotoxic agent. A humanized variant of the MAb CTM01, which binds to PEM, has been identified which has an affinity two- to three-fold greater than its parent murine MAb but retains the same specificity (J.R. Adair et al., unpublished). Calicheamicin conjugates of the humanized and murine forms have been compared for binding to tumour cell lines expressing PEM, for internalization and cell killing in vitro and for tumour regression in animal models. The higher affinity humanized form performs better for all three parameters (L. Hinman, personal communication).

In summary, there are not yet sufficient data to demonstrate conclusively a general correlation between affinity and efficacy for therapeutic MAbs or their conjugates in animal models or in patients. Indeed, ethical considerations make it very difficult to carry out such comparisons in the clinic. Such demonstrations will require thorough head-to-head efficacy comparisons in animal models of MAbs with different affinities but which recognize the same epitope and have equivalent pharmacokinetics and biodistribution. Recently murine B72.3 and a humanized variant with a 100-fold lower affinity have been compared for ability to localize to tumours in mice (D. King, personal communication). The murine antibody showed somewhat greater localization. Although definitive studies on the importance of affinity remain to be done, it is very likely that for most therapeutic applications MAbs with minimum dissociation constants of 10^{-9} to 10^{-10} M will be essential to achieve efficacy and to permit economically realistic doses. It is therefore extremely important to retain at least most of the MAbs affinity for its antigen through the antibody engineering and conjugation procedures required to render it suitable for therapeutic use.

One of the important advantages of MAbs and MAb conjugates over conventional low molecular weight drugs is their specificity for the target molecule. This is very likely to be reflected in a lower failure rate of therapeutic MAbs in development at the stage of toxicology. Specificity is of particular importance for anti-cancer MAbs because unfortunately no antigens have yet been identified which are expressed exclusively on tumour cells.

This is why the term 'tumour-specific antigen' has largely been replaced by the more accurate 'tumour-associated antigen'. Expression of the antigen on normal tissues is a major potential problem for tumour therapy with MAbs and MAb conjugates, since it can lead to dose-limiting toxicities. It is therefore important when selecting such MAbs for development to include a step in the screening cascade which evaluates binding of the MAb to normal tissues. Immuno-histochemical studies are usually performed which indicate the ability of the MAb to bind to tumours and to a range of normal tissues. MAbs can then be chosen which bind antigens that are highly expressed on tumours and also on a large proportion of cells within any one tumour. The most suitable of these MAbs will be those for which the antigen shows little expression on normal tissues — especially those likely to be involved in dose-limiting toxicities — or shows expression on a much smaller proportion of the cells. In some favourable cases the antigen may be expressed on important normal tissues but may be in a cellular location that is much less accessible to a therapeutic MAb in the circulation than is the antigen on the surface of tumour cells. The most frequent normal tissue expressing the TAA is of course the tissue of origin of the tumour. In many cases the TAA is much more highly expressed on tumour cells than on these normal cells, and this may be reflected in the levels of MAb uptake by tumour and normal tissues at relatively low doses. There are many TAAs for which it is suggested that the tumour form of the antigen is structurally different from that found on normal cells. Considerable effort is presently going into identifying MAbs recognizing specifically the aberrantly glycosylated forms of glycosphingolipid and glycoprotein TAAs suggested to be present on tumour cells (Hakomori, 1991a, b).

A related potential problem of specificity is caused by the tendency of many TAAs to be shed from the tumour into the circulation. In some cases this circulating antigen may interfere with localization of the MAb to the tumour, especially at low doses. MAb–antigen complex formation in the circulation may cause toxicity when the MAb carries a cytotoxic agent through deposition of the agent in the organs of clearance of the complex. In one targeting study with colorectal cancer patients a particular anti-CEA MAb was shown to localize efficiently to 42 out of 43 tumours examined (Boxer *et al.*, 1992). In the remaining case the patient had a high level of circulating CEA. On the other hand the presence of circulating PEM was shown to increase levels of immune complexes in the circulation and to enhance tumour localization in colorectal cancer patients administered the anti-PEM antibody ICR2 (Davidson *et al.*, 1991). It is thus far from clear yet whether circulating antigen will be a general problem for cancer therapy with MAbs or MAb conjugates. In cases where circulating antigen is shown to interfere with MAb targeting, the specificity of MAbs is such that it may well be possible to identify and use MAbs capable of binding preferentially to the tumour-bound form of the antigen. The MAb CTM01, for example, appears to bind preferentially to tumour-bound PEM rather than to PEM shed into the circulation (T. Baker, personal communication).

Clinical data with naked rodent MAbs

The clinical use of naked rodent MAbs has so far largely focused on the treatment of cancer, and on suppression of immune responses involved in auto-immune disease, graft versus host disease (GVHD) and transplant rejection. Naked rodent MAbs have in general proven very ineffective in cancer therapy, with only 23 partial remissions and three complete remissions reported among the initial 185 patients included in 25 clinical trials (Catane and Longo, 1988). This is partly because most of these MAbs were not directed against cell surface structures with functions required for tumour cell proliferation, partly because HAMA responses prevented repeated administration, and partly because most rodent antibodies are very inefficient at recruiting human immune effector mechanisms. (Murine IgG2as and rat IgG2bs are rather more efficient in this respect than other isotypes.) Some partial responses have been observed in B-cell lymphoma patients treated with MAbs directed to B-cell Ig idiotypes (Meeker et al., 1985). The MAb most widely used clinically is OKT3, which binds to the CD3 antigen of the T-cell receptor (TCR) complex that is expressed on virtually all circulating T-cells. OKT3 has been approved by the FDA for the treatment of acute renal allograft rejection on the basis of its superiority in randomized clinical trials (Ortho Multi-Center Transplant Study Group, 1985) over conventional, broad spectrum immunosuppressive agents (93% reversal of acute rejection episodes for OKT3 compared to 75% for conventional agents). Despite being itself an immunosuppressive agent, murine OKT3 elicits HAMA responses in patients (see later), and also leads to toxicity problems arising from cytokine release which accompanies T-cell activation in response to binding of the MAb. OKT3 therapy also leads to much broader immunosuppression than is desirable, with increased incidence of viral infections and B-cell neoplasms. Anti-Tac is another rodent MAb which is intended for use in clinical studies of renal allograft rejection and which appears much more promising. This MAb blocks the binding of IL-2 to the IL-2 receptor, which is expressed on T-cells participating in allograft rejection, in certain auto-immune disorders and in one type of acute T-cell leukaemia (ATL). Of 20 ATL patients treated with anti-Tac seven showed remission lasting from one to at least 17 months (Waldmann, 1989, 1991a, b). Murine anti-Tac is presently in clinical evaluation for acute allograft rejection, and has been successfully humanized (see below).

Approaches to overcoming rodent MAb immunogenicity

There is a great deal of evidence demonstrating that the administration of antibodies from rodents (and other species) to humans results in an immune response in the great majority of patients, which limits the use of such antibodies to one or perhaps two doses (Lind et al., 1991, and references therein). For mouse antibodies the response has been termed the 'HAMA' (human anti-mouse antibody) response. Administration of further doses leads to accelerated clearance and in many cases to complete abrogation of

efficacy. It can also lead to allergic reactions and in severe cases to anaphylactic shock. Clearance of the complexes which form between the administered MAb and the HAMA antibodies can also occur through routes which deposit cytotoxic agents carried by the former in undesirable locations, giving toxicity in the organs of clearance or in the reticulo-endothelial system (RES). In some cases the HAMA titre increases with the kinetics expected of a secondary response, consistent with the presence of a pre-existing antibody with anti-mouse specificity (Schroff *et al.*, 1985; Shawler *et al.*, 1985; Courtenay-Luck *et al.*, 1987; Khazaeli *et al.*, 1991). Antibody detected during the early phase of the response tends to be directed to the Fc portion of the antibody, but later on reactivities outside this region — and especially to the variable region — can be detected. In some cases components of the response are directed to the antigen binding site, termed 'anti-idiotype' responses. It has been clearly demonstrated that HAMA interferes with tumour localization by anti-TAA antibodies (see for example Ledermann *et al.*, 1988; Goldenberg, Sharkey and Ford, 1987) and with the immunosuppressive effect of OKT3 in the course of acute allograft rejection (Chatenoud *et al.*, 1986).

Most antibody-based therapies are very unlikely to achieve success with a single dose. The only exceptions to this are likely to be in acute indications such as septic shock, for which it is possible that a single dose of an anti-TNF or anti-IL1 antibody can neutralize sufficient of these cytokines to prevent the serious (and usually fatal) organ damage which otherwise occurs. It is abundantly clear that it will not be possible in general to eradicate solid tumours with a single dose of an antibody or an antibody conjugate. Many different approaches are therefore being taken to allow repeated administration of therapeutic antibodies. One obvious approach is to isolate and use human rather than foreign antibodies. Although there are some human antibodies presently in clinical evaluation — notably the anti-LPS antibody 'Centoxin' for treatment of septic shock — the technology does not yet exist routinely to isolate human antibodies of suitable affinity and specificity. Recent technology developments which may eventually lead to more routine isolation of therapeutically useful human MAbs are summarized at the end of this review. In the short term the use of at least the binding site from a non-human antibody remains the only generally applicable method of exploiting the specificity and affinity of the antibody–antigen interaction for therapy.

Several approaches have been suggested and attempted for overcoming the HAMA response to allow repeated administration of therapeutic mouse MAbs. One is to use conventional immunosuppressants at sub-toxic doses to reduce the patient's ability to mount an effective immune response. Co-treatment with corticosteroids and azathioprine, for example, was shown to delay and diminish the HAMA response to murine OKT3 (Chatenoud *et al.*, 1986). Similarly co-treatment with cyclosporin A was shown to reduce and delay the HAMA response of colorectal cancer patients treated with the radiolabelled anti-CEA antibody A5B7 (Ledermann *et al.*, 1988). The effect was sufficient to allow four doses of the conjugate to be administered. Tumour uptake of the conjugate increased with each dose in the cyclosporin co-treated patients but not in those who were not given the immunosuppres-

sant. Although the use of conventional immunosuppressants in these cases allowed more than a single administration the number of effective doses was still quite small. Such general immunosuppression is clearly undesirable in any case since it leads to the risk of infection and since the involvement of the patients immune system may well often be required in order to achieve cures. It is also possible that one consequence of general immunosuppression will be accelerated cancer progression.

A second approach to circumventing the HAMA response involves a sequential multi-antibody treatment regime in which each time a response to the therapeutic antibody is detected treatment switches to the administration of a different antibody from a different species, or from the same species but with a different isotype and idiotype (Jonker and Den Brok, 1987). Although this approach is technically feasible, and has been used with radiolabelled polyclonal antibodies for treatment of hepatoma and lymphoma (Order, Klein and Leichner, 1987b), it is economically very unattractive because of the costs associated with developing several different MAbs as therapeutic agents. The use of antibody fragments lacking some of the immunogenic regions of antibodies has also been suggested, but such fragments retain some immunogenicity and have pharmacokinetics and biodistribution which are inappropriate for many MAb-based therapies. Several approaches have been proposed which would aim to induce tolerance to the therapeutic rodent MAb. These include pre-administration or co-administration of an anti-CD4 antibody intended to ablate T-helper cell involvement for treatment of auto-immune disease (Mathieson et al., 1990), and administration of small doses or very large doses of the therapeutic MAb (Khazaeli et al., 1988; Sears et al., 1987; Blottiere et al., 1991), strategies which are commonly used to induce tolerance in animals. Another approach involves the covalent attachment to the MAb of polyethylene glycol (PEG), which has been reported to diminish the immune response in patients to several other highly immunogenic proteins such as bovine adenine deaminase for treatment of adenine deaminase deficiency, and bacterial asparaginase for treatment of childhood leukaemia. The therapeutic value of PEG-modified proteins has been recently reviewed (Nucci, Shorr and Abuchowski, 1991). As yet there are no reports on the use of PEG to overcome HAMA in patients treated with rodent MAbs but PEGylation of a mouse MAb has been shown to render it non-immunogenic in rabbits (Kitamura et al., 1991). The mechanism of these effects is not yet clear but it seems likely that PEG is capable of both shielding epitopes from the immune system and inducing specific tolerance to the PEGylated protein (Katre, 1990). The humanization of MAbs, however, is undoubtedly the most generally applicable and economically viable approach to overcoming the HAMA response.

Construction, expression and manufacture of humanized whole antibodies

Antibody humanization involves replacing as many as possible of the non-human residues of the antibody with those from a human antibody. The constant regions of a rodent MAb can readily be humanized by replacing the

DNA sequences encoding the mouse heavy and light chains with equivalent sequences from human constant regions. The resulting recombinant antibodies with rodent variable domains and human constant domains have become known as 'chimeric' antibodies. The conserved three-dimensional structure of antibodies together with the organization of the proteins into domains and of the genes into exons make this a relatively straightforward and reproducible procedure to accomplish. However, the available evidence on the usefulness of such rodent–huimric antibodies in clinical studies (outlined below) suggests that they will in general show only partly reduced immunogenicity, and although chimerization may expand the therapeutic opportunities for MAbs somewhat, it is already clear that a significant proportion of patients mount an immune response to the mouse variable region. An extension of the humanization process has therefore been developed in which only the residues required for antigen binding are transferred from the parent non-human MAb to an acceptor human antibody framework. Several slightly different approaches to generating such fully humanized MAbs have been taken and are described below.

CLONING OF VARIABLE REGIONS AND CONSTRUCTION OF CHIMERIC ANTIBODIES

Several different procedures have been developed for the construction and expression of chimeric genes. In the most commonly used procedure (historically) genomic DNA from a hybridoma producing the MAb of choice is cloned, usually in a phage λ vector (exemplified by Sun *et al.*, 1986; Hutzell *et al.*, 1991). Using Southern blotting techniques it is possible to identify the expected size of a restriction fragment which will include the correctly rearranged VJ and VDJ sequences and to perform size selection of the genomic DNA digest to reduce the complexity of the phage library. The desired sequence can be identified with restriction fragment probes covering the J minigene regions or with oligonucleotides specific to the VJ and VDJ recombinational events. Sequence information about the VJ and VDJ joining regions can be obtained by sequencing first strand complementary DNA (cDNA) made from the hybridoma messenger RNA (mRNA). The VJ or VDJ exon along with the signal sequence exon and the Ig promoter and in some cases the Ig enhancer can then also be isolated as a single DNA fragment using suitable restriction sites. This DNA is inserted 5′ of the required human constant region DNA and the resultant chimeric gene is assembled in a suitable vector for expression, typically pSV2*neo* and pSV2*gpt* or derivatives (Mulligan and Berg, 1980, 1981). In this form of construct expression of the antibody genes is usually achieved by transfection into a myeloma cell line which has been selected for loss of expression of one or both of the resident antibody chains, for example Sp2/0 or NS0. Such vectors can, however, also be used for expression in non-myeloid cell types such as CHO cells (Neumaier *et al.*, 1990), which is perhaps surprising since the Ig promoters and enhancers have been regarded as lymphoid cell-specific. A potential disadvantage of this genomic cloning procedure is that hybridoma

lines often harbour aberrantly rearranged variable region gene segments, some of which are transcribed. For example Sp2/0 cells possess an aberrant light chain, κ138 (Cabilly and Riggs, 1985), which is transcribed. Both the genomic sequence and cDNA have been inadvertantly cloned during chimeric antibody construction (Cabilly et al., 1984; Hoogenboom et al., 1990; Neumaier et al., 1990; our unpublished observations). It is therefore important to be able to characterize the potential clones quickly to avoid wasting significant effort on such incorrect genes.

The second commonly used procedure involves construction of a cDNA library from mRNA of the chosen hybridoma. This library can be prepared using Oligo-dT as primer for first strand synthesis on the polyA-containing mRNA to produce full length dDNAs for the heavy and light chains. Alternatively specific primers can be used which are complementary to sequences in the 5' region of the first constant domain, or even in the J sequences, in order to clone the variable regions only. The variable region cDNAs are then fused to DNA encoding the constant domains of the chosen human antibody. The constant regions can be in the form of cDNA (Liu et al., 1987a, b, c) or genomic DNA (Whittle et al., 1987). In each of these cases the variable region DNA becomes fused to the CH1 domain DNA of the human constant region in such a way that none of the coding sequence is lost or altered. Alternatively chimeric antibody construction can be achieved by the use of oligonucleotide linkers which extend from a convenient restriction site in or near the 3' of V_H and V_L and which recreate the coding sequence. Sequences are included for a splice donor site immediately 3' to the J sequence and also for a short section of intron sequence up to a convenient restriction site for cloning of the adapted V-regions 5' to the required constant region genes. The chimeric antibody genes can also be expressed from heterologous promoters (see Gillies, Lo and Weslowski, 1989 action on chimeric 14.18 below). This cDNA approach allows the chimeric genes to be expressed using promoter/enhancer systems other than the Ig promoter and using myeloid or non-myeloid mammalian host cells (Liu et al., 1987a; Whittle et al., 1987; Colcher et al., 1989; Gillies, Lo and Weslowski, 1989; Bebbington, 1991; King et al., 1992a). A potential complication for this cDNA approach is that mRNAs for antibody chains which are derived from the fusion partners and which are still transcribed in the hybridomas (Cabilly and Riggs, 1985) are often cloned along with those of the desired antibody (Queen et al., 1989; Gorman et al., 1991; J. Adair, unpublished).

Recently a third general approach has been developed which is becoming the most commonly used because of its greater speed. It involves using the polymerase chain reaction (PCR) procedure to amplify the antibody sequences. In this method primers are used that allow amplification of a DNA sequence derived from a first-strand cDNA synthesis. The primers used are designed to be complementary to regions in the signal sequence (Jones and Bendig, 1991) or the first framework region (Gavilondo-Cowley et al., 1990; Orlandi et al., 1989), and in the J region or constant region of the antibody (Gavilondo-Cowley et al., 1990; Jones and Bendig, 1991; Orlandi et al., 1989). At each priming position in the signal and variable regions a level of

redundancy needs to be incorporated into the primer design to allow for the sequence differences seen between antibodies. The primers also incorporate restriction sites suitable for subsequent rapid cloning of the amplified sequences into expression vectors. The primers can be designed to allow subsequent assembly of the chimeric gene as cDNAs, as genomic forms or as any convenient intermediate conformation. cDNAs derived from fusion partner mRNAs are also co-isolated by this PCR cloning approach (J. Adair, unpublished) but primers which anneal specifically to the κ138 or MOPC-21 light chains (the ones most commonly arising from the fusion partners) can be used as probes to rapidly screen them out of the PCR libraries.

Antibody gene sequences can be rapidly cloned from a very small number of cells with this PCR amplification approach. Indeed, the amplification of variable gene sequences from single hybridoma cells has recently been reported (Liu, Creadon and Wysocki, 1992). Great care must be taken with the PCR procedure, however, to avoid cloning errors due to inadvertent cross-contamination of the reactions. It is also necessary to ensure that the sequences cloned do not contain replication errors incorporated during the error-prone amplification procedure, and this is usually accomplished by sequencing several independently amplified clones. The most significant evidence that the correct sequences have been cloned is the demonstration that when expressed they give antigen binding activity equivalent to the rodent MAb.

It is also technically feasible to construct chimeric antibodies by homologous recombination in hybridoma cells (Fell *et al.*, 1989; Holzschu and Daiss, 1989). In this process a plasmid containing the genes for the human constant region of choice is targeted to the immunoglobulin locus in the hybridoma cell and replaces the murine constant region so that the hybridoma expresses a mouse variable–human constant region chimeric antibody. The disadvantages of this approach are the poor efficiency of the gene-targeting process (especially considering that it must be performed for both heavy and light chains), its applicability only to chimeric rather than fully humanized antibodies, and also the restriction of the expression level for the chimeric antibody to that of the murine form in the parental hybridoma, which may not be sufficient for an economically viable process.

DESIGN AND CONSTRUCTION OF FULLY HUMANIZED ANTIBODIES

Results (described below) on a number of rodent–human chimeric antibodies in clinical studies show that in general they are less immunogenic than their parent murine MAbs but are nevertheless still immunogenic in a substantial proportion of patients. (It is conceivable that monkey–human chimerics — so-called 'primatized antibodies' (Newman *et al.*, 1992) — may prove less immunogenic in humans than rodent–human chimerics.) Characterization of the HAMA responses observed in some of these clinical studies with rodent–human chimeric antibodies indicates an anti-V region response with components directed against both the antigen binding site and the framework. Technology has therefore been developed to construct more fully

humanized MAbs. Several different approaches have been taken, termed 'CDR grafting' (Jones et al., 1986; Verhoeyen, Milstein and Winter, 1988), 'reshaping' (Riechmann et al., 1988; Tempest et al., 1991), 'hyperchimerization' (Queen et al., 1989), 'civilization' (Kurrle et al., 1990; Shearman et al., 1991b) and 'veneering' (Law et al., 1991) or 'surface replacement' (Padlan, 1991). These all involve redesigning the variable region so that the amino acids comprising the non-human binding site are integrated into the framework of a human antibody variable region. This can now be accomplished routinely with retention of most (or all) of the antigen binding activity of the non-human MAb. This is a considerable feat of protein engineering, and its feasibility is a consequence of the close structural and sequence relationship between IgGs from different species.

In order to accomplish this full humanization process two important decisions need to be taken, namely the choice of human framework and the extent of rodent variable region sequence to be transferred. The choice of human framework for the humanization process is often based on the desire to use an antibody for which a structure has been determined by X-ray crystallography, for example NEWM or KOL for the heavy chain and REI for the light chain (referenced in Kabat et al., 1987), so that some positional information is available about framework amino acids. Alternative strategies favour: use of a light and heavy chain from the same human antibody, for example EU (referenced in Kabat et al., 1987); use of a representative example from the various human subgroups (the heavy chains EU, NEWM and KOL are each examples of the three major human heavy chain subgroups; and use of a human variable domain which has high homology to that of the rodent antibody in question. A human κ light chain (for example REI or EU) is usually chosen, reflecting the bias towards the use of κ chains in mice and the high degree of homology between mouse and human κ chain sequences.

The other important decision to be made concerns the extent of the mouse sequences that are to be transferred. An examination of the database of structures of antibodies and antigen–antibody complexes suggests that only a small number of antibody residues make direct contact with antigen (Amit et al., 1986; Colman et al., 1987; Sheriff et al., 1987; Padlan et al., 1989; Boulot et al., 1990; Poljak, 1991). The residues chosen for transfer can be identified in a number of ways. Firstly, as described earlier, the antigen binding surface is predominantly located on a series of loops, three per domain, which extend from the β-barrel framework.

In most but not all cases the CDRs correspond to, but extend a short way beyond, these structural loop regions. Secondly, residues not identified in the structural loops or hypervariable regions may contribute to antigen binding directly or indirectly by affecting binding site topology, by inducing a stable packing of the individual variable domains, or by stabilizing the inter-variable domain interaction. Such residues may be identified for example by superimposing the sequences for a given antibody on a known structure (and if necessary adjusting loop lengths) to produce a molecular model of the Fv and then evaluating particular residues for their contribution (e.g. Queen et al.,

1989). Alternatively such residues can be identified by sequence alignment analysis and noting 'idiosyncratic' residues, followed by examination of their structural location and likely effects (Riechmann *et al.*, 1988; Gussow and Seemann, 1991), or if time and cost permit by determination of a structure for the murine variable regions by X-ray crystallography and inspection of the structure for interacting non-CDR residues. Another general approach involves making the assumption that only surface residues are involved in immunogenicity, replacing only surface residues which are not in the antigen binding region with human positional equivalents (Padlan, 1991).

Once the relevant sequence choices have been made the humanized variable region DNA can be generated by any of several procedures: by gene synthesis using suitable overlapping oligonucleotides (exemplified by Jones *et al.*, 1986); by using long, alternating oligonucleotides and filling the single strand gaps with T4 DNA polymerase (Queen *et al.*, 1989) or by PCR procedures to gap-fill and amplify the synthetic DNA (Daugherty *et al.*, 1991); or by using simultaneous or sequential site-directed mutagenesis of existing DNA sequences (Riechmann *et al.*, 1988; Verhoeyen, Milstein and Winter, 1988; Gorman *et al.*, 1991). Some groups favour designing the humanized variable sequences to reflect the codon preferences observed for antibody variable domains, since these tend to differ from those of antibody constant regions and other eukaryotic genes (Grantham and Perrin, 1986). Suitable restriction enzyme recognition sites are often included in the design of the humanized sequences to facilitate easy replacement of sections of the gene around the CDRs in case further changes are required to achieve affinity similar to that of the parent rodent MAb. Once the humanized variable region sequences have been generated they can be converted to humanized whole IgG genes for expression in mammalian cells by the procedures outlined above for chimeric antibodies. A few examples will now be given of the various approaches which have been taken to fully humanize rodent MAbs.

Humanization by reshaping

B1.8.   The first description of the production of more fully humanized MAbs was published in 1986 (Jones *et al.*, 1986) and described the formation of a hybrid antibody with murine light chain and humanized heavy chain from the B1.8, which recognizes the hapten 4-hydroxy-3-nitrophenacetyl caproic acid (NP-cap). The heavy chain was humanized by replacing the CDRs — residues 31–35, 50–65 and 95–102 inclusive as defined by Wu and Kabat (1970) and Kabat *et al.* (1987) — of the heavy chain of the human antibody NEWM (for which a high resolution structure is available) with those of the B1.8 heavy chain, and using an IgE constant region. The humanized variable region was generated by assembly of the double-stranded form from long synthetic oligonucleotides and was cloned by substitution into convenient restriction sites of the B1.8 heavy chain variable region sequence with a view to achieving transcription, mRNA processing and translation of the humanized

variable sequences in a manner similar to those of the murine variable domain gene. The expression vector was based on pSV2*gpt* and used the murine Ig promoter/enhancer. It was introduced into J558L cells for co-expression with the resident light chain, which is known to associate with the murine B1.8 heavy chain to create a binding site for NP-cap. The dissociation constant of the resultant hybrid antibody was shown to be 1.9 µM compared to 1.2 µM for the murine antibody, equivalent to 63% relative potency. Interestingly a monoclonal anti-idiotype antibody against B1.8 was not able to bind to the hybrid antibody and a second anti-idiotype antibody bound less well. Polyclonal rabbit anti-B1.8 idiotypic serum also recognized the humanized antibody less well than the murine antibody. This result, albeit based on reactivity towards a hapten antigen, suggested that the humanization strategy might indeed prove useful as an approach to alleviating HAMA responses.

D1.3.   Humanization and reconstitution of activity for the B1.8 heavy chain stimulated interest in the humanization of an antibody recognizing a protein antigen (most antigens of clinical interest being proteins). A useful model is the lysozyme/anti-lysozyme system for which several murine MAbs are well characterized and for which structures of MAb–lysozyme complexes have been deduced from X-ray crystallography (Amit *et al.*, 1986; reviewed in Poljak, 1991). Initially only the heavy chain was humanized (Verhoeyen, Milstein and Winter, 1988) because the X-ray structure of the antigen–antibody complex (Amit *et al.*, 1986) suggested that 9 out of the 12 hydrogen bond contacts to the antigen made by D1.3 are through the heavy chain. The light chain has since also been humanized (Riechmann, Foote and Winter, 1988; Foote and Winter, 1992). The heavy chain was humanized by substituting the CDRs, as defined by Kabat, into the NEWM heavy chain framework of the humanized B1.8 V_H gene, and using an IgG2 constant region. The CDRs were replaced by sequential mutagenesis using long single-stranded oligonucleotides on M13 templates. The variable region was re-introduced into the expression vector and transfected into J558L cells. The J558L light chain in the hybrid antibody produced was then replaced *in vitro* by the D1.3 light chain. The binding of the resulting antibody to lysozyme was compared to the murine antibody in a competition binding assay and was judged to be around 10-fold less (Verhoeyen, Milstein and Winter, 1988). A number of possible reasons for the loss of binding affinity were suggested, including the lack of a potential contact between residue 32 in CDR1 and a residue in the adjacent structural loop at position 27. Also the crystal structure of the D1.3-lysozyme complex shows that the antigen makes contact with the threonine at residue 30 in the loop region immediately N-terminal to CDR1 on the heavy chain (Amit *et al.*, 1986). The light chain was later humanized using the human REI Bence–Jones protein sequence as the framework (Riechmann, Foote and Winter, 1988; Foote and Winter, 1992) and using CDRs from the mouse antibody as defined by Kabat *et al.* (1987).

Subsequently work with other antibodies (see below) has established the requirement to consider non-CDR residues in the transfer of binding activity, and Foote and Winter (1992) have recently disclosed a further series of

experiments in which the affinity of the humanized D1.3 has been improved by a number of mutations in the framework. The affinities of the murine and humanized antibodies were first measured. The K_d for D1.3 was stated to be 3.7 nM with a K_{on} of $1\cdot4 \times 10^6$ s^{-1} M^{-1}, while that for the humanized D1.3 (HuLys0) was 260 nM (K_{on} of $0\cdot7 \times 10^6$ s^{-1} M^{-1}) (K_{on} is the rate constant for formation of the antibody–antigen complex). Residue 71 on the light chain is a packing residue which interacts with light chain CDR1. When this residue was altered from the phenylalanine in the humanized framework to tyrosine as found in the murine sequence, the resultant antibody showed an improved K_d of 64 nM, with an improvement in K_{on} to $1\cdot8 \times 10^6$ s^{-1} M^{-1}. A number of sequential changes were then made to the heavy chain (using the $\gamma1$ gene) which resulted in affinity improvements without affecting K_{on}. In the first heavy chain change the serine at residue 27 in the NEWM framework was altered to the phenylalanine found in the murine sequence as suggested from the initial study. The resultant antibody (HuLys6) has a K_d of 48 nM (K_{on} $1\cdot48 \times 10^6$ s^{-1} m^{-1}). Residue 71 on the heavy chain has been proposed as a key residue involved in the positioning of heavy chain CDR2 (Chothia and Lesk, 1987; Chothia *et al.*, 1989; Tramontano, Chothia and Lesk, 1990). Replacement of the human residue (valine) with the murine residue (lysine) to give HuLys10 caused a further improvement of affinity to 18 nM. Finally, the sequences in the surface loop adjacent to heavy chain CDR1, at amino acids 28–30 inclusive, were replaced. This resulted in the sequence threonine–phenylalanine–serine being replaced by serine–leucine–threonine. The resultant antibody (HuLys11) showed a further improvement in affinity to 14 nM, a relative potency of 26.4% for the final antibody compared to 1.33% for the first humanized form. Several MAbs of therapeutic interest have now been fully humanized successfully with the reshaping approach, notably the MAbs YTH34.5HL, RSV19, BW431/26 and H17E2. Humanization and other studies with these four MAbs are described later.

Humanization by hyperchimerization

The studies with B1.8 and D1.3 suggested it is possible to transfer binding activity from a rodent MAb to a human framework with reasonable success. An alternative procedure involves using the human sequence of greatest sequence homology as the acceptor framework. Within this framework idiosyncratic residues are generally also replaced with those more commonly found in human antibodies. If structural information is not available for the rodent MAb (as is usually the case) a computer model of its Fv is then generated and used to identify non-CDR residues which may interact with the CDR sequences, either because they are adjacent to the CDRs in the linear sequence or are within 3Å of amino acids in the CDRs (Queen and Selick, 1990). Some or all such residues of the rodent antibody are then included in the design of the humanized variable domains. The humanized antibodies designed by this procedure share very significant homology to the parent non-human MAb, which may help to give the former a greater proportion of the latter's antigen binding activity. This approach has been termed 'hyper-

chimerization' and has been used to humanize several rodent MAbs of clinical interest, notably anti-Tac, Fd79, Fd138–80, YTH12.5, M195 and 4D5 (see later). Humanization and other studies on these MAbs are described later, but in all these cases humanized variants were identified which showed *in vitro* potencies relative to the parent rodent MAbs of at least 33%. For both M195 and 4D5 humanized variants were identified with relative potencies apparently greater than the parent rodent MAbs.

Comparative humanization by reshaping and hyperchimerization

A few MAbs have now been humanized by both reshaping and hyperchimerization, enabling the two strategies to be compared from the viewpoint of affinity retention.

0.5β. Maeda *et al.* (1991) have humanized the murine antibody 0.5β (Matsushita *et al.*, 1988). This MAb recognizes an epitope on the gp 120 glycoprotein from the virus HTLV-III$_B$, neutralizes viral infection *in vivo* and inhibits syncitium formation between HIV-infected cells and uninfected CD4$^+$ cells *in vitro*. Chimeric antibodies were produced using the genomic cloning approach and following transfection into myeloma cells. For the humanization study expression in COS cells from the hCMV immediate early promoter/enhancer was used (Whittle *et al.*, 1987) for rapid analysis of newly constructed antibody genes. Chimeric antibodies were first reconstructed for expression by isolation of the V regions by PCR extraction from the original clones with simultaneous introduction of 5' and 3' cloning sites and cloning into pSV2 based vectors containing the Pstl-m fragment of hCMV (Boshart *et al.*, 1985) and either the human κ or IgG1 constant region sequences for light and heavy chain, respectively.

Only a single humanized V$_L$ was designed and constructed. It was based on the modified REI sequence used by Riechmann and co-workers (Riechmann, Foote and Winter, 1988; Riechmann *et al.*, 1988) and used the CDRs from the murine antibody as defined by Kabat *et al.* (1987). The 0.5β CDRs were substituted for the D1.3 CDRs by simultaneous site-directed mutagenesis. When this humanized V$_L$ sequence (RVL) was assembled in the expression vector and co-expressed in combination with the chimeric heavy (cH) expression plasmid the antibody produced, RVL/cH, was found to bind (in an ELISA format direct binding assay) to a peptide of the appropriate region of gp 120 from HTLV-III$_B$ as well as the chimeric 0.5β antibody. Humanized variants of the heavy chain variable region were designed and constructed using both reshaping and hyperchimerization strategies. For the former NEWM was used as the human framework, but was modified so that packing residue 27 was phenylalanine as used by Riechmann *et al.* (1988) and Gussow and Seemann (1991), rather than the 0.5β residue, tyrosine. The CDRs were as defined by Kabat *et al.* (1987) and were introduced into the humanized D1.3 sequence (Verhoeyen, Milstein and Winter, 1988) by simultaneous site-directed mutagenesis. Three reshaped heavy chains were made: the first RVHa had no other framework changes except residue 27; the second RVHb

had the murine residues at 23, 24, 25, 27 (tyrosine), 30 and 94; the third RVHd had in addition murine residues at 37, 38, 40 and 66–70 inclusive. When these variable region sequences were assembled with the IgG1 constant regions in the expression vector and co-expressed with the chimeric light (cL) gene expression vector no antigen binding activity could be detected. The RVHd chain differs from the cH chain sequence by only 28 residues in the V region.

The hyperchimerization approach was therefore also used in an attempt to identify a humanized variant with antigen binding activity. Human subgroup I (Kabat *et al.*, 1987) was identified as having the most homologous consensus sequence and rather than choosing a particular highly homologous sequence from within this subgroup the consensus sequence itself was used as the framework region. The subgroup I consensus sequence had been previously constructed by gene synthesis (Kettleborough *et al.*, 1991) and the 0.5β CDRs were substituted by site-directed mutagenesis. For RVHc in addition to the CDRs the 0.5β specific amino acids were used at positions 30, 71 and 94, based on their identification by Chothia *et al.* (Chothia and Leck, 1987; Chothia *et al.*, 1989) as being potentially involved in the generation of the canonical forms of the CDRs. Antibody RL/RVHc showed detectable binding to the HTLV-III$_B$ peptide but had negligible competitive ability for the HTLV-III$_B$ peptide against the murine antibody. Two further variants, RVHe and RVHf, were therefore made. RVHe had, in addition to the murine framework residues in RVHc, the murine amino acid at 37, 38, 40, 48 and 66–69 inclusive, while RVHf had, in addition to the murine framework residues in RVHc, the murine residues at 37, 38, 40 and 48. The RL/RVHe antibody was also able to bind to the peptide and to compete with the murine antibody for the viral peptide, although with an eight-fold lower potency. In a viral neutralization assay involving inhibition of syncitium formation the RL/RVHe antibody showed two-fold lower potency than either the murine or chimeric forms. The RL/RHf form had 17-fold lower potency in this assay.

CAMPATH-9. Anti-CD4 MAbs are being intensively studied as immuno-suppressive agents. Despite causing immunosuppression, rodent and chimeric MAbs have nevertheless been shown to be immunogenic themselves. Gorman *et al.* (1991) have humanized a rat anti-CD4 MAb called CAMPATH-9. The V_L and V_H sequences were isolated by cloning in M13 vectors after PCR amplification by a modification of the PCR procedure of Orlandi *et al.* (1989), using a specific forward primer for the heavy chain. Chimeric IgG1/κ antibody genes were constructed using the vectors described by Page and Sydenham (1991), which use the β-actin promoter. Expression was achieved in CHO cells following DHFR selection. The first approach to full humaniza-tion involved reshaping with the REI and NEW sequences as light and heavy chain frameworks, respectively, and with phenylalanine at position 27 on the heavy chain as used for YTH34.5L (see later) rather than the leucine found in the rodent sequence. Similarly a threonine was used at position 30 rather than the serine found in the CAMPATH-9 and NEW sequences. The reshaped variable regions were constructed by simultaneous site-directed mutagenesis

of the rat cDNAs, and the full length IgG1/κ antibodies constructed and expressed as for the chimeric form. This first humanized variant bound poorly to the CD4 antigen presented on the surface of a rat T-cell line transfected with a construct for expression of human CD4. The REI and NEW frameworks had 67% and 47% sequence homology respectively with the light and heavy chain of the rodent MAb, and it was suggested that the NEW framework was insufficiently homologous for retention of good antigen binding activity. A second humanized heavy chain was therefore constructed using the KOL framework, which is 72% homologous to that of CAMPATH-9, and also the rat residue (threonine) at position 108. Antibody with this second humanized variant bound to the cell line presenting the antigen with approximately 31% relative potency compared to the chimeric form.

Humanization by 'civilization'

Another procedure for antibody humanization has recently been described (Kurrle *et al.*, 1990) which has been called 'civilization'. It is similar to the hyperchimerization procedure but uses a different means of predicting non-CDR residues which may contribute to affinity. The procedure involves first selecting heavy and light chains from the same human antibody to be the frameworks, with a view to retaining the relevant domain interactions. The human antibody chosen is the one with the most homology to the rodent antibody for both heavy and light chains. The human J-regions most closely homologous to that of the rodent antibody are also used. A first generation civilized antibody, CIV1, is then designed and built using the CDR definitions of Kabat *et al.* (1987). If CIV1 fails to show suitable affinity a number of iterative steps are used to incorporate an increasing number of non-human residues until adequate affinity is recovered. First, sequences within four amino acids on each side of the CDRs are examined and any rodent residues which differ in size or charge between human and non-human are substituted into CIV1 to give CIV2. The rationale for this is that the CDRs and the loop structures observed in crystal structures can vary by up to four residues. If this proves unsuccessful a third variant CIV3 is made in which all the rodent residues within four amino acids each side of the CDRs are substituted into CIV1. Finally information from X-ray crystal structures of homologous antibodies is used to identify any other residues close to or potentially contacting the CDRs in either the rodent MAb or the human acceptor framework, and the non-human residues are also incorporated into the design of the humanized antibody.

The only antibody humanized thus far by this procedure is the murine MAb BMA031, which recognizes an epitope on the human T-cell receptor TCR-a/b. This receptor is present on all mature lymphocytes and — in association with the CD3 complex — is responsible for signal transduction after antigen binding as the first step in T-cell activation. BMA031 is therefore being evaluated for immuno-modulatory interventions, and chimeric and humanized variants have been constructed (Shearman *et al.*, 1991a, b). The human

EU framework was found to have 67% sequence identity with the BMA031 heavy chain and 63% homology with its light chain, and was therefore chosen as the human framework. The human J_H4 and J_k4 J regions were also chosen on the basis of sequence homology. A CIV1 humanized construct was made by assembling the humanized V sequences from oligonucleotides and using them to replace the murine V region of a IgG1 chimeric form. The CIV1 antibody, produced after co-transfection of heavy and light chain expression plasmids into SP2/0 cells, was unable to bind antigen. A CIV2 variant was therefore made with an extra five murine residues substituted into the heavy chain and two into the light chain. This variant was also inactive in antigen binding assays. A CIV3 variant was then made with an extra seven murine residues in the heavy chain and an extra three in the light chain compared to CIV2. The CIV3 variant showed good antigen binding, with an affinity two-to three-fold lower than the murine and chimeric forms.

Humanization by 'surface replacement'

In this procedure, which was suggested by Padlan (1991) and is also termed 'veneering', as many solvent-accessible residues of the rodent antibody as possible are replaced by residues from a human antibody. The solvent-inaccessible residues, CDRs, inter-domain contact residues, and residues immediately flanking the CDRs all remain rodent. This strategy is intended to mimic the surface of a human antibody while retaining all of the packing and interface interactions from the rodent antibody, which may aid in retention of full antigen binding activity. Recognition of the humanized antibody by antigen-processing cells should be minimized, reducing the possibility of presentation to T-cells of the foreign peptides generated by processing of the hybrid variable regions. The choice of surface residues is determined by homology matching between the rodent antibody variable domains and those of available human sequences (either individual or consensus human sequences). The solvent-accessible residues are identified by inspection of the high resolution structures of the human antibody KOL or the murine antibody J539. Padlan (1991) also notes other regions of the antibody which may be relevant to humanization: buried residues which make contact with the CDRs and are different between the murine and human antibodies (in such cases the rodent residue is used); the N-terminal regions, which are positioned near the CDRs for both domains and may play a role in antigen binding; electrostatic interactions may also play a part, even at long distance.

There are no examples yet published of antibodies humanized by this procedure, but a very similar procedure has been used to humanize antibody 1B4. This murine MAb 1B4 binds to CD18, the β2 chain of the leukocyte integrin heterodimers. It has been humanized (Daugherty *et al.*, 1991) with a view to using the MAb for treatment of inflammatory responses in indications such as graft rejection and GVHD. Two light chain frameworks were used in the humanization studies, REI and LEN, which have 69% and 81% sequence identity with 1B4. Similarly three different heavy chain frameworks were used, these being NEW, GAL and JON with 55%, 82% and 78% identity to

1B4, respectively. κ and IgG4 constant regions were used for the light and heavy chains, respectively. These frameworks were chosen to examine the effects on affinity retention of sequence identity, especially for the non-solvent exposed regions. The REI-based humanized light chain, when co-expressed with the chimeric heavy chain, bound antigen as well as the murine MAb in cell-based assays with the antigen on stimulated polymorpho-nuclear lymphocytes (PMNs). When the REI-based humanized light chain was co-expressed with the NEW, GAL and JON-based humanized heavy chains the resulting antibodies showed potencies of 6·5%, 31%, and 8·8%, respectively, relative to the murine MAb. Two features of JON are worth noting in this context: first, it has a potential site for glycosylation, which used may influence antigen binding; second, JON has two more residues than GAL adjacent (in resolved antibody structures) to CDRs which are different from murine 1B4. The second humanized light chain, with the LEN frame-work, was also co-expressed with the GAL-based heavy chain. The resulting antibody showed the same potency relative to murine 1B4 as the REI/GAL-based humanized antibody, so no significant effect could be observed of using the more homologous light chain framework. Finally, a variant of the GAL-based heavy chain was constructed with three further mouse residues incorporated, at position 16 — which is solvent accessible — and 47 and 109 — which are buried residues. This GAL-based variant was co-expressed with the REI-based light chain to give an antibody with 75% relative potency.

Further examples of full antibody humanization are described in later sections, and conclusions on humanization strategies are drawn at the end of the review.

EXPRESSION AND MANUFACTURE OF HUMANIZED ANTIBODIES

The development of recombinant MAb-based therapies can be regarded as a three-stage process. In the early stage only small quantities (μg) of the engineered MAb are required for *in vitro* tests of its ability to bind to purified antigen or to cells presenting the antigen. In the second stage larger quantities (10s to 100s of mg) of somewhat purer material are required, typically to perform more demanding bioassays, *in vitro* cell-killing studies and *in vivo* efficacy studies in animal models. In the final stage large quantities (g) of material purified by rigorous (and usually costly) procedures to therapeutic grade are required to allow development of manufacturing processes accept-able to regulatory authorities, for animal toxicology studies and finally for evaluation in clinical studies. In general the second and third stages will require more material for highly engineered MAb conjugates compared to naked whole antibodies. The issues involved in producing MAbs for clinical studies by processes which comply with good manufacturing practice (GMP) have been summarized by Bright, Adair and Secher (1991), and the regula-tory considerations relevant to producing recombinant MAbs or other recombinant proteins by Brown (1990). It is important that the final manufac-turing process is efficient, safe and economically viable on a large scale. The priority in the early stages of MAb development, on the other hand, is

producing the material rapidly and easily such that many engineered variants can be compared by *in vitro* tests. The development of several different expression systems for engineered MAbs in part reflects these different priorities at different stages of therapeutic MAb development. *Table 1* shows the wide range of approaches that have been taken to express recombinant MAbs. They have been expressed in mammalian cells, insect cells, plant cells, yeast and *E. coli*. Most of these systems are mentioned in the following recent reviews: Morrison and Oi (1989); Gold (1990); Gorman (1990); Morrison and Schlom (1990); Bebbington (1991); Pluckthun (1991); Yarranton and Mountain (1992). While whole antibodies can be expressed in soluble form and glycosylated in the appropriate position in yeast, insect cells and plant cells, these expression hosts do not give the pattern of sugars when glycosylated that is found in non-recombinant MAbs or those expressed in mammalian cell systems (Kukuruzinska, Bergh and Knackson, 1987; Hasemann and Capra, 1990; Hein, 1990). Such glycosylation is important for effector functions (Horwitz *et al.*, 1988) and structural integrity of the antibody. Whole antibodies cannot readily be expressed in soluble or glycosylated form in *E. coli*. In this present review we therefore focus now on mammalian cell systems, which are at present the preferred route for production of whole antibodies, and later on *E. coli* systems, which are rapidly becoming the preferred route for production of antibody fragments.

Mammalian cell expression

The most commonly and successfully used cell lines for the production of engineered whole antibodies are the myeloid lines SP2/0 (Shulman, Wilde and Kohler, 1978) and NS0 (Galfre and Milstein, 1981; Bebbington *et al.*, 1992), and the non-myeloid line CHO (Bebbington, 1991). Features which are advantageous for large-scale production of antibody by these or other cell lines are: an efficient means of inserting the expression plasmid(s) into the host cell; a means of maintaining the plasmid(s) stably within the cell after transfection; a means of selecting for cell lines in which multiple copies of the antibody genes have become integrated into the genome; a strong promoter/ enhancer sequence to direct transcription; a means of ensuring efficient initiation of translation; a means of ensuring approximately equal expression of heavy and light chains; for CHO cells a means of inducing suspension-adapted rather than attached cell growth; and a means of inducing the cells to grow to high biomass in serum-free medium. Many of these technical difficulties have now been overcome. Techniques such as fusion of the recipient cells to bacterial protoplasts (Sandri-Goldin *et al.*, 1981), DEAE-dextran treatment (Queen and Baltimore, 1983), calcium phosphate DNA precipitation (Graham and Van der Eb, 1973; Wigler *et al.*, 1979), electroporation (Toneguzzo, Hayday and Keating, 1986), and lipofection (Felgner *et al.*, 1987), can be used to generate stably transfected cell lines. Electroporation and calcium phosphate DNA precipitation are presently the most commonly used procedures, and can be used to generate stable cell lines with frequencies of around 10^{-3}–10^{-6} per cell. For myeloma cells electroporation is

Table 1. Systems for antibody expression

| Cell type | IgG | | | | F(ab')$_2$, Fab | | | | Fv, scFv | | | |
| | Transient* | | Stable* | | Transient | | Stable | | Transient | | Stable | |
	Small scale† (<100μg)	Large scale† (>100μg)	Small scale (1-100mg)	Large scale (>100mg)	Small scale (<100μg)	Large scale (>100μg)	Small scale (1-100mg)	Large scale (>100mg)	Small scale (<100μg)	Large scale (>100μg)	Small scale (1-100mg)	Large scale (>100mg)
Eukaryotic												
mammalian												
Myeloid												
NS0			+	+			+	+				
SP2/0.Ag8			+	+			+					
X63Ag8.653			+									
YO (rat)			+								+	
YB20 (rat)			+									
Non-myeloid												
CHO-K1 (hamster)	+	+	+	+			+	+			+	
CHO L761h (hamster)	+	+	+		+							
BHK (hamster)		+	+									
CV-1 (monkey)	+			+								
COS (monkey)	+	+			+				+			
293 (human)	+	+										
HeLa (human)	+											
Yeast												
S. cerevisiae			+	+			+	+				
S. pombe			+									
Insect												
S. frugiperda	+	+										
Amphibian												
X. laevis	+											
Plant												
N. tobaccum	+		+	+								
A. mediterranea	+											
Prokaryotic												
E.coli												
Intracellular			+				+	+			+	+
Extracellular							+	+			+	+

*Transient expression here means the introduction of antibody genes on a suitable expression vector immediately prior to expression and expression over a short (usually 1-7d) period. This should be compared with *Stable expression* which implies the stable introduction of the antibody genes on a suitable expression vector. Stably transformed or transfected cells may subsequently be used to produce antibody over varying time periods.

† Yields from small- and large-scale procedures are given in parentheses.

Engineering antibodies for therapy   27

almost always used because calcium phosphate precipitation is very inefficient with them.

Development of such stable cell lines requires the use of selectable markers and several dominant markers are in common use. For example, resistance to mycophenolic acid, which inhibits the conversion of inosinic acid (IMP) to xanthylic acid (XMP), can be conferred on mammalian cells by the presence within the cells of the bacterial enzyme 5-phospho-α-D-ribose-1 diphosphate:xanthine phosphoribosyl transferase (XGPRT). This enzyme is encoded by the *gpt* gene, the use of which has been reviewed by Bebbington (1991) and which features in the generic and widely used expression vector pSV2*gpt* (Mulligan and Berg, 1980, 1981). A second marker that is commonly used is *neo*, which confers resistance to the aminoglycoside G418 (Davies and Jiminez, 1980), a structural analogue of the antibiotics neomycin, kanamycin and gentamycin that also blocks eukaryotic protein synthesis (Southern and Berg, 1982; reviewed in Morrison and Oi, 1984). A third marker often used in mammalian cell expression systems is hygromycin resistance, conferred by the product of the *E. coli hph* gene (Gritz and Davies, 1983; Gorman, 1990).

These markers can be used in combination, which offers the flexibility of introducing the genes for the antibody light and heavy chains by sequential or co-transfection of the genes on separate plasmids (Gorman, 1990; Bebbington, 1991; *see*). Sequential transfection is of particular value if several variants of the antibody are to be made using the same antigen binding domains, e.g. an Ig isotype and/or allotype series, Fab', or Fab or antibody–enzyme/toxin hybrids. Then a stable cell line producing suitable levels of the common light chain can be generated into which a second plasmid capable of expressing a series of heavy chain variants can be introduced (Beidler *et al.*, 1988; Colcher *et al.*, 1989; King *et al.*, 1992a). It is important when using the sequential transfection approach to introduce the light chain constructs first. This is because many heavy chains are not released from the endoplasmic reticulum (ER) until they associate with the appropriate light chains because they associate with the abundant ER protein grp78 (Hendershot, Ting and Lee, 1988), and accumulation of some heavy chains in this location is toxic to the cells (Kohler, 1980).

More recently vectors in which both light and heavy chain genes can be co-expressed following introduction on the same plasmid have been developed and now tend to be preferred because they give a better chance of achieving more balanced synthesis of the two chains (reviewed in Bebbington, 1991). Care must be taken with the design of such single vector expression constructs, however, to ensure expression of one chain is not reduced by promoter occlusion by upstream transcription (Bebbington, 1991). This possibility can be avoided by placing a transcription terminator from an Ig gene between the two transcription units (C. Bebbington, personal communication). In these plasmids dominant markers such as dihydrofolate reductase (DHFR) or glutamine synthetase (GS) (reviewed in Bebbington and Hentschel, 1987) are used which — after the initial transfection — permit the selection of cells which have undergone amplification of the selected marker. Amplification is selected by methotrexate (MTX) resistance for the

27 of 142
Celltrion, Inc., Exhibit 1067

DHFR marker, and by methionine sulphoximine (MSX) for the GS marker. Co-amplification of the antibody genes usually occurs in such cells, which often gives higher yields of antibody per cell.

The choice of promoter used for expressing the antibody genes has, in the past, been partly dictated by the way in which the antibody genes were isolated. Genomic cloning of antibody genes usually has the added benefit that the Ig promoters from the hybridoma are also cloned and can be used to drive expression of the reconstructed genes (*see Table 3*). However, expression of cloned antibody genes (whether murine, chimeric or fully humanized) from their natural promoters usually gives expression levels which are much lower than those of the murine genes in the original hybridoma. This is because DNA sequences quite distant from the exons are normally required for expression of Ig genes, and these are not usually present on the cloned fragments. When the antibody genes are isolated as cDNAs it is therefore advantageous to express them from heterologous promoters, and many strong viral and cellular promoters have been tested. High level expression of antibody can be achieved in myeloid cells with the human cytomegalovirus major immediate early promoter/enhancer (hCMV; Colcher *et al.*, 1989), the Rous sarcoma virus promoter (RSV; C. Bebbington, personal communication), and the mouse metallothionein promoter (mMT; Gillies, Lo and Wesolowski, 1989). High expression in non-myeloid cells can be achieved by the use of the hCMV and β-actin (Page and Sydenham, 1991) promoters.

Many highly expressed mammalian genes share a common sequence immediately preceding the translation start codon (Kozak, 1987). In some cases expression levels for recombinant antibody genes can be increased two- to five-fold by including this sequence in the expression constructs (J.R. Adair and C. Bebbington, unpublished observations).

As suggested earlier, the priority in the early stage of therapeutic MAb development is producing the material rapidly and easily such that many engineered variants can be compared by *in vitro* tests. This applies especially to antibody humanization, for which loss of affinity in many of the humanized variants is very common. Transient expression systems are usually used at this stage rather than those giving stable cell lines, since the isolation of the latter is a relatively slow process taking at least one month following transfection. The most commonly used transient systems are COS cells (Whittle *et al.*, 1987), CHO L761h cells (Cockett, Bebbington and Yarranton, 1991) and 293 cells (Daugherty *et al.*, 1991; DeMartino *et al.*, 1991; Carter *et al.*, 1992b). COS cells, which are monkey kidney cells transformed with a replication origin-defective SV40 virus, support the replication of a wide range of bacterial plasmids providing these carry a short sequence from SV40 which includes its replication origin, and providing also that certain sequences from the widely used bacterial colE1 replicon are removed because these poison replication in the mammalian cells. CHO-L761h and 293 cells contain an adenovirus E1A transactivator gene which can stimulate expression from several promoters. Any of these can be used to produce recombinant antibodies secreted into the culture medium at levels of 1 μg ml^{-1} or greater

within two to three days of transfection. The 293 system has been reported to yield 15 μg ml^{-1} (Carter *et al.*, 1992b).

It is now routine to isolate stable cell lines, especially myeloma lines, with yields of recombinant whole antibody as great or better than those of murine MAbs from hybridoma cultures (*Table 3*). Accumulated yields of greater than 200 mg l^{-1} have been achieved using the GS amplification system in CHO-K1 cells (Bebbington, 1991) and 0·5–1·0 mg l^{-1} has been achieved using the GS amplification system in NS0 cells (Bebbington *et al.*, 1992; H. Brand *et al.*, unpublished data). Recombinant antibody production processes using both of these types of cell line have been scaled up to manufacturing scale production using 2000 l fermentors to obtain GMP quality material for clinical trials. It is important to stress, however, the importance of media and fermentation development in achieving such high yields. Seven- to ten-fold yield improvements are commonly achieved by these development activities (Bebbington *et al.*, 1992). It is also important to stress that maximal production rates for recombinant cell lines vary between different antibodies (Bebbington, 1991). Mutant light chains (Nakaki, Deans and Lee, 1989) and heavy chains (Hendershot, Ting and Lee, 1987) vary significantly in the extent of their interaction with the ER protein grp78, with consequences for their secretion rate. Engineered proteins may also be retained by the secretory pathway and directed for degradation rather than secretion, for reasons that are as yet not clearly understood (Klausner and Sitia, 1990; Dorner, Bole and Kaufman, 1987).

As yet there are few reports of direct comparisons of the properties of recombinant antibodies made in different mammalian cell types. Chimeric B72.3 made in CHO and NS0 cells appears indistinguishable in terms of *in vitro* antigen binding activity and biodistribution in mice (D. King, personal communication). It is nevertheless possible that glycosylation patterns for some MAbs will be affected by both the polypeptide conformation and the host cell type, as they are for other recombinant proteins (Hamaguchi *et al.*, 1989; Parekh *et al.*, 1989; Takeuchi *et al.*, 1989). The significance of subtle differences in attached carbohydrate structure for pharmacokinetics and biodistribution in patients remains to be determined.

The identification and development of such high yielding cell lines suitable for recombinant antibody production on the manufacturing scale is a lengthy (9–12 months from transfection) and labour-intensive process. Various approaches are being explored towards deriving processes which are faster and require less effort. The use of *trans*-activator genes to further enhance expression from strong promoters (Cockett, Bebbington and Yarranton, 1991) can permit levels of recombinant protein production on a small scale with low copy number vectors that approach those obtained with amplified cell lines. It is not yet clear whether culture of cell lines carrying such *trans*-activators can readily be scaled up. Another promising approach involves attempts to direct the recombinant antibody gene efficiently to chromosomal locations which are highly transcribed, in particular to the Ig loci themselves. Such gene-targeting approaches have been reviewed by Plon and Groudine (1991). They involve integration of the engineered antibody

gene into the desired chromosomal locus by homologous recombination with flanking sequences. The feasibility of this approach has been indicated by the chimerization of a mouse antibody chain by targeting a human constant region into a mouse Ig gene (Fell *et al.*, 1989; Holzschu and Daiss, 1989). An alternative approach involves attempts to clone large 'dominant control regions' (or 'locus organizers') from the mouse genome which contain all the sequences required for very high level expression irrespective of the eventual chromosomal location of the transfected antibody genes (Plon and Groudine, 1991).

Approaches to manipulating antibody pharmacokinetics and biodistribution

The design of therapeutic strategies with MAbs and MAb conjugates must take into consideration their pharmacokinetics and biodistribution. In virtually all animal model and patient studies on MAbs clearance of the antibody from the blood shows two component kinetics, comprising a shorter α-phase and longer β-phase. In general the decline in the blood level in the α-phase represents distribution of the antibody from the blood into the rest of the tissues, and the decline in the β-phase represents its subsequent clearance from the body. In man there is considerable patient-to-patient variation in the $t_{1/2}$ (half-life) of the β-phase, only some of which is likely to be caused by variation in magnitude of the HAMA response. Considerable data are available for chimeric MAbs in man, but very little for fully humanized MAbs. Upon first administration (which is relatively unaffected by HAMA responses) chimeric antibodies show a $t_{1/2}\alpha$ of 12–20 h and a $t_{1/2}\beta$ of 2–10 d when administered i.v. The only data available for fully humanized antibodies concerns H17E2, for which a $t_{1/2}\beta$ of 73 h is reported (Hird, Snook and Kosmas, 1991). Fully humanized MAbs will no doubt prove in general to have half-lives which are similar to or longer than those of chimeric MAbs. For several MAbs (B72.3, L6, 17.1A, 14.18 and H17E2) pharmacokinetic data are available for both murine and humanized forms (fully humanized for H17E2, chimeric for the others, see below for details). In each case the $t_{1/2}\beta$ of the humanized form proved to be at least two- to three-fold longer than that of the murine MAb.

The pharmacokinetics of humanized IgGs are not the most appropriate for many therapies. This is especially true for tumour diagnosis and therapy with Mab–radioisotope conjugates. For diagnosis with such conjugates long half-lives limit the tumour-to-background ratio and hence the sensitivity of lesion detection. For therapy they lead to long-term irradiation of the normal tissues and hence to dose-limiting toxicity, this being almost always myelosuppression resulting from bone marrow irradiation. Several approaches are being taken to manipulate the pharmacokinetics of MAbs, and these usually also affect their biodistribution. The approaches include: the use of particular isotypes; the use of antibody fragments; PEGylation; galactosylation; the use of *in vivo* or *ex vivo* clearing regimes.

Limited data from animal and clinical studies suggest there may be significant differences in $t_{12}\beta$ between the different Ig isotypes (reviewed by

Mariani and Strober, 1990). IgG1 and IgG4 chimeric variants of B72.3, for example, have been compared for pharmacokinetics in cynomolgus monkeys (Hutzell *et al.*, 1991). The IgG4 variant showed a $t_{12}\beta$ about three-fold longer than the IgG1 equivalent (260 and 90 h, respectively). No such directly comparative studies have been performed in humans, but the available data are consistent with the $\gamma4$ isotype persisting longer in the circulation (LoBuglio *et al.*, 1989; Merdith *et al.*, 1991; Saleh *et al.*, 1992a). There are comparable data from studies with nude mice, however, which have led to different conclusions. Pharmacokinetic studies with IgG1, IgG2, IgG3 and IgG4 chimeric variants of B72.3 have been performed with tumour-bearing nude mice (A. Phipps *et al.*, unpublished). In these animals the IgG1 and IgG2 variants were observed to persist longer in the circulation than the IgG3 and IgG4 equivalents. The difference in clearance time between the IgG1 and IgG4 variants led to greater tumour loading for the former. It should be noted that, although human tumour xenografts in nude mice are the most commonly used animal models for efficiacy studies with anti-cancer MAbs, the available comparative data suggests that pharmacokinetic behaviour in monkeys is much more predictive for humans than that in nude mice. It is possible that shorter half-lives for humanized antibodies could be achieved by using IgM rather than IgG constant regions. The $t_{12}\beta$ of Centoxin, one of the few IgM MAbs for which pharmacokinetic data are available, is typically about 30 h in clinically stable cancer patients (Khazaeli *et al.*, 1990). Efficient production of recombinant IgMs, however, is presently more difficult than that of IgGs, partly because of the need to co-express J chains in the chosen host cell line.

Galactose conjugation of antibodies allows them to be recognized by the asialo-glycoprotein (ASGP) receptor in the liver, giving rapid clearance from the blood through that organ (Mattes, 1987). Galactosylation can be accomplished with retention of ability to bind antigen and to mediate recruitment of immune effectors (Ong *et al.*, 1991). The procedure was originally developed to enhance regional immunotherapy, and was shown for example to allow high tumour:non-tumour ratios to be achieved in mice with tumours in the peritoneal cavity when a radiolabelled antibody was injected. The prospects of using it to manipulate clearance time of radiolabelled antibodies injected i.v. for tumour therapy are now being explored. The optimal time in the circulation for such conjugates is 24–48 h, a time period which allows high tumour loading while minimizing irradiation of the bone marrow and other organs. Recently it has been demonstrated that high tumour:non-tumour ratios together with high tumour loading can be achieved in animal models following i.v. administration by co-treatment with an inhibitor which binds competitively to the ASGP receptor, and can delay clearance of the galactosylated MAb for 2–3 d. After this time the conjugate is cleared rapidly through the liver. This approach may have some clinical utility, but will be restricted to MAbs conjugated to cytotoxic agents which can be rapidly eliminated by the liver without damaging it, as appears to be the case for the isotope [125]I (Ong *et al.*, 1991). A galactosylated anti-enzyme antibody has

been used in a clinical study to achieve rapid clearance of a MAb–enzyme fusion (Bagshawe *et al.*, 1992, see later).

Two alternative approaches have been suggested to allow maintenance of high blood levels of MAb conjugates for 1–3 d followed by rapid clearance. One involves removing the conjugate after the desired time period by administration of a second 'clearing' antibody which binds to the first antibody, giving immune complexes which are cleared rapidly through the liver, spleen and perhaps other organs. This method has been demonstrated to be capable of reducing background and enhancing detection of tumours with radiolabelled antibodies (Bradwell *et al.*, 1983; Begent *et al.*, 1981; Goldenberg, Goldenberg and Primus, 1987). Two drawbacks of this approach are the time and cost implications of developing a second antibody and the possibility that clearance of immune complexes through several organs might give dose-limiting toxicity in one of them. A similar approach, with similar drawbacks, involves the use of biotinylated MAb conjugates and their subsequent clearing by the administration of avidin (Klibanov *et al.*, 1988; Sinitsyn *et al.*, 1989). Extracorporeal immunoadsorption (ECIA) is a similar but more sophisticated approach to manipulating clearance times in which the MAb or MAb conjugate is removed from the blood *ex vivo* at the desired time by the use of an affinity column. The affinity reagent can be the antigen or an anti-antibody, and the procedure requires processing the blood through an extracorporeal shunt. In the only clinical study in which this procedure has yet been used it was demonstrated that 55–80% of the circulating ^{111}In-labelled antibody could be removed, with very significant dose-sparing to the bone marrow and other organs, without compromising the dose delivered to the tumours, in this case to lung and breast tumours (Johnson *et al.*, 1991). The obvious drawback to this approach is its complexity and expense in the clinical setting.

The simplest and most generally applicable approach to manipulating antibody pharmacokinetics is the use of antibody fragments. It is clear that Fab and Fv fragments and their derivatives are cleared more rapidly from the circulation than whole antibodies. These fragments distribute much more rapidly from the blood to the tissues, which is an advantage for MAb-based therapies in which the target antigen is not in the circulation, and especially for tumour imaging and therapy with antibody–isotope conjugates. As mentioned above, whole antibodies injected i.v. distribute from the blood to the tissues with a half-life of 12–20 h, then clear from the body with a half-life of 2–10 d, in both animal models and in man. Results from animal models suggest that F(ab')$_2$ fragments are cleared from the body with a half-life of 5–10 h (Brown *et al.*, 1990; Milenic *et al.*, 1991; Molthoff *et al.*, 1992), and Fab or Fv fragments with half-lives between a few minutes and one hour (Milenic *et al.*, 1991; Owens *et al.*, 1991a; King *et al.*, 1992b). In one mouse study (Covell *et al.*, 1986) it was calculated that the mean residence times in the body for IgG, F(ab')$_2$ and Fab' forms of the same murine antibody were 8·5, 0·5 and 0·2 d, respectively. The F(ab')$_2$ was calculated to give 17-fold less exposure of the body to the emissions of the isotope used, in this case [131]I.

Two crucially important parameters in the design and prospects for success

of imaging and therapy with antibody–isotope conjugates are: the tumour loading, which refers to the proportion of the administered dose that binds to the tumour and is usually expressed in terms of per cent injected dose per gram of tumour ($\%i.d.g^{-1}$); and the tumour:non-tumour ratio (sometimes called the tumour localization index), which is a measure of the dose delivered to the tumour compared to the exposure of the other organs. The most important tumour:non-tumour ratio is usually that of tumour:blood, since irradiation of the bone marrow from the MAb–isotope conjugate in the circulation usually gives the dose-limiting toxicity. The factors which determine these two parameters include the residence time of the MAb in the circulation, the degree of vascularization of the tumour, the density of the antigen and the avidity of the MAb for the latter. Whole IgGs persist longer in the circulation and give the best tumour loading, but more rapid clearance of fragments gives better tumour:non-tumour ratios. The choice of optimal MAb form for isotope delivery in patients is thus a complex issue. The most detailed comparisons of different antibody forms so far have concerned IgGs and their F(ab')$_2$s (Buchegger *et al.*, 1986; Colapinto *et al.*, 1988; Endo, Kamma and Ogata, 1988; Pervez *et al.*, 1988; Brown *et al.*, 1990; Gerretsen *et al.*, 1991; Molthoff *et al.*, 1992), largely because this fragment was the easiest one to prepare by non-recombinant means. In general F(ab')$_2$s have been found to give tumour loading in these animal models two- to ten-fold lower than IgGs, but tumour:non-tumour ratios five- to ten-fold higher. A few similar studies have recently been performed for Fab', Fv and scFv fragments of the anti-tumour MAbs B72.3 and CC49 (Milenic *et al.*, 1991; Owens *et al.*, 1991a; King *et al.*, 1992b; Yokota *et al.*, 1992). All three fragments show very rapid distribution after injection, with (for example) the CC49 scFv showing a $t_{1/2}\alpha$ of 3–4 min in both mice and monkeys (Milenic *et al.*, 1991). All three fragments also showed very rapid clearance from the circulation, with (for example) only 5% and 0·8% $i.d.g^{-1}$ remaining in the blood after 1 h for the B72.3 chimeric Fab' and Fv, respectively (King *et al.*, 1992b). The rapid clearance was reflected in relatively poor tumour loading. The B72.3 chimeric Fab' showed no significant benefit in tumour:blood ratios over the chimeric F(ab')$_2$, despite its more rapid clearance. The B72.3 Fv on the other hand, showed tumour:blood ratios at all time points which were superior to those of the F(ab')$_2$. The CC49 scFv showed tumour:non-tumour ratios which were equal to or superior to those of IgG, F(ab')$_2$ and Fab' forms of this antibody at all time points (Milenic *et al.*, 1991).

Many groups have concluded that for tumour imaging and diagnosis the reduced loading of the F(ab')$_2$ or Fab' compared to the IgG is likely to be more than compensated by their reduced background, and therefore isotope conjugates of these fragments are likely to give more sensitive tumour detection that those of whole antibody. Fab' and F(ab')$_2$ fragments are now quite widely used for tumour detection in patients by immunoscintigraphy (Delaloye *et al.*, 1986; Bischof-Delaloye *et al.*, 1989). Recently three ^{123}I-labelled forms of the anti-CEA antibody IMMU-4, these being murine IgG, F(ab')$_2$ and Fab', were compared in a randomized, double-blind Phase II study to determine the optimal antibody form for colorectal cancer detection

(Serafini *et al.*, 1989). All three forms gave similar lesion detection rates. The Fab′ form is being used in further trials because it was found to be less immunogenic.

The choice of antibody form for tumour therapy with antibody-isotope conjugates is even more complicated. Rapidly cleared fragments such as the Fab′ or scFv give tumour loading which is adequate for successful tumour detection, but not for successful tumour therapy. Thus only IgG and F(ab′)$_2$s have been compared for therapeutic effects in the nude mouse xenograft model. In most of these studies IgG and the fragment have been compared by injecting equal activities of the radiolabelled proteins. Interpretation of results with such protocols is complicated by the dependence of tumour loading on many different factors, including size and vascularity of the tumour, retention of antigen binding through radiolabelling, retention time on the tumour and immune response by the animal. Recently, though, a comparison of IgG and F(ab′)$_2$ fragments of murine anti-CEA antibodies has been reported (Buchegger *et al.*, 1990) in which efforts were made to give equivalent radiation doses with the antibody forms. ^{131}I-labelled F(ab′)$_2$s were observed to give much better therapeutic efficiency together with similar or reduced toxicity than ^{131}I-labelled IgGs. Although nude mice carrying human tumour xenografts constitute the best available animal model for testing efficacy of new anti-cancer drugs, and for testing anti-cancer MAb conjugates in particular, it has many very significant differences from the clinical situation for human cancer. The pharmacokinetics of MAbs often differ between the mouse and man, no allowance is made for HAMA responses, and uptake of the conjugate by normal tissues which express the antigen is generally not a problem in mice since the antibodies rarely cross-react with the equivalent murine antigens. In addition, xenograft tumours in nude mice are a great deal better vascularized than human tumours, and therefore give dramatically higher tumour loading. It is common, for example, to achieve 20–30% i.d.g^{-1} with IgGs in the nude mouse system, while figures of 0·01% i.d.g^{-1} have rarely been achieved in human patients. Nevertheless it is extremely likely that antibody fragments will ultimately prove superior to whole antibody for tumour radioimmunotherapy.

It is likely also that the optimal $t_{1/2}\beta$ for tumour therapy with antibody–isotope conjugates will prove to be somewhere in between those of F(ab′)$_2$ fragments (5–10 h) and IgG (up to 10 d). A $t_{1/2}\beta$ of 24–48 h will probably give high tumour loading with the highest tumour:non-tumour dose ratios per dose. The more rapid clearance of humanized fragments compared to humanized IgG will also allow more sub-toxic doses to be administered per unit time. The metabolism of F(ab′)$_2$ fragments occurs partly by cleavage of the disulphide between the Fab monomers. More stable bivalent Fab fragments have been constructed by chemical cross-linking with half-lives falling in the desired range (D. King, personal communication). Unfortunately monovalent and bivalent Fab fragments have less favourable biodistribution than whole IgG. Like Fvs and scFvs (King *et al.*, 1992b; Yokota *et al.*, 1992), these fragments are cleared through the kidney. This probably would

not preclude their use in humans with ^{131}I as the therapeutic isotope, since this is readily released from cells as iodinated amino acids or after de-iodination (Geissler, Andersen and Press, 1991). Kidney localization and accumulation of metallic isotopes such as ^{90}Y, however, would probably give dose-limiting toxicity.

The recent studies with CC49 fragments (Yokota *et al.*, 1992) suggest one advantage in biodistribution for small fragments, in that tumour penetration was observed to be size dependent. Thus autoradiographic analysis on tumours from nude mice which were sytemically administered ^{125}I-labelled IgG, $F(ab')_2$, Fab' or scFv showed that most of the IgG was concentrated in the region immediately adjacent to the blood vessels, while the scFv was much more evenly distributed. The Fab fragments showed properties in between the scFv and IgG. The ScFv achieved maximum penetration within 30 min of administration, while the IgG took 48–96 h to achieve this degree of penetration. This is a very significant result, and one which complicates further the choice of most suitable fragment for tumour therapy. The ideal fragment would probably be as small as possible to give maximum tumour penetration, but give overall tumour loading equivalent to IgG. Tumour loading is determined partly by clearance time and partly by avidity. Because Fab and scFv fragments are monovalent they have antigen binding activity *in vitro* 7- to 10-fold lower than the bivalent IgG and $F(ab')_2$. The avidity disadvantage of Fab and scFv fragments can readily be overcome by engineering onto them a hinge sequence with a site for chemical cross-linking, and bivalent and trivalent forms of both Fab' and scFv have been made with this approach (Owens *et al.*, 1991b). The bivalent fragments have been shown to bind antigen as well as IgG *in vitro*, and both trivalent fragments to bind antigen approximately three-fold better than IgG.

PEGylation of several proteins has been shown to prolong half-life in the circulation and biological effectiveness in animal models and in man, as well as to reduce their immunogenicity (Nucci, Shorr and Abuchowski, 1991). PEGylation of a murine antibody has been shown to affect both clearance times and biodistribution for both IgG and a $F(ab')_2$ fragment (Kitamura *et al.*, 1991). The PEGylated $F(ab')_2$ showed reduced uptake by the kidney and liver, and its slower clearance gave higher tumour loading. It is conceivable that a PEGylated multi-scFv will ultimately prove to give the optimal combination of clearance time, avidity and tumour penetration.

Recent evidence implicates the CH2 domain in controlling clearance of IgGs. Deletion of this domain from a chimeric IgG1 antibody recognizing the ganglioside GD2 (which is expressed on human tumours of neuroectodermal origin) conferred upon the antibody pharmacokinetic properties in nude mice very similar to those reported for $F(ab')_2$ fragments, i.e. more rapid distribution from the blood followed by more rapid body clearance, and superior tumour:non-tumour ratios (Mueller, Reisfeld and Gillies, 1990). Although the CH2 deletion removes the only site in the antibody for N-linked glycosylation, Mueller and co-workers (Mueller, Reisfeld and Gillies, 1990) suggest it is unlikely that this is responsible for the dramatic difference in pharmacokinetics since a site-specific substitution of the glycosylated residue

(asparagine at position 297) in another chimeric antibody had no effect on its pharmacokinetics (Tao and Morrison, 1989). Mueller and co-workers therefore suggest that the CH2 deletion alters pharmacokinetics through conformational effects on the whole molecule. On the other hand an aglycosylated IgG4 variant of chimeric B72.3 has been found to be cleared faster than the glycosylated form in mice (Rhind *et al.*, 1990).

Therapeutic MAbs and MAb conjugates are most frequently administered i.v., which is clearly the preferred route for most therapies — certainly for therapy of most cancers, especially those for which the disease is disseminated. Intra-cavity, intra-tumoral, intra-lymphatic and intra-arterial administration are all feasible and are capable of giving much more limited biodistribution. Intra-peritoneal administration of MAb–isotope conjugates targeting antigens expressed on ovarian carcinoma is the best-studied example, and has been shown to give slower localization to the ovarian tumours but with greater penetration (Ward *et al.*, 1987).

In summary, it is clear that the use of antibody fragments is the most promising and generally applicable approach to manipulating pharmacokinetics and biodistribution of MAbs and MAb conjugates. It is extremely likely that for many therapies fragments will give superior therapeutic ratios, and this applies particularly to tumour therapy. Because the nude mouse xenograft model has only limited predictive value for the human clinical situation, it will be some years yet before optimal fragments are clearly identified.

Construction, expression and manufacture of antibody fragments

The advent of PCR technology has accelerated both the primary cloning of antibody variable sequences to make chimeric forms (as described above) and subsequent engineering of the genes for production of antibody fragments. The two most suitable expression systems for either small- or large-scale production of such fragments are mammalian cells and *E. coli*. Fabs, F(ab')$_2$s, Fvs and scFvs can be made in either system. High yields have been achieved for Fabs in both mammalian cells (King *et al.*, 1992a; Adair *et al.*, 1993) and *E. coli* (Better, Horwitz and Robinson, 1990; Carter *et al.*, 1992a). High yields of Fvs have been achieved in *E. coli* and low yields in mammalian cells (King *et al.*, 1992b, 1993; Adair *et al.*, 1993). For mammalian cells yields in excess of 100 mg l^{-1} of Fabs can routinely be achieved (H. Caskey-Finney, personal communication). Although yields of Fabs and Fvs in *E. coli* in excess of 500 mg l^{-1} have been reported (Better, Horwitz and Robinson, 1990; Carter *et al.*, 1992a) there is considerable variation in yield between fragments of different antibodies (Adair, Mountain, Weir *et al.*, manuscript in preparation). Overall *E. coli* is now the expression host of choice for Fvs and scFvs, on both small and large scale, while for Fabs the preferred system varies between antibodies.

Two types of *E. coli* expression system are in common use, giving either secretion or intracellular accumulation of product. Both types of system have been used to produce antibody fragments. Bird *et al.* (1988), Field, Yarranton

and Rees (1989) and Buchner and Rudolph (1991) describe examples of intracellular expression. As with most recombinant mammalian proteins produced intracellularly in *E. coli*, antibody fragments accumulate as insoluble inclusion bodies with the protein in a denatured and aggregated state. Because the refolding of such proteins to give material which is soluble and active in antigen binding is an inefficient process, secretion systems are preferred for the production of antibody fragments. The impact of the discovery that antibody fragments can readily be made in active form in such *E. coli* secretion systems has recently been reviewed (Pluckthun, 1991; Skerra, Pfitzinger and Pluckthun, 1991; Ward, 1992). A number of plasmid-based systems for secretion of antibody fragments from *E. coli* have been developed. The features required if such systems are to be suitable for large-scale manufacture are: a suitable signal sequence to direct the heavy and light chains of the fragments into the secretion pathway; a controllable promoter and efficient translation initiation region to direct transcription and translation; a strong transcription terminator to prevent read-through interference on plasmid genes involved in plasmid replication and stability; a plasmid replicon which is not prone to segregational or structural instability; a plasmid architecture which allows the expression of both chains of the Fab or scFv to be transcribed from the same promoter as a bi-cistronic mRNA; a host strain which is relatively resistant to cell lysis by the antibody fragments as they accumulate in the periplasm; and fermentation processes allowing controlled growth to high biomass, preferably in a defined medium and without the need for antibiotics to maintain the plasmid within the cells.

Secretion to the periplasm requires the use of a signal sequence (reviewed in Briggs and Gierasch, 1986) and although the secretion of an antibody light chain has been accomplished using its natural signal sequence (Zemel-Dreasen and Zamir, 1984), it is preferable to use one from an efficiently exported bacterial protein, such as *pelB* (Better *et al.*, 1988) *ompA* (Skerra and Pluckthun, 1989), *phoA* (Skerra dn Pluckthun, 1989), or *stll* (Carter *et al.*, 1992a). Secretion of heterologous proteins is deleterious for *E. coli* and leads to strong selection pressure in favour of cells which have lost the whole expression plasmid (segregational instability) or which have deletions of the antibody gene insert (structural instability). For this reason it is essential to use a tightly controlled promoter to prevent expression during most of the growth phase of the culture. Antibody fragments can readily be translocated across the inner membrane of *E. coli*, but they also have a tendency towards insolubility in the periplasm. The extent of this problem varies markedly between fragments of different antibodies and between different fragments of the same antibody. As fragments accumulate in the periplasm they tend to permeabilize the outer membrane and leak into the culture medium, along with other periplasmic proteins. At higher concentrations in the periplasm they cause cell lysis and release of many cellular proteins into the culture medium. It is highly advantageous to use promoters which allow partial induction to allow a production rate that is optimal for secretion and folding with minimal cell lysis. In most cases the best yields are given by sub-maximal production rates which allow continued cell growth after induction and allow

the most favourable balance between secretion and cell lysis. Promoters which have been used include: the *lac*UV5 promoter/operator (p/o; Back-mann, Ptashne and Gibert, 1976) controlled by IPTG induction; the wild-type *lac* p/o (Charnay *et al.*, 1978) controlled by IPTG induction (Skerra and Pluckthun, 1989); the *Salmonella typhimurium araB* promoter controlled by arabinose induction (Better *et al.*, 1988); the $\lambda O_R/P_R$ (Queen, 1983) controlled by temperature-induced inactivation of the λcl857 repressor (Colcher *et al.*, 1991); the *tac* promoter (Amann, Brosius and Ptashne, 1983) controlled by IPTG induction (King *et al.*, 1992b, 1993; Owens *et al.*, 1991 a, b; Yarranton and Mountain, 1992); the *phoA* promoter, which is induced by phosphate starvation (Carter *et al.*, 1992a); and the *trpE* p/o (Yarranton and Mountain, 1992) controlled by temperature-induced plasmid copy number increase which titrates the chromosomally expressed *trp* repressor (Wright, Humphreys and Yarranton, 1986). Of these the temperature and IPTG induced systems allow the most flexible control. (Yarranton and Mountain, 1992).

Transcription terminators which have been used include the *lpp* terminator (Skerra, Pfitzinger and Pluckthun, 1991), the *rrnB* terminator (King *et al.*, 1992b; Owens *et al.*, 1991a, b) and the phage λt_o terminator (Carter *et al.*, 1992a; Shalaby *et al.*, 1992). All of these give highly efficient termination. When mammalian genes fail to express or express poorly in *E. coli* the problem is very often caused by poor translation initiation. This is usually attributable to an unfavourable secondary structure in the mRNA which conceals the ribosome binding site and can be avoided by translational coupling of the antibody cistrons to an efficiently translated cistron (Yarranton and Mountain, 1992).

The secreted antibody fragments can conveniently be recovered in assembled, active form from either the periplasm or the culture medium. The former allows recovery in a smaller volume while the latter requires fewer steps. Much greater yields can be achieved in fermentors because they allow greater control over the parameters that govern cell physiology than shake flasks. For example Better, Horwitz and Robinson (1990) reported yield improvements for the production of cL6 Fab from 0.5–1.0 mg l^{-1} in shake flasks to 500 mg l^{-1} in fermentors. Structural and segregational instability have been shown to be a problem for high copy number expression plasmids such as the pUC-based vectors at fermentor scale. This is especially true when using a defined medium, which is advantageous for GMP processes because it obviates the need to demonstrate clearance of multiple media components, but which tends to exacerbate plasmid instability problems. Stability is a particularly important issue with large-scale production of fragments for clinical use, because demonstrating reproducible stability is an important requirement for GMP compliance. Dual origin vectors have been developed which allow cell growth to high biomass at low copy number with very tight control over fragment expression followed by induction of expression for the production phase by temperature increase (Yarranton and Mountain, 1992). A *tac* promoter-based vector has been developed which also allows very tight control over expression in the growth phase (Yarranton and Mountain, 1992).

For GMP compliance with the latter it is advantageous to induce expression by switching carbon source from glucose to lactose because IPTG is expensive and usually contains the carcinogenic substance dioxane, clearance of which must be demonstrated from the final antibody fragment product.

In summary, it is possible to make large quantities of antibody fragments suitable for clinical studies using either *E. coli* or mammalian cell systems. Development times are shorter for *E. coli* systems, which in the long term will also prove to give economic advantages on the very large scale required to satisfy large markets.

Approaches to cell killing with antibodies

Binding to or neutralizing the target antigen is not sufficient to give a therapeutic effect for many antibody-based therapies — killing the target cell is required. This is particularly the case for cancer therapy with antibodies, for which essentially five cell-killing strategies are being developed. Four of these involve respectively radioisotopes, protein toxins, low molecular weight cytotoxic drugs, and enzyme-activated prodrugs as the cell-killing agent. Because human tumours are usually poorly vascularized their uptake of MAbs or MAb conjugates is very low compared to the uptake observed in nude mice with human tumour xenografts. For this reason only extremely potent cell-killing agents can kill tumours to which they are targeted by anti-TAA antibodies. The fifth strategy involves using the antibody constant regions to recruit immune effector mechanisms to mediate cell killing.

RADIOISOTOPE CONJUGATES

The use of antibody–isotope conjugates for tumour imaging and therapy has a long history, the first human therapy studies being performed with ^{131}I-labelled polyclonal antibodies in 1957. It has proved much easier to deliver doses to the tumour which are adequate for tumour detection than to deliver doses which give therapeutic benefit, and the vast majority of clinical studies with antibody–isotope conjugates have been for radioimmunodetection (RAID). The present status of RAID has recently been reviewed (Goldenberg *et al.*, 1991a, b; Larson, 1991). Since this present review is concerned with therapy rather than diagnosis, we will not review RAID again here, but it is worth noting that RAID is now regarded as a very safe and valuable adjunct to more anatomical detection methods such as CT (computerized tomography) and MRI (magnetic resonance imaging), and that in many cases RAID has enabled the detection of occult tumours (i.e. otherwise undetectable). The use of engineered antibody fragments with superior pharmacokinetics, together with the more widespread use of more suitable isotopes (such as ^{99m}Tc) is very likely to improve RAID further in the next few years, and several ^{111}In and ^{99m}Tc-antibody conjugates are close to being launched as approved products for tumour imaging.

The selection of isotopes for therapy is a very complicated issue, depending largely on the following factors: the size of tumours being targeted; the range

and intensity of the radiation delivered; the ease with which the cell can repair the DNA damage caused by the radiation (and which is the means of cell killing); the availability of suitably pure isotope; the availability of chelators and conjugation technology giving adequate *in vivo* stability for the conjugate and biodistribution of the isotope; and the ease of patient handling. Thus, for example, β-emitters such as ^{131}I and ^{90}Y have relatively long effective path lengths and are capable of killing adjacent tumour cells which do not express the antigen or are inaccessible. This so-called 'cross-fire' or 'bystander killing' effect is an important advantage of isotope over other cell-killing agents. For killing individual cells or micrometastases α-emitters such as ^{211}At, ^{212}Bi or ^{212}Pb have the theoretical advantage of delivering a higher energy dose with a shorter path length, but these isotopes are not yet readily available. Similarly ^{67}Cu is a high-energy β-emitter with a path length between ^{131}I and ^{90}Y which is also unavailable. Considering all these factors, the most promising isotopes at the present time are ^{131}I, and the longer range β-emitters ^{90}Y and ^{188}Re, which have path lengths in the millimetre range. The great majority of clinical therapy studies with antibody–isotope conjugates thus far have used ^{131}I, largely because of its availability and simple conjugation methodology. ^{90}Y offers the advantages of greater energy per emission, greater β-component, more suitable half-life and easier patient handling, but the disadvantage of requiring much more efficient chelators because it is a bone-seeking isotope. Suitable macrocyclic chelators have now been developed (Farnsworth *et al.*, 1991; Maraveyas and Epenetos, 1991; Beeley and Ansell, 1992) and ^{90}Y is likely to prove the most effective isotope for antibody-based therapy over the next few years.

Radioisotopes are usually linked to antibodies by random attachment after modification of the antibodies at various amino acid side chains (reviewed by Beeley and Ansell, 1992). Site-specific attachment has been accomplished, however, by engineering suitable residues into the antibody in the desired positions. For example several IgG4 chimeric variants of B72.3 have been constructed with surface serine or threonine residues in the CH1 domain substituted by cysteines (Lyons *et al.*, 1990). All these variants could be expressed in mammalian cells with appropriate folding and assembly, and those with introduced cysteines in concave regions of the antibody surface could be labelled site-specifically by conjugating ^{125}I via a maleimide linkage to the thiols. The resulting conjugates showed localization to tumours in tumour-bearing nude mice (Lyons *et al.*, 1990). In an alternative approach site-specific labelling with ^{111}In or ^{90}Y at the hinge region has been accomplished by incorporating an appropriate macrocycle chelator into a bifunctional maleimide linker used to chemically cross-link two chimeric B72.3 Fabs to give a F(ab)$_2$ (Rhind, 1990; Rhind, Millican and Millar, 1990). The hinge region in this case was derived originally from an IgG4 antibody and contained two cysteines. One of these cysteines was substituted with a serine to reduce the complexity of the cross-linked Fabs. The resulting chemically cross-linked F(ab)$_2$ showed slower blood clearance than the normal F(ab)$_2$ cross-linked by disulphide bonds (D.J. King *et al.*, unpublished observations).

Very few data are available yet on the use of humanized antibody–isotope

conjugates for cancer therapy. Recently, however, encouraging data have emerged from early clinical studies with murine antibodies on patients with haematologic malignancies, i.e. leukaemia and lymphoma. From the viewpoint of therapy with antibody–isotope conjugates such cancer types have the advantage of much easier access for the conjugate to the tumour cells than is the case for solid tumours, but the disadvantage that the target cells are located largely within the most radiation-sensitive normal organ, the bone marrow. Tumour regressions have been observed in one clinical study of T-cell lymphoma (Rosen *et al.*, 1987) and four of B-cell lymphoma with murine MAbs carrying [131]I. The B-cell lymphoma trials concerned the MAbs MB1 (Eary *et al.*, 1990), OKB7 (Scheinberg *et al.*, 1990; Czuczman *et al.*, 1991), Lym-1 (de Nardo *et al.*, 1988) and LL2 (Goldenberg *et al.*, 1991a, b). Some of these remissions occurred with doses of the conjugate as low as 6 mCi (Goldenberg *et al.*, 1991a, b). In one study complete remissions lasting 4–29 months have been observed (Bernstein *et al.*, 1991). All of these patients also suffered myelosuppression and most were given infusions of bone marrow cells. No toxicity in organs other than the bone marrow was observed, suggesting higher doses could be administered. In a Phase I study of acute myelogenous leukaemia (AML) administration of an ^{131}I conjugate of a murine antibody recognizing CD33 on the surface of leukaemic blast cells was shown to give very rapid and effective imaging of all areas of leukaemia involvement at low doses. In a dose escalation study high doses were shown to kill 99% of the leukaemic blast cells but also gave complete bone marrow ablation resulting in the need for bone marrow transplants (Scheinberg *et al.*, 1991). Killing this proportion of the leukaemic cells was not sufficient to be curative — the patients relapsed after about 3 months and it was not possible to administer further doses because of HAMA responses to the murine antibody. For these haematologic malignancies it is very likely that MAb conjugates with short path length isotopes such as α-emitters and Auger electron emitters, or cytotoxic drugs, would be more likely to permit killing of the cancer cells without simultaneous destruction of normal bone marrow cells.

For the major solid tumour types (lung, breast, colorectal and prostate cancer) there are few reports yet of tumour responses in patients administered antibody–isotope conjugates. Partial responses have been achieved for 50% of hepatoma patients with a multi-modality therapy which involved external beam irradiation, chemotherapy and ^{131}I-labelled anti-ferritin antibody, which gives very high uptake by the liver (Order *et al.*, 1985). Perhaps the most interesting data so far concern a Phase I study of metastatic colorectal carcinoma with ^{131}I-labelled CC49 as sole therapy (Divgi *et al.*, 1992). In five out of 18 patients given relatively low doses the treatment gave disease stabilization, and one of these had a minor tumour regression.

There are several cases for which tumour loading data in patients indicate that a single or very few administrations of ^{131}I-labelled murine whole antibody have delivered a dose of 10–20 Gy to the tumour (Langmuir, 1992). Although different solid tumour types differ in their radiosensitivity, it has been calculated on the basis of external beam irradiation that an average

tumour killing dose will be around 60 Gy, and that the maximum tolerated dose to the bone marrow is around 3 Gy (Mach, Pelegrin and Buchegger, 1991). It is likely that for many solid tumour types MAbs will eventually prove to be capable of delivering a killing dose to the tumour without serious damage to the bone marrow, given that humanized antibodies will allow repeat dosing, that antibody fragments will allow superior tumour:non-tumour dose ratios and tumour penetration, that new developments in chelator technology now allow the use of superior isotopes such as [90]Y. In all the clinical studies with ^{90}Y-MAb conjugates until very recently release of the isotope from the conjugate has led to low dose limitation through myelosup-pression (Order et al., 1988; Haller et al., 1992; Hird et al., 1991a). It is also likely that tumour loading and consequently efficacy with MAb conjugates in patients will improve through the use of MAbs which recognize antigens that are more highly and more homogeneously expressed than many of those used so far. It is now much more widely accepted that such MAb conjugates are more likely to be of clinical benefit in patients with minimal residual and disseminated disease (i.e. after removal of most of the tumour bulk by surgery or other means) rather than in patients with large solid tumours. Other approaches being taken to improve therapeutic ratios for antibody–iso-tope conjugates include radioprotection of bone marrow by systemic injection of cytokines (Neta, Douches and Oppenheim, 1986) and increasing tumour blood flow and vascular permeability to improve tumour loading (Jain, 1990).

PROTEIN TOXIN CONJUGATES

Several highly potent protein toxins are being evaluated as cell-killing agents targeted to cancer cells by MAbs. The most notable are the bacterial toxins Diphtheria toxin (DTX) and Pseudomonas exotoxin (PE), and the plant toxins ricin and abrin. DTX and PE inactivate the translation elongation factor EF-2, while ricin and abrin are enzymes that inactivate ribosomes. This mode of cell killing requires internalization of the toxins into the target cell, which restricts the selection of the MAb part of the immunotoxins to those which recognize internalizing antigens. It has been estimated that less than 10 molecules of ricin in the cytoplasm is sufficient to kill the cell, but many more must be targeted to the cell surface because internalization and translocation are inefficient. Immunotoxins have been described which are intended for therapy of most human cancers and which show potent and selective killing of tumour cells in vitro and in animal models (reviewed by Wawrzynczak, 1991). Several are undergoing clinical evaluation.

   Although immunotoxins are a simple and attractive concept, the first generation of them have proved relatively ineffective in patients. Several problems have been encountered, notably that of achieving adequate target selectivity. Toxins usually have subunits or domains which are responsible for non-specific cell binding and which must be removed to avoid killing non-target cells. Removal of the ricin B chain, however, to reduce non-specific binding, also reduces the capacity of the A chain to translocate across the vesicle of the endosome and therefore reduces potency. The carbohydrate

components of plant toxins also promote binding to cells of the RES. Such toxins may require to be unglycosylated to avoid rapid clearance and killing of non-target cells. Another problem encountered with immunotoxins in patients has been instability *in vivo* of the linkage, usually disulphide bonds, between MAb and toxin. This problem has been partially solved by introducing methyl or phenyl groups adjacent to the disulphides to restrict access to them, or by expressing the antibody–toxin as a genetic fusion, i.e. with co-expression of the toxin and one antibody chain as a single polypeptide.

Promising results have been achieved for several immunotoxins in animal models, with treatments delaying tumour appearance and growth rate and in some cases giving complete cures. The performance of immunotoxins in clinical studies has, however, been disappointing. In the first 10 clinical trials 127 patients with ovarian, breast, colorectal or lymphoid tumours were treated, but only two complete remissions and eight partial remissions were observed (Waldmann, 1991b). Serious toxicity was frequently observed, most often a capillary leak syndrome. In some breast and ovarian trials severe neurological toxicity occurred because of cross-reactivity of the MAb with a central nervous system (CNS) antigen (Gould *et al.*, 1989). More recent trials with modified immunotoxins are giving somewhat more promising results: 60–70% of B-cell lymphoma patients with minimal residual disease showed partial or transient responses when administered an anti-CD19 MAb conjugated to ricin with galactose-binding sites sterically blocked (Grossbard *et al.*, 1992); 22 of 32 evaluable patients with GVHD showed a favourable early response in at least one organ when administered an anti-CD5 MAb conjugated to ricin A chain (Byers *et al.*, 1990); and about 45% of B-cell lymphoma patients administered an anti-CD22 MAb conjugated to ricin A chain showed partial responses (Thorpe, 1992). Although much effort is presently going into engineering toxins to give superior combinations of specificity and potency, most immunotoxins have a very serious long-term drawback — they are foreign proteins which are highly immunogenic in patients. Indeed such immunogenicity has already been reported to limit the effectiveness of ricin immunotoxins in clinical studies (Thorpe, 1992). Effective therapy with such immunotoxins will be restricted to indications for which a single dose is effective unless their immunogenicity can be overcome by co-treatment with immunosuppressive agents, by PEGylation, or by removing immunodominant epitopes through protein engineering techniques without compromising potency. Recent studies suggest it may be possible instead to use a human protein as a toxin, namely a human RNase which kills cells only after internalization and which will hopefully not be immunogenic in patients (Rybak *et al.*, 1992).

DRUG CONJUGATES

Cytotoxic and cytostatic drugs have been in clinical use as anti-cancer agents for about 50 years now. Until the recent advent of MAbs, MAb–conjugates and 'biological' therapeutic agents (e.g. cytokines) such drugs represented the only systemic treatment for patients with widely disseminated metastatic

disease. The limitation of such drugs is their very poor selectivity for tumour cells over other rapidly dividing cells, especially in the bone marrow, which is usually the organ giving dose-limiting toxicity. It is for this reason that there has been little improvement in clinical results with chemotherapy over the last 25 years. Improving the therapeutic ratio for such drugs by targeting them to tumour cells with MAbs in a form which is inactive until internalized into the target cells is therefore a very attractive concept. Intensive efforts have been made over the last 5–10 years to develop such MAb–drug conjugates for clinical evaluation. Antibody conjugates with many different drugs, including methotrexate, chlorambucil, adriamycin, daunorubicin, vindesine, α-amanitin, mitomycin C and neocarzinostatin have been shown to give targeted cell killing *in vitro* and in some cases in animal tumour models. As yet, though, there are very few reports of clinical studies with such conjugates and no tumour responses have been achieved (Starling and Mastro, 1992). The main problems have been firstly in identifying drugs which are sufficiently potent to kill cells, given the inefficiency of antibody uptake by human solid tumours and that only a minor proportion of the activated drug reaches its intracellular site of action, and secondly in identifying linkers for conjugating drug onto MAbs to give linkages which are stable extracellularly in the circulation but are readily cleaved after uptake of the conjugate to liberate the active form of the drug.

In the last few years several types of cytotoxic drug have been identified which have greatly superior potency than the commonly used chemotherapeutic agents and which may prove to be more suitable for MAb-targeted cancer therapy. These are principally the enedienes (reviewed by Nicolaou *et al.*, 1992), the maytansinoids (Chari *et al.*, 1992) and the tricothecenes (Liu, 1989). The enedienes kill cells by damaging DNA and must therefore be trafficked to the nucleus after uptake of the conjugate, while the maytansinoids block microtubule polymerization and depolymerization, and the tricothecenes are inhibitors of protein synthesis. Some of these new cytotoxic agents are as potent as ricin, but since they are of much lower molecular weight they will be much less immunogenic, and also much less likely to be inactivated in endosomes. Like the immunotoxins, cell killing by MAb conjugates with such agents requires internalization into the target cells, which will restrict the choice of antigen somewhat. Suitable linkages and procedures for MAb conjugation have been developed for one of the most promising of these new agents, the enediene calicheamicin (Lee *et al.*, 1987a, b), which cleaves DNA after binding in the minor groove (Drak *et al.*, 1991). Very promising tumour regression results have been obtained in animal models for internalizing MAbs carrying calicheamicin, with complete cures of large tumours, long-term survival and therapeutic ratios five-fold better than can be achieved with non-targeted cytotoxic agents (Hinman *et al.*, 1993). Clinical studies with AML and ovarian cancer patients using these agents are beginning in 1993.

ANTIBODY-DIRECTED ENZYME PRODRUG THERAPY

ADEPT (antibody-directed enzyme prodrug therapy, also called 'ISEC' or immunosensitized enzymatic chemotherapy) is an another approach which aims to use antibody targeting to improve therapeutic ratios for cytotoxic agents. In ADEPT the MAb is used to target an enzyme to tumours which is capable of converting a non-toxic prodrug administered systemically into an active form at the tumour site (Searle *et al.*, 1986). This is an elegant concept for which the advantages are that lower tumour loading may be required because the enzyme acts catalytically, and that unlike MAb–drug conjugates bystander cell killing should occur. The drawbacks are that active drug is generated outside the cell where it can diffuse away and also kill non-target cells, and that to avoid toxicity the enzyme must not occur naturally in humans, or at least not in extracellular locations. The only enzymes used successfully in animal models so far have been bacterial ones such as carboxypeptidase G2 (Searle *et al.*, 1986; Bagshawe *et al.*, 1992) or alkaline phosphatase (Senter *et al.*, 1988), which are highly immunogenic in man. Complete tumour eradication in animals has been achieved with this approach (Senter *et al.*, 1988). The first clinical study with this approach has used a $F(ab')_2$ fragment of a murine anti-CEA antibody conjugated to carboxypeptidase G2 to give activation of a prodrug to an alkylating agent (Bagshawe *et al.*, 1992). After administration of the antibody–enzyme fusion sufficient time was allowed to give good tumour loading, then before administration of the prodrug a galactosylated anti-enzyme antibody was used to clear the fusion from the circulation rapidly through the liver. Some objective radiologic responses were reported but immune responses to both antibody and enzyme appeared very rapidly. ADEPT, like immunotoxin therapy, will require serious immunogenicity problems to be overcome to stand a realistic chance of success in the clinic.

RECRUITMENT OF IMMUNE EFFECTOR FUNCTIONS

Naturally occurring IgG antibodies can be regarded as bifunctional agents, with the N-terminal variable region responsible for antigen binding and sequences within the C-terminal part responsible for determining interactions with the various cell types which participate in immune responses. Recognition of these effector signals on antibodies by specific cell surface receptors on cytotoxic cells (lymphokine-activated killer cells, monocytes, granulocytes and macrophages) can result in antibody-dependent cellular cytotoxicity (ADCC). Recognition of other effector signals by components of the complement cascade can lead to antibody-dependent complement-mediated cytotoxicity (ADCMC). Both ADCC and ADCMC can result in killing of the cells presenting the antigen. Various strategies are being evaluated to harness these effector functions, especially for cancer therapy. One attraction of these approaches is that naked antibodies are much simpler and cheaper to produce than MAb–cytotoxic agent conjugates. Most rodent MAbs are very inefficient at recruiting human effector functions, though mouse IgG2as and rat

IgG2bs are more efficient than other isotypes. Considerable effort has been put into comparing the human IgG isotypes for ability to mediate cell killing by ADCC and ADCMC, especially since recombinant DNA technology has allowed chimeric or fully humanized isotype series to be constructed and prepared (Bruggeman *et al.*, 1987; Bindon *et al.*, 1988; Bindon, Hale and Waldmann, 1990; Lund *et al.*, 1990, 1991, 1992; Canfield and Morrison, 1991; Sandlie and Michaelsen, 1991). For technical reasons it is difficult to compare the results of different studies on *in vitro* cell killing, but the data are consistent with the conclusion that antibodies with the human IgG1 isotype are most efficient at mediating ADCC, with IgG3s less efficient and IgG2s and IgG4s very inefficient. The human IgG1 and IgG3 isotypes both also appear to be much more effective in mediating ADCMC than IgG2s or IgG4s, at least under conditions of low antigen density. In general the ability of MAbs to mediate ADCMC depends very significantly on antigen access and density. It is likely that the constitution of the hinge, its interaction with the CH1 domain and the segmental flexibility this allows partly determine efficiency in mediating ADCMC . Alterations or removal of the carbohydrate portions of IgGs have been shown to diminish both ADCC (Dorai *et al.*, 1991; Sarmay *et al.*, 1992) and ADCMC (Dorai *et al.*, 1991; Tao and Morrison, 1989). Some types of target cell may be able to prevent lysis through ADCMC even when complement is loaded on the cell surface — proteins in the regulator of complement activation cluster (Liszewski, Post and Atkinson, 1991) and homologous restriction factors (Brooimans *et al.*, 1992) appear to inhibit the process. It is therefore difficult to predict whether a given humanized antibody will efficiently mediate ADCMC in patients. Nevertheless it can be concluded that therapeutic strategies with naked humanized MAbs which rely on recruitment of immune effector mechanisms for cell killing are much more likely to succeed if IgG1 or IgG3 constant regions are employed. In practice most groups choose those of IgG1s because IgG3s are difficult to purify, tend to aggregate *in vitro* and can be difficult to handle. The humanized IgG1 versions of the CAMPATH-1, L6 and anti-Tac MAbs have been shown to mediate ADCC more effectively than the parent rodent antibodies (see below). In cases where interaction with effector cells is not desired, then IgG2 or IgG4 should be used. Once the choice of isotype is made, then the allotype predominating in the patient group representing the major market should also be chosen.

A second approach to the exploitation of ADCC involves so-called 'bispecific' or 'bifunctional' antibodies which link the target cell directly to trigger molecules on the effector cells (reviewed in Fanger and Guyre, 1991), and which are under development for treatment of cancer and infectious diseases. To be effective the bispecific antibodies must retarget the cytotoxic cells from their natural ligands to those identified by the MAb, and must activate the cytotoxic cells without the normal histocompatibility complex (MHC). The most promising candidates aim to recruit cytotoxic T-cells by binding to their CD3 antigen, or to recruit natural killer cells by binding to their CD16 FcγRIII receptor. In the case of T-cell recruitment it is hoped that in addition to direct killing of cells presenting the TAA there may be release

of cytokines from the T-cells to give therapeutically beneficial effects on tumour cells which are sterically inaccessible to the antibody. Bispecific MAbs with one antigen binding site for either of these specificities together with one for a TAA have been shown to give killing of tumour cells *in vitro* by peripheral blood mononuclear cells, and to give regression of human tumours in nude mouse models (Garrido *et al.*, 1990; Nelson *et al.*, 1990; Mezzanzza-nica *et al.*, 1991; Damanet *et al.*, 1991). Encouraging results have also been obtained on killing of HIV–infected cells *in vitro* with this approach (Zarling *et al.*, 1988). Although bispecific MAbs are a recent development they have been used in several Phase I clinical studies, with interesting results. In one trial infusions of activated T-lymphocytes targeted with anti-CD3/anti-TAA antibodies gave considerable local lysis of lung, ovarian or breast tumor cells (DeLeij *et al.*, 1991). In another, IL2-activated PBLs targeted by an anti-CD3/anti-TAA antibody were administered intracranially to glioma patients (Nitta *et al.*, 1990). After two years 76% of patients treated with the antibody-targeted lymphocytes were tumour free, compared to 33% of those given non-targeted lymphocytes.

Bispecific antibodies can be produced by chemical cross-linking of two monovalent Fab fragments or by fusion of the two parent hybridomas to give 'quadromas', (though the latter are usually highly unstable). Potentially the bispecific antibody approach is capable of recruiting cytotoxic cells more efficiently than monospecific IgG1s, but an obvious drawback is the drop in avidity for tumour cells which must result from monovalent binding to the TAA. A second approach to improving the efficiency of cytotoxic cell recruitment to tumours involves using anti-TAA antibodies conjugated to or genetically fused to cytokines. Such conjugates or fusions have been gener-ated for the human cytokines IL-2, IL-5 and IL-8, but the best studied cases concern IL-2. Antibody targeting of IL-2 to tumour cells has been shown to stimulate killing of those cells *in vitro* by cytotoxic T-cells (Fell *et al.*, 1991; Gillies *et al.*, 1992). A third variant involves co-administration of MAb and cytokines. It is likely, though, that the usefulness of such approaches in patients will be limited by the systemic toxicity of the IL-2 portion of the molecules.

Chimeric antibodies in research and clinical development

In the first half of this review we have covered the design, construction and production of engineered antibodies for therapy. In this second half we review the construction, evaluation *in vitro* and in animals, and evaluation in early clinical studies, of six rodent–human chimeric antibodies. Four of these (17-1A, B72.3, 14.18 and L6) are anti-tumour antibodies, and the other two (SK3 and M-T412) anti-CD4 antibodies. The studies reported illustrate the various approaches that have been taken to generate useful forms of chimeric antibodies and most of the pertinent problems and issues involved in designing and producing MAb-based therapeutics. We also summarize in *Tables 2, 3* and *4* information on the other chimeric antibodies which were in the literature but not in clinical studies at the time of writing. We then survey

the development of more fully humanized antibodies, thirteen in total, for which pre-clinical and clinical data have been disclosed to date.

17-1A

Studies with 17-1A focused largely on isotype choice for efficient recruitment of ADCC, and the conclusions drawn have influenced this decision for many other MAbs. MAb17-1A was one of the earliest MAbs generated for diagnostic and therapeutic purposes (reviewed in Herlyn *et al.*, 1986). The MAb is a murine IgG2a/κ, recognizes a 33 kDa protein antigen on adenocarcinoma cells of gastro-intestinal origin (Gottlinger *et al.*, 1986) and was originally raised by immunization of mice with SW1083 carcinoma cells. The antibody has a relatively low affinity of 0.7×10^8 M^{-1} for antigen presented on the SW948 carcinoma. The antigen is not shed *in vitro* by carcinoma cells, unlike, for example, CEA-bearing cells. The antibody has been shown to inhibit tumour growth in nude mice bearing SW948 xenografts, an effect most probably mediated by murine macrophages (Herlyn *et al.*, 1980). The MAb has been used to image tumours in patients (Herlyn *et al.*, 1983; Schlimock *et al.*, 1987) and also with therapeutic intention (Koprowski *et al.*, 1984; Sears *et al.*, 1984; Khazaeli *et al.*, 1988; LoBuglio *et al.*, 1988; Frodin, Lefvert and Mellstedt, 1990). In all of the studies most of the patients developed a HAMA response of IgG type and most also showed an IgM response. High doses of antibody led to reduced HAMA but not to tolerance for the MAb (Sears *et al.*, 1987). In the high dose studies the HAMA response did not significantly affect the β half-life of the MAb.

Construction and expression of chimeric antibodies

Initially an IgG3 chimera (c17-1A(γ3)) was constructed (Sun *et al.*, 1986, 1987). The heavy and light chain genes were isolated from a cDNA library and used as probes to isolate the corresponding genomic sequences. The genomic light chain variable gene with the Ig promoter was cloned 5′ to the human κ constant region in a pSV2*neo*-drived vector. The heavy chain variable gene and Ig promoter was cloned into a pSV2*gpt*-derived vector 5′ to the human IgG3 constant region sequences. The vectors were co-introduced into Sp2/0 cells by protoplast fusion and a cell line, SG3/5, was identified which produced 20 mg l^{-1} of antibody. Subsequently the IgG1, IgG2 and IgG4 isotypes were constructed and expressed in Sp2/0 cells (Steplewski *et al.*, 1988) with production in the 10–15 mg l^{-1} range. Subsequently Fogler *et al.* (1989) prepared a chimeric IgM form of 17-1A. The genomic heavy variable region was linked to the genomic Igμ constant region sequences, including the membrane exons, and cloned in a pSV2*gpt*-derived vector. The chimeric light chain in the expression vector as described above and the IgM vector were introduced into Sp2/0 cells sequentially, light chain first, by electroporation. A yield of 5 mg l^{-1} of antibody was produced from stable cell lines.

Table 2A. Chimeric and humanized model antibodies*

Name	Specificity	Human isotype	Reference
Mouse-human chimeric model antibodies			
S107	PC	IgG1, 2	Morrison *et al.* (1984), Tan, Oi and Morrison (1985)
Sp6	TNP	IgM	Boulianne, Hozumi and Shulman (1984), Boulianne *et al.* (1987)
B1.8	NP	IgG1, 2, 3, 4, IgE, IgM IgA, IgD	Neuberger *et al.* (1985), Bruggemann *et al.* (1987; 1989b), Bindon *et al.* (1988, 1990), Duncan and Winter (1988), Duncan *et al.* (1988), Garred, Michaelsen and Aase (1989), Sandlie *et al.* (1989), Walker *et al.* (1989a, b), Garred *et al.* (1990), Jefferis, Lund and Pound (1990), Lund *et al.* (1990, 1991, 1992), Michaelsen, Garred and Aase, (1991), Sandlie and Michaelsen (1991), Michaelsen *et al.* (1992) Sarmay *et al.* (1992)
TEPC15	TEPC15	IgG1	Takeda *et al.* (1985)
DNS1	DNS	IgG1, 2, 3, 4, IgE	Dangl *et al.* (1988), Tao and Morrison, (1989), Shopes *et al.* (1990), Tan *et al.* (1990), Weetall *et al.* (1990), Canfield and Morrison (1991), Tao, Canfield and Morrison (1991), Shin *et al.* (1992)
T14, T17	TGAL	IgG1	Horgan, Brown and Pincus (1990)
Humanized model antibodies			
B1.8	NP	IgG2	Jones *et al.* (1986)
D1.3	Hen egg lysozyme	IgG1	Riechmann, Foote and Winter (1988), Verhoeyen, Milstein and Winter (1988), Foote and Winter (1992)

* Murine unless stated.

Engineering antibodies for therapy

49

Celltrion, Inc., Exhibit 1067

Table 2B. Chimeric and humanized therapeutic antibodies*

Name	Isotype[†]	Specificity	Human isotype	Reference
Mouse-human chimeric therapeutic antibodies				
17-1A	IgG2a/κ	Carcinoma associated antigen	IgG1, 2, 3, 4/κ IgM/κ	Sun *et al.* (1986, 1987), Shaw *et al.* (1987, 1988), Masucci *et al.* (1988, 1990), Shaw, Khazaeli and LoBuglio (1988), Steplewski *et al.* (1988), Fogler *et al.* (1989), LoBuglio *et al.* (1989), Buchsbaum *et al.* (1990), Trang et al. (1990), Masucci and Mellstedt (1991), Meredith *et al.* (1991, 1992a)
B6.2	IgG1/κ	Colorectal carcinoma	IgG1/κ	Sahagan *et al.* (1986), Brown *et al.* (1987)
L6	IgG2a/κ	Pancarcinoma	IgG1/κ	Liu *et al.* (1987b), Hellstrom *et al.* (1990), DeNardo *et al.* (1991a, b, c), Goodman (1991)
B72.3	IgG1/κ	TAG-72 (mucin)	IgG4/κ	Whittle *et al.* (1987), Colcher *et al.* (1989), Begent *et al.* (1990), Baker *et al.* (1991), LoBuglio *et al.* (1991), Owens *et al.* (1991a, b), Khazaeli *et al.* (1992) King *et al.* (1992a, b, 1993), Meredith *et al.* (1992a, b), Adair *et al.* (1993), Angal *et al.* (1993)
			IgG1/κ, IgG4/κ	Primus *et al.* (1990), Hutzell *et al.* (1991)
			IgG1, 2, 3 ,4/κ	A. Phipps *et al.* unpublished.
CEM231.6.7	IgG1/κ	CEA	IgG1/κ	Beidler *et al.* (1988)
CE25/B7	IgG1/κ	CEA	IgG4/κ	Bischoff-Delaloye *et al.* (1989a, b), Hardman *et al.* (1989)
T84.66	IgG1/κ	CEA	IgG1/κ	Neumaier *et al.* (1990)
2.7.1G.10	IgG1/κ	CEA	IgG1/κ	Koga *et al.* (1990)
MBr1	IgM/κ	Mammary carcinoma	IgG1/κ	Orlandi *et al.* (1989, 1991)
NRML-05	n.s.	Melanoma associated antigen	IgG4/κ	Marchitto *et al.* (1989)
14.18	IgG3/κ	Disialoganglioside GD2	IgG1/κ	Gillies, Lo and Wesolowski (1989), Gillies *et al.* (1989, 1991, 1992), Gillies and Wesolowski (1990), Mueller *et al.* (1990), Mueller, Reisfeld and Gillies (1990), Barker *et al.* (1991), Dorai *et al.* (1991), Saleh *et al.* (1992a)
MRK16	IgG2a/κ	Multi-drug transporter P-glycoprotein	IgG1/κ	Hamada *et al.* (1990)
BA-Br1	IgG1/κ	Pan-adenocarcinoma	IgG1/κ	Hank *et al.* (1990), Liao *et al.* (1990), Robinson *et al.* (1991)
BA-Br3	IgG1/λ	Carcinoma	IgG1/λ	Robinson *et al.* (1991)

YH206	IgM/κ	Adenocarcinoma associated antigen	IgG1/κ	Nakashima, Nishimura and Watanabe (1991)
KM-10	IgG1/κ	Pan-carcinoma	IgG1/κ	Better, Horwitz and Robinson (1990)
NL-1	IgG2a/κ	cALLA (CD10)	IgG1/κ	Nishimura et al. (1987), Yokoyama, Nishimura and Watanabe (1987), Saga et al. (1990)
2H7	IgG2b/κ	CD20	IgG1/κ	Liu et al. (1987c)
RFT2	IgG2a/κ	CD7	IgG1/κ	Heinrich et al. (1989), Akbar et al. (1991)
E6	IgG1/κ	Transferrin receptor (CD71)	IgG1/κ	Hoogenboom et al. (1990)
SK3	IgG2a/κ	CD4	IgG1/κ	Knox et al. (1991)
BAT123	IgG1/κ	HTLV-III$_B$ gp120	IgG1/κ	Liou et al. (1989), Sun et al. (1991)
2H1	n.s.	HbsAg	IgG1/κ	Li et al. (1990)
15-C5	IgG1/κ	Human fibrin fragment D	igG1/κ	Bulens et al. (1991)
M-T412	IgG2a/κ	CD4	IgG1/κ	Choy et al. (1991), Ghrayeb, Knight and Looney (1991), Prinz et al. (1991)
M-T151	IgG2a/κ	CD4	IgG1/κ	Weissenhorn et al. (1991)
AHT107	IgG1/κ	IL2 receptor p55 protein (CD25)	IgG1/κ	Rose et al. (1992a)
AHT54	IgG1/κ	IL2 receptor p55 protein(CD25)	IgG1/κ	Rose et al. (1992b)

Continued

Engineering antibodies for therapy 51

Table 2B continued

Name	Isotype[†]	Specificity	Human isotype	Reference
Humanized therapeutic antibodies				
YTH34.5HL	IgG2b/κ(rat)	CAMPATH-1 (CDw52)	IgG1/κ	Hale *et al.* (1988), Riechmann *et al.* (1988), Mathieson *et al.* (1990), Page and Sydenham (1991), Clark (1992), Crowe (1992), Isaacs *et al.* (1992)
Anti-Tac	IgG2a/κ	IL2 receptor p55 protein(CD25)	IgG1/κ	Queen *et al.* (1989), Queen and Selick (1990), Brown *et al.* (1991), Hakimi *et al.* (1991), Junghans *et al.* (1991)
HuRSV19	IgG2a/κ	Respiratory syncytial virus glycoprotein	IgG1/κ	Taylor *et al.* (1991), Tempest *et al.* (1991)
Fd79	IgG1/κ	Herpes simplex virus glycoprotein B	IgG1/κ	Co *et al.* (1991)
Fd138-80	IgG2a/κ	Herpes simplex virus glycoprotein D	IgG1/κ	Co *et al.* (1991)
BMA031	IgG2b/κ	T cell Receptor α/β	IgG1/κ	Kurrle *et al.* (1990), Shearman *et al.* (1991a, b)
H17E2	IgG1/κ	PLAP	IgG1/κ	Verhoeyen *et al.* (1991)
Campath-9	IgG2b/κ(rat)	CD4	IgG1/κ	Gorman *et al.* (1991)
1B4	IgG2a/κ	CD18	IgG1/κ	DeMartino *et al.* (1991), Daugherty *et al.* (1991), Law, Mark and Williamson (1991)
0.5β	IgG1/κ	HIV gp120	IgG1/κ	Maeda *et al.* (1991)
425	IgG2a/κ	hEGFR	IgG1/κ	Kettleborough *et al.* (1991)
YTH12.5	IgG2b/λ(rat)	CD3	IgG1/λ	Routledge *et al.* (1991)
M195	IgG2a/κ	CD33	IgG1, 3/κ	Co *et al.* (1992)
BW431/26	IgG1/κ	CEA	IgG3/κ	Gussow and Seemann (1991), Bosslet *et al.* (1992)
4D5	IgG2a/κ	p185[HER2]	IgG1, Fab', Fab/κ	Carter *et al.* (1992a, b), Kelley *et al.* (1992), Shalaby *et al.* (1992)
UCHT1	IgG2a/κ	CD3	Fab/κ	Shalaby *et al.* (1992)
OKT3	IgG2a/κ	CD3	IgG4/κ	Adair, Emtage and Athwal (1991), Jolliffe *et al.* (1991), Alegre *et al.* (1992), Woodle *et al.* (1992)

[*] Murine unless stated.
[†] n.s., not stated.

Table 3. Expression of chimeric and humanized therapeutic antibodies*

Name	Isotype	Gene format	Vector format	Promoter	Transfection procedure	Host	Amplification	Yield from cell line
Expression of chimeric antibodies								
17-1A	IgG1, 2 3, 4	Genomic	Separate	Ig	Protoplast fusion/ Co-transfection	Sp2/0	No	10-20 mg l^{-1}
	IgM	Genomic	Separate	Ig	Electroporation/ Sequential	Sp2/0	No	5 mg l^{-1}
B72.3	IgG1	Genomic	Separate	Ig	Electroporation/ Sequential	Sp2/0	No	10-20 mg l^{-1}
	IgG1	Genomic	Single	Ig	Protoplast fusion	Sp2/0	No	50-60 mg l^{-1}
	IgG1	Genomic	Single	Ig	Protoplast fusion	Sp2/0	Yes (DHFR)	120-150 mg l^{-1}
	IgG1, 2 3, 4	cDNA/ Genomic fusion	Separate	hCMV-MIE	DEAE dextran/ Co-transfection	COS (Transient)	No	0.5-2 µg ml^{-1}
	IgG1, 2, 3,	cDNA/ Genomic fusion	Separate	hCMV-MIE	Electroporation/ Sequential	CHO	No	≤20 mg l^{-1}
	IgG4		Separate	hCMV-MIE	Electroporation/ Sequential	CHO	No	35-80 mg l^{-1}
	IgG4		Single	hCMV-MIE	Electroporation	CHO	Yes (GS)	>200 mg l^{-1}
	IgG4		Single	hCMV-MIE	Electroporation	NS0	Yes (GS)	>560 mg l^{-1}
	cFab'	cDNA	Separate	hCMV-MIE	Electroporation/ Sequential	CHO	No	20-120 mg l^{-1}
	cFab'	cDNA	Single	TAC	CaCl$_2$	*E.coli*	No	50-100 mg l^{-1}
	Fv		Single	hCMV-MIE	Electroporation	CHO	No	2 mg l^{-1}
	Fv		Single	TAC	CaCl$_2$	*E.coli*	No	40->500 mg l^{-1}
CEM231.6.7	IgG1	Genomic	Separate	Ig	Electroporation/ Sequential	Sp2/0	No	>100 mg l^{-1}
	IgG1	Genomic	Separate	Ig	Electroporation/ Sequential	P3.653	No	1-5 mg l^{-1} (10^{6}cells)$^{-1}$
CE25/B7	IgG4	Genomic	Separate	Ig	Electroporation/ Sequential	Sp2/0	No	1 mg l^{-1}
	IgG4	Genomic	Single	Ig	Electroporation	Sp2/0	No	15 mg l^{-1}
T84.66	IgG1	Genomic	Separate	Ig	Electroporation/ Co-transfection	Sp2/0	No	150-240 µg l^{-1} (10^{7}cells)$^{-1}$

Continued

A. MOUNTAIN AND J.R. ADAIR

Table 3 continued

Name	Isotype	Gene format	Vector format	Promoter	Transfection procedure	Host	Amplification	Yield from cell line
T84.66	IgG1	Genomic	Separate	Ig	Lipofection	CHO	No	15-70 µg l^{-1} (10^7cells)$^{-1}$
2,7.1G.10	IgG1	Genomic	Separate	Ig	Electroporation/ Sequential	P3.X63. 653.Ag8	No	n.s.
B6.2	IgG1	Genomic	Separate	Ig	Electroporation/ Co-transfection	Sp2/0 P3.X63. 653.Ag8 J558L	No No No	1 µg (10^6cells)$^{-1}$ (24 h)$^{-1}$ n.s. n.s.
	scFv			$\lambda O_L/P_R$	CaCl$_2$	E.coli N99 (cl857)	No	3 mg l^{-1}
L6	IgG1	cDNA	Single	SV40E	Electroporation	Sp2/0	No	<1 mg l^{-1}
	IgG1	cDNA	Separate	PGK	Co-transfection	S cerevisiae	No	<0,1 mg l^{-1}
	cFab	cDNA	Single	SV40E	Electroporation	Sp2/0	No	0,1-0,2 mg l^{-1}
	cFab		Single	araB	CaCl$_2$	Ecoli	No	2->500 mg l^{-1}
MBr1	IgG1	Genomic	Separate	Ig	Electroporation/ Co-transfection	NS0	No	n.s.
NRML-05	IgG4	Genomic	Separate	Ig	n.s.	n.s.	n.s.	n.s.
14.18	IgG1	Genomic	Single	Mouse metallothionein(MT1)	Protoplast fusion	Sp2/0	No	120-180 mg l^{-1}
MRK16	IgG1	Genomic	Separate	Ig	Electroporation/ Co-transfection	Sp2/0	No	5-10 mg l^{-1}
BA-Br1(ING1)	IgG1	cDNA	Separate	ALV LTR	Electroporation/ Sequential	Sp2/0	No	n.s.
BABr3(ING2)	IgG1/λ	cDNA	Separate	ALV LTR	Electroporation/ Sequential	Sp2/0	No	n.s.
YH206	IgG1	Genomic	Separate	Ig	Electroporation/ Sequential	X63.Ag8.653	No	n.s.
KM-10	IgG1	cDNA	Separate	ALV LTR	Electroporation/ Sequential	Sp2/0	No	21 mg l^{-1}
	cFab	cDNA	Single	PGK		S. cerevisiae	No	100 µg l^{-1}
	cFab	cDNA	Single	araB	CaCl$_2$	Ecoli W3110	No	n.s.
NL-1	IgG1	Genomic	Separate	Ig	Protoplast fusion (H)/ DEAE dextran (L)/ Sequential	X63.Ag8.653	No	10-30 mg l^{-1}

Table 3 continued

2H7	IgG1	cDNA	Separate	SV40E	Electroporation/ Co-transfection	Sp2/0	No	1-1.5 mg l^{-1}
RFT2	IgG1	Genomic	Separate	Ig	Electroporation/ Co-transfection	Sp2/0	No	
E6	IgG1	cDNA/ Genomic fusion	Separate	Ig	Electroporation/ Sequential	Sp2/0	No	1 mg l^{-1}
SK3	IgG1	Genomic	Separate	Ig	Protoplast fusion	n.s.	No	n.s.
Anti-Tac	IgG1	Genomic	Separate	Ig	Electroporation/ Sequential	Sp2/0	No	$\leq$3 μg (10^{6}cells)$^{-1}$ (24 h)$^{-1}$
BMA031	IgG1	Genomic	Separate	Ig	Electroporation/ Co-transfection	Sp2/0	No	1.2-7 μg (10^{6}cells)$^{-1}$ (24 h)$^{-1}$ (35 mg l^{-1})
	IgG4	Genomic	Separate	Ig	Electroporation/ Co-transfection	Sp2/0	No	1.2-7 μg (10^{6}cells)$^{-1}$ (24 h)$^{-1}$ (15 mg l^{-1})
BAT123	IgG1	Genomic	Separate	Ig	CaPO$_4$/Co-transfection	Sp2/0	No	20 mg l^{-1}
2H1	IgG1	Genomic	Separate	Ig	Electroporation/ Co-transfection	Sp2/0	No	1.2-7 μg (10^{6}cells)$^{-1}$ (24 h)$^{-1}$
15-C5	IgG1	Genomic	Separate	hCMV-MIE	CaPO$_4$/Co-transfection	CHO	No	80-200 ng (10^{6}cells)$^{-1}$ (24 h)$^{-1}$
	IgG1	Genomic	Separate	hCMV-MIE	CaPO$_4$/Co-transfection	CHO (Transient)	No	25-50 ng (10^{6}cells)$^{-1}$ (24 h)$^{-1}$
M-T412	IgG1	Genomic	Separate		Electroporation/ Co-transfection	Sp2/0	No	))28 mg l^{-1}
	IgG1	Genomic	Single		Electroporation	Sp2/0	No	)
MT151	IgG1	Genomic	Separate	Ig	Electroporation	Sp2/0	No	10 μg (10^{6}cells)$^{-1}$ (24 h)$^{-1}$
AHT107	IgG1	Genomic	Separate	hCMV/HIV	Electroporation/ Co-transfection	CHO	No	n.s.
AHT54	IgG1	cDNA	Separate	RSV	Electroporation/ Co-transfection	CHO	No	n.s.
4D5	Fab'	cDNA	Single	phoA	CaCl$_2$	E.coli25F2	No	0.1-0.2 g l^{-1}
	Fab	cDNA	Single	phoA	CaCl$_2$	E.coli25F2	No	50-200 mg l^{-1}
UCHT1	Fab'	cDNA	Single	phoA	CaCl$_2$	E.coli25F2	No	40 mg l^{-1}

Expression of humanized antibodies

YTH34.5HL	IgG1	Genomic	Separate	Ig	Electroporation/ Co-transfection	YO(rat)	No	5mg l^{-1}
	IgG1	cDNA	Separate	β-actin	CaPO$_4$/Co-transfection	CHO	YES (DHFR)	200mg l^{-1}
Anti-Tac	IgG1	Genomic	Separate	Ig	Electroporation/ Sequential	Sp2/0	No	$\leq$3 μg (10^{6}cells)$^{-1}$ (24 h)$^{-1}$ (8 mg/L)

Table 3 continued

Name	Isotype	Gene format	Vector format	Promoter	Transfection procedure	Host	Amplification	Yield from cell line
HuRSV19	IgG1	Genomic	Separate	Ig	Electroporation/ Co-transfection	YB2O (rat)	No	5 mg l^{-1}
Fd79	IgG1	Genomic	Separate	Ig	Electroporation/ Co-transfection	Sp2/0	No	n.s.
Fd138-80	IgG1	Genomic	Separate	Ig	Electroporation/ Co-transfection	Sp2/0	No	n.s.
BMA031	IgG1	Genomic	Separate	Ig	Electroporation/ Co-transfection	Sp2/0	No	7 µg $(10^6 cells)^{-1} (24 h)^{-1}$
H17E2	IgG1	Genomic	Separate	Ig	Electroporation/ Co-transfection	NS0	No	10 mg l^{-1}
Campath-9	IgG1	Genomic	Separate	β–actin	CaPO$_4$/Co-transfection	CHO	No	n.s.
1B4	IgG1	Genomic	Separate	Ad-MLP	CaPO$_4$/Co-transfection	293 (Transient)	No	385-650 ng ml^{-1}
	IgG1	Genomic	Separate	HIV-LTR	CaPO$_4$/Co-transfection	293 (Transient)	No	13.75 µg ml^{-1}
0.5β	IgG1	Genomic	Separate	hCMV-MIE	Electroporation/ Co-transfection	COS (Transient)	No	n.s.
425	IgG1	Genomic	Separate	hCMV-MIE	Electroporation/ Co-transfection	COS (Transient)	No	n.s.
YTH12.5	IgG1	Genomic	Separate	β-actin	DOTMA Co-transfection	CHO	No	n.s.
M195	IgG1	Genomic	Separate	hCMV-MIE	Electroporation Co-transfection	Sp2/0	No	3-8 µg $(10^6 cells)^{-1} (24 h)^{-1}$
BW431/26	IgG1	Genomic	Separate	SV40	CaPo$_4$/n.s.	BHK	YES (DHFR)	15 mg l^{-1}
OKT3	IgG4	cDNA/ Genomic fusion	Separate	hCMV-MIE	CaPO$_4$/ Co-transfection	COS (Transient)	No	0.5-1 µg ml^{-1}
4D5	IgG1	cDNA	Separate	hCMV-MIE	n.s./Co-transfection	293 (Transient)	No	7-15 µg ml^{-1}
	Fab'	cDNA	Single	phoA	CaCl$_2$	E.coli25F2	No	1-2 g l^{-1}
	Fab	cDNA	Single	phoA	CaCl$_2$	E.coli25F2	No	0.5-2 g l^{-1}
UCHT1	Fab'	cDNA	Single	phoA	CaCl$_2$	E.coli25F2	No	0.4-0.6g l^{-1}

* n.s. not stated

Table 4. Chimeric and humanized therapeutic antibodies — effector functions of chimeric antibodies

Antibody[*]	Isotype	ADCC	ADCMC[†]	Human isotype	ADCC	ADCMC[†]
Chimeric antibodies						
17-1A	IgG2a/κ	+[§]		IgG1/κ	+	-
				IgG2/κ	-	-
				IgG3/κ	+	-
				IgG4/κ	+	-
				IgM/κ	+/-	+
L6	IgG2a/κ	+	+	IgG1/κ	+	+
				IgG1/κ(Yeast)	+	-
B72.3	IgG1/κ	-	n.s.[¶]	IgG1/κ	+	n.s.
				IgG4/κ	+	n.s.
2.7.1G.10	IgG1/κ	-	-	IgG1/κ	+	+
MBr1	IgM/κ	-	n.s.	IgG1/κ	+	n.s.
14.18	IgG2a/κ	+	+	IgG1/κ	+	+
				IgG1ΔCH2/κ	-	-
				IgG1(Sγ1)/κ	-	+/-
				IgG1ΔCHO/κ	-	+/-
BA-Br1(ING-1)	IgG1/κ	-	-	IgG1/κ	+	+
BA-Br3(ING-2)	IgG1/λ	-	-	IgG1/λ	-	-
KM-10	IgG1/κ	-	-	IgG1/κ	+	-
NL-1	IgG2a/κ	+	+	IgG1/κ	+	+
2H7	IgG2b/κ	-	-	IgG1/κ	+	+
RFT2	IgG2a/κ	+	-	IgG1/κ	+	+
BAT123	IgG1/κ	+	n.s.	IgG1/κ	+	n.s.
AHT107	IgG1/κ	-	n.s.	IgG1/κ	+	n.s.
AHT54	IgG1/k	-	n.s.	IgG1/κ	+	n.s

Continued   *57*

Engineering antibodies for therapy

Table 4 continued

Antibody[*]	Isotype	ADCC	ADCMC[†]	Human isotype	ADCC	ADCMC[†]
Chimeric antibodies						
YTH-34.5HL	IgG2b/κ(rat)	+	-	IgG1/κ	+	+
				IgG2/κ	-	+/-
				IgG3/κ	+	+
				IgG4/κ	-	-
Anti-Tac	IgG2a/κ	-	-	IgG1/κ	+	-
				IgG3/κ	-	-
BMA031	IgG2b/κ	-	-	IgG1/κ	+	-
				IgG4/κ	+	-
Campath-9	IgG2b/κ(rat)	-	n.s.	IgG1/κ	+	n.s.
Humanized antibodies						
YTH34.5HL	IgG2b/κ(rat)	+	-	IgG1/κ	+	+
Anti-Tac	IgG2a/κ	-	-	IgG1/κ	+	-
BMA031	IgG2b/κ	-	-	IgG1/κ	+	n.s.
Campath-9	IgG2b/κ(rat)	-	n.s.	IgG1/κ	+	n.s.
YTH12.5	IgG2b/λ	+	+	IgG1/κ(monovalent)	+	+
				IgG1/κ(bivalent)	+	-

* murine unless stated
† Human complement
¶. n.s. no data described
§ + means that the antibody was considered to show activity in the assays described. For quantitative comparisons of the rodent and chimeric/humanized antibodies refer to the text or to the references provided in Table 2.

Pre-clinical studies

Relative antigen binding ability.   The chimeric IgG3 (c17-1A(γ3)) was first shown to compete equivalently with murine 17-1A (m17-1A) for antigen on SW1116 and HT29 carcinoma cells (Shaw *et al.*, 1987). The c17–1A(μ) competed equivalently on a weight for weight basis with c17-1A(γ1) for antigen on HT29 colon carcinoma cells (Fogler *et al.*, 1989). On a molar basis the K_As were calculated to be $3 \cdot 4 \times 10^7$ M^{-1} and $1 \cdot 6 \times 10^8$ M^{-1} for the c17-1A(γ1) and c17–1A(μ), respectively.

ADCC.   Initial experiments suggested that m17-1A may exert an effect on xenografts via interaction with macrophages, and considerable effort has been expended in examining the various human IgG isotypes to establish whether this applies to chimeric forms (Shaw *et al.*, 1987, 1988; Shaw, Khazaeli and LoBuglio, 1988; Masucci *et al.*, 1988, 1990; Steplewski *et al.*, 1988; Fogler *et al.*, 1989; Masucci and Mellstedt, 1991). These experiments used a variety of effector cells, target cells, assay formats, effector cell:target cell (E:T) ratios, antibody concentrations and in some cases included exogenous lymphokines. Nevertheless a consistent theme emerged, in that the c17-1A(γ1) led to ADCC equivalent to or superior to the m17-1A antibody and was generally better than the other isotypes at effecting ADCC. In most of the experiments the other IgG isotypes and the IgM showed much less activity but were still clearly active compared to the no antibody controls. Between experiments the rank order of the isotypes varied. For example the c17-1A(γ2) was shown to be equivalent to the c17-1A(γ1) and m17-1A in an 18 h ^{111}In release assay using SW948 carcinoma cells as targets and lymphocytes as effectors, but to be only half as effective with unfractionated PBMC (Steplewski *et al.*, 1988). The c17-1A(γ3) and c17-1A(γ4) antibodies were relatively ineffective in these assays. In a 4 h ^{51}Cr release assay the c17-1A(γ3) demonstrated equivalent activity to the c17-1A(γ1) (Shaw *et al.*, 1988; Shaw, Khazaeli and LoBuglio, 1988) with either lymphocytes or monocytes as effectors, with the lymphocyte preparation showing the higher levels of ADCC with all of the antibodies. When monocytes (NK cell-depleted) were allowed to differentiate into macrophages by 48 h incubation in the presence or absence of IFNγ to further stimulate the expression of FcR, ADCC was stimulated with all of the antibodies and the order of reactivity was IgG1 > m17-1A > IgG4 > IgG2 = IgG3. The lysis effected by the C17-1A(γ1) was 50% of maximal levels with untreated macrophages and 80% with treated macrophages. ADCC effected by c17-1A(γ4) with IFNγ-treated macrophages was equal to that of c17-1A(γ1) using untreated macrophages, while the c17-1A(γ2) or c17-1A(γ3) ADCC with IFNγ-treated macrophages was equal to ADCC with m17-1A and untreated macrophages (Steplewski *et al.*, 1988).

In general when PBMC were subfractionated into lymphocytes and monocytes a given level of ADCC could usually be achieved by the lymphocyte preparations with a lower E:T ratio than for the monocyte preparations. It was also shown that NK cell-depleted monocytes were poorer at ADCC than

undepleted monocytes. NK cell-depleted monocytes, allowed to differentiate into macrophages in the presence of IFN-γ, gave higher levels of ADCC than non-treated macrophages. Further, when monocytes were titrated back into a lymphocyte population ADCC, in an 18 h assay format, increased. The activity of the c17-1A(γ1) was shown to be further increased by pre-treatment of the effector cells with a variety of lymphokines. In particular a combination of rIL-2, GM-CSF and TNF-α led to a five-fold improvement in c17-1A(γ1)-directed lysis of SW948 carcinoma cells using PBMC as effectors (Masucci and Mellstedt, 1991). These experiments suggest that *in vivo* c17-1A(γ1) assisted by lymphokine co-treatment may be a useful means of activating anti-tumour ADCC.

ADCMC.   Neither m17-1A nor any of the chimeric IgG antibodies were shown to effect ADCMC using human complement in short incubation assays (Shaw, Khazaeli and LoBuglio, 1988). However the c17-1A(μ) was efficient at ADCMC if the time of incubation was extended to 18–24 h (Fogler *et al.*, 1989).

Animal studies.   In a nude mouse xenograft experiment SW948 carcinoma cells were injected subcutaneously (s.c.) and 100 μg of antibody was given intra-peritoneally (i.p.) on five consecutive days beginning at the time of inoculation of the cells (Steplewski *et al.*, 1988). The m17-1A and, perhaps surprisingly, c17-1A(γ4), showed the greatest degree of inhibition of tumour growth. The c17-1A(γ1) was slightly inferior and the c17-1A(γ2) or c17-1A(γ3) showed little inhibition of tumour growth compared to a non-specific antibody control. The c17-1A(γ1) and c17-1A(γ4) were shown to be able to interact with murine macrophages *in vitro* to cause ADCC of the SW948 cells. The c17-1A(γ3) showed intermediate lysis while the c17-1A(γ2) was inactive.

Buchsbaum *et al.* (1990) examined the ability of the c17-1A antibodies to localize to s.c. LS174T tumours in a nude mouse xenograft model. Initially ^{125}I-labelled antibodies were injected into nude mice without tumours and the relative blood half-lives were measured. After i.p. injection of antibody $t_{1/2}\beta$ values for the m17-1A, m17-1A(Fab')$_2$, c17-1A(γ1), c17-1A(γ2) and c17-1A(γ4) were 7·5, 0·5, 5·2, 6·9, and 1·9 d, respectively. Antibody injected into tumour-bearing mice gave tumour:blood ratios for m17-1A, m17-1A(Fab')$_2$, c17-1A(γ1), c17-1A(γ2) and c17-1A(γ4) of 2·7, 12·2, 3·2, 2·0 and 1·4, respectively. The m17-1A achieved 12% i.d.g^{-1} compared to 20·5%, 10% and 4·9% for the c17-1A(γ1), c17-1A(γ2) and c17-1A(γ4), respectively, at 4 d post-injection.

Different results were seen when the SW948 carcinoma cell line was used to generate the xenografts. Here the tumour:blood ratios for the m17-1A and c17-1A(γ1) were similar (1·5 versus 1·6) with 1·4 and 0·7 for the c17-1A(γ2) and c17-1A(γ4) antibodies. Antibody localized to the tumour at 4 d was 11% i.d.g^{-1} for the m17-1A and 7·3%, 6·5% and 2·9% i.d.g^{-1} for the c17-1A(γ1), c17-1A(γ2) and c17-1A(γ4), respectively.

To measure tumour regression m17-1A or c17-1A(γ1) were injected i.p. 9 d after s.c. injection of LS174T cells. A single injection of 26 μg (300 μCi)

of ^{125}I-labelled m17-1A caused a significant reduction in tumour growth compared to unlabelled antibody. When 3 doses were given (30, 15 and 12 μg, and all approx 300 μCi) on days 9, 16 and 28 post-injection of LS174T cells a marked reduction in tumour growth was seen. This level of effect was seen when a single dose (65 μg, 300 μCi) of c17-1A(γ1) was given on day 9 after the injection of the LS174T cells.

Clinical studies

The first published study of the clinical use of a chimeric antibody described a Phase I trial using c17-1A(γ1) (LoBuglio *et al.*, 1989; Trang *et al.*, 1990). In this study 10 patients with metastatic colorectal adenocarcinoma, each of whom had an anticipated survival of at least three months and who had had no other therapy within six weeks of the study, were given c17-1A(γ1). Two patients received a single intravenous infusion of 10 mg and two a single dose of 40 mg, while three patients received three doses of 10 mg at 0, 14 and 28 days, and a final group of three patients received three doses of 40 mg at 0, 14 and 28 days. It was demonstrated that the plasma disappearance curves of c17-1A(γ1) could best be described by a two-component decay with mean $t_{1/2}\alpha$ of 17·5 h (range 13–23 h) and $t_{1/2}\beta$ of 100·5 h (range 65–139 h). For patients receiving multiple doses of antibody the repeat pharmacokinetics were very similar with each patient showing different, but reproducible, $t_{1/2}\beta$ values. Only one patient, who had received three 40 mg infusions, showed a small but statistically significant rise in anti-chimeric antibody titre. In this case the serum was able to bind 5–7 ng ml^{-1} of chimeric antibody compared to > 100 ng ml^{-1} binding usually seen in response to treatment with murine 17-1A. This response was detected 5 and 8 weeks after the final exposure to c17-1A(γ1). Whether the antibody response was IgG or IgM was not determined. The antibody response could be competed by murine 17-1A and therefore it was concluded that this response was against the variable region of the c17-1A(γ1). The serum from this patient was not able to interfere with the binding of ^{125}I-labelled murine 17-1A to the cell line SW1116, which expresses the epitope recognized by 17-1A. It was concluded that the anti-chimeric antibody in the serum recognizes framework regions on the variable domains of the antibody. No allergic or other toxic side-effects were noted in the study.

The main intended therapeutic strategy with c17-1A has involved the use of naked antibody to kill tumours by recruitment of immune effector functions. However c17-1A-isotope conjugates have also been considered, and the original study by LoBuglio *et al.* (1989) was followed by a second study in which six patients with metastatic colon cancer were treated with 2 mg of ^{131}I-labelled c17-1A(γ1) (5·61–8·31 mCi) by i.v. infusion (Meredith *et al.*, 1991). The pharmacokinetics of the clearance of the antibody from the blood could be described by a two component model with $t_{1/2}\alpha$ of 19·7 ± 2·9 h and $t_{1/2}\beta$ of 106·4 ± 14 h. One patient showed a much longer $t_{1/2}\beta$ of 172 h, compared to the range for the other patients of 71·4–106·7 h.

None of the patients developed an anti-antibody response against this

single, low dose of c17-1A over a 12 week period of examination. While the issue of immunogenicity may have been resolved for this antibody the long circulating half-life may be unacceptable for a MAb–isotope conjugate. In this clinical study the dose to the bone marrow was estimated to be 0.76–1.05 rad mCi^{-1}. The long half-life of the chimeric antibody in the circulation delayed tumour imaging of tumour to 4–7 d after infusion, though all known lesions less than 4 cm were detected. Subsequently a further nine patients have been administered c17-1A. None have developed a HAMA response giving a current total of 1 response in 25 patients, with the single response a weak one (A. LoBuglio, personal communication).

B72.3

B72.3 is a murine IgG1/κ generated by immunizing mice with a membrane-enriched fraction of a carcinoma metastasis (Colcher *et al.*, 1981). B72.3 reacts with an O-linked sialosyl-2-6α-*N*-acetyl-galactosylaminyl structure (Gold and Mattes, 1988), within a glycoprotein mucin-like complex (TAG72) of high molecular weight (220–400 kDa). This TAA is expressed on 50–80% of gastrointestinal, ovarian, breast and prostate tumours. The association constant of B72.3 for antigen (K_a) has been measured at 2.54×10^9 M^{-1} (Muraro *et al.*, 1988) and is one of a number of murine anti-TAG72 antibodies which have a heavy variable sequence apparently derived from a common germ-line gene, V_HTAG (Mezes, Gourlie and Mark, 1990). B72.3 shows no significant reactivity with normal adult liver, spleen, heart, breast, uterus, bone marrow, colon, stomach, salivary gland, lymph node, or kidney (Nuti *et al.*, 1982; Stramignoni *et al.*, 1983) but has been shown to be reactive with secretory endometrium (Thor *et al.*, 1987) and transitional colonic epithelium (Xu *et al.*, 1989). The antigen recognized by B72.3 is also expressed in some human fetal tissues, including fetal gut and therefore the antigen has been defined as an onco-fetal antigen (Thor *et al.*, 1986).

MAb B72.3 has been used successfully to target carcinomas *in situ* using radiolabelled preparations of the antibody (reviewed by Schlom *et al.*, 1990; Colcher *et al.*, 1991) and B72.3 is in clinical studies as an imaging agent for colorectal carcinoma and ovarian and prostate adenocarcinoma (Fradd, 1992). Although the murine MAb has been administered to over 1000 patients, its usefulness for cancer therapy is limited by HAMA responses, which have been observed in the great majority of patients (see, for example, Khazaeli *et al.*, 1992).

Construction and expression of chimeric antibodies and fragments

Chimeric forms of B72.3 have been constructed by cDNA (Whittle *et al.*, 1987; Gillies, Lo and Wesolowski, 1989; King *et al.*, 1992a), by genomic DNA (Primus *et al.*, 1990; Hutzell *et al.*, 1991) and by PCR cloning approaches (Xiang, Roder and Hozumi, 1990). In the first approach (Whittle *et al.*, 1987) the variable region cDNA sequences were fused to the Cκ and CH1 exon sequences to give hybrid cDNA/genomic format genes. Initially the IgG4

isotype was chosen because the cB72.3 was viewed as a carrier for radioisotopes and therefore the ability to interact efficiently with cells via FcR to cause ADCC and with complement for ADCMC was regarded as undesirable. These chimeric genes were inserted into expression vectors for expression in CHO-K1 cells using the hCMV promoter/enhancer, and SV40 polyadenylation signals (Stephens and Cockett, 1989; Cockett, Bebbington and Yarranton, 1990; reviewed in Bebbington, 1991). Initially the correct assembly and antigen binding activity of the chimeric antibody was examined by transient expression in COS cells (Whittle *et al.*, 1987). Stable cell lines were generated by sequential transfection by electroporation into CHO-K1 cells (Colcher *et al.*, 1989). Cell lines expressing 35–40 mg l^{-1} from attached cells in shake flasks and 80–100 mg l^{-1} in suspension culture in air-lift fermentors and in serum-free medium were obtained from these low copy number cell lines.

The cB72.3 heavy chains with IgG1, IgG2 and IgG3 isotypes were subsequently constructed and after insertion into expression vectors these were transfected into the chimeric light chain-producing cell line (A. Phipps *et al.*, unpublished; *see also Table 3*). Much higher level production of cB72.3(γ4) antibody has been achieved in CHO-K1 cells (Bebbington, 1991) and in NS0 cells (Bebbington *et al.*, 1992) by placing both chimeric genes, each with the hCMV-MIE and SV40 polyadenylation signals, on the same vector along with the amplifiable glutamine synthetase (GS) selectable marker. Expression levels of 200–400 mg l^{-1} in CHO-K1 have been achieved in batch fermentations using enriched, serum-free, media (Bebbington, 1991), while expression in NS0 cells has led to yields of 560 mg l^{-1} of cB72.3(γ4). A single round of selection for amplification was required to achieve these high productivities and the cell line continued to yield the same high level of antibody when grown for extended periods in the absence of MSX (used to select vector amplification).

Gillies, Lo and Wesolowski (1989), in a second approach, cloned and reconstructed the B72.3 V regions into genomic exon format to produce a cB72.3(γ1) antibody. Variable region sequences were isolated from cDNA and were modified by the use of oligonucleotide linkers which extended from a convenient restriction site near the 3' of V_H and V_L and which recreated the coding sequence and included sequences for a splice donor site immediately 3' to the J-regions of V_H and V_L and intron sequence from an Ig leader-V region intron extending up to a convenient restriction site for cloning of the adapted V regions 5' to the constant region genes. The V regions were cloned into expression vectors with a mutant DHFR gene as the selectable marker, allowing for subsequent gene amplification of the transfected DNA. The chimeric antibody genes were arranged so as to be transcribed from natural or synthetic Ig promoter/enhancers. The chimeric genes were assembled into a single vector for transfection into Sp2/0-Ag14 cells by protoplast fusion. Cell lines producing 120–150 μg ml^{-1} were obtained using MTX selection. The B72.3 variable region sequences have also been isolated from genomic DNA using J region restriction fragment probes (Hutzell *et al.*, 1991). The genomic V region sequences were cloned into pSV2△H*gpt*-HuG1 and pSV2△H*neo*-

HuK vectors (Oi and Morrison, 1986). The vectors were transfected sequentially (light chain vector first) into Sp2/0 cells by electroporation. A stable cell line producing 10–20 mg l^{-1} in low serum medium was established. Finally the heavy chain V region sequence has been recloned from cDNA by Xiang, Roder and Hozumi (1990) using the PCR approach (Orlandi et al., 1989) and using the published sequence (Whittle et al., 1987) to provide the amplimer sequences in the signal sequence and J regions. The V sequence was assembled to be expressed from the Ig promoter and was linked to the human IgG1 genomic constant region sequences. The vector, with *neo* selection, was inserted by electroporation into a heavy chain loss variant of the B72.3 hybridoma to give a stable cell line expressing 2 mg l^{-1} of mouse light chain/chimeric heavy chain antibody.

All four IgG isotypes of cB72.3 are secreted as fully assembled tetramers from CHO, NS0 and Sp2/0 cells. Colcher et al. (1989) and King et al. (1992a) have shown that a proportion of cB72.3(γ4) does not form the inter-heavy chain disulphide bridges. All IgG4s appear to show this phenomenon (see for example Fasler, Skarvill and Lutz, 1983; Bruggemann et al., 1987; Tan et al., 1990). The presence of a proportion of incorrectly bridged IgG4 may have a bearing on the *in vivo* properties observed for IgG4 (see below). Therefore a modified cB72.3(γ4) has been generated which has a single amino acid substitution in the hinge (serine 241 to proline) which makes the core hinge sequence the same as that in IgG1 and IgG2 (Angal et al., 1993). Expression of this cB72.3(γ4P) variant leads to a homogeneous antibody with full binding properties and improved biodistribution (see below).

Fab' and Fv forms (*Figure 2*) of the B72.3 have been constructed and expressed in CHO cells (Gillies and Wesolowski, 1990; Owens et al., 1991a; King et al., 1992a, b, 1993) and in *E. coli* (Owens et al., 1991a; King et al., 1992b, 1993) to provide material for biodistribution and pharmacokinetic analyses. For Fab' expression the cIgG4 heavy chain gene was converted into an Fd' gene by using synthetic oligonucleotide adapters to attach the hinge sequence exon directly to the CH1 exon and following this sequence with inframe stop codons and a cloning site. This reconstructed chimeric Fd'(γ4) gene was then placed in the heavy chain expression vector (Whittle et al., 1987) and transfected into the chimeric light chain-expressing cell line (King et al., 1992a). Cell lines were obtained which expressed 20 mg l^{-1} cB72.3 Fab'(γ4) in small-scale culture, and 120 mg l^{-1} in fermentors after adaptation of the cells suspension growth in low serum medium. The chimeric light and Fd'(γ4) chain genes have also been expressed in *E. coli* using the *tac* promoter with IPTG induction and the ompA signal sequence to direct secretion (A. Mountain et al., unpublished data). Yields of a few mg l^{-1} secreted into the culture medium were observed.

Very little, <10%, of the Fab' secreted from the CHO cell lines is secreted or associated in the supernatant to form F(ab')$_2$. The hinge thiols appear to be in an oxidized form, which may be a prerequisite for efficient secretion of antibody (Alberini et al., 1990). However the thiols can be regenerated with β-mercaptoethylamine and F(ab')$_2$ efficiently formed. Similarly the *E. coli*-expressed material is produced in the culture supernatant as Fab' and has

been processed to give $F(ab')_2$ (D. King *et al.*, unpublished data).

A $\gamma 1$ variant of the cB72.3Fab has also been produced (Gillies and Wesolowski, 1990). In this case the CH2 and CH3 exons and CH2-CH3 intron of the cB72.3($\gamma 1$) heavy chain gene were replaced by a small synthetic DNA fragment coding for a tripeptide and a stop codon and bounded by splice donor and acceptor sequences. When expressed in Sp2/0 cells Fab' was produced but no $F(ab')_2$ was detected in the culture supernatant. In order to examine the role of the hinge in the spontaneous formation of $F(ab')_2$ a series of a further 13 cB72.3 Fd' genes were constructed as described above for the Fd'($\gamma 4$) construct (Bodmer, Adair and Whittle, 1989). For this purpose DNA hinge sequences were synthesized derived from IgG1–4, but also including different length variants of the IgG3 hinge and a derivative of the IgG4 hinge in which the second cysteine was converted to an alanine, ($\gamma 4 \triangle$cys). Each hinge DNA sequence was attached either to its cognate CH1 domain sequence (e.g. IgG1 CH1 with IgG1 hinge) or to the IgG4 CH1 domain. Therefore it was possible to examine the effect on spontaneous $F(ab')_2$ formation of having between 1 and 11 cysteines in the hinge, and also the effect of the CH1 domain on the $F(ab')_2$ formation. The genes were assembled in expression vectors with the hCMV promoter/enhancer and co-expressed with cB72.3 light chain in COS cells. Fab' prepared using Fd' genes with four or more hinge cysteines showed significant amounts of $F(ab')_2$ formation, whereas Fd' genes derived from IgG1 and IgG4 gave rise predominantly to Fab' formation (J. Adair, M. Bodmer and N. Whittle, unpublished).

Fab' prepared using the Fd' gene with the ($\gamma 4 \triangle$cys) can be cross-linked efficiently *in vitro* using bifunctional maleimide cross-linking reagents to produce chemically cross-linked $F(ab')_2$ (*Figure 2*) which shows improved pharmacokinetic properties over normally disulphide bridged $F(ab')_2$ (Rhind, 1990; Rhind, Millican and Millar, 1990b; D.J. King *et al.*, unpublished data). Dimeric truncated IgG has also been formed by a different means (Gillies and Wesolowski, 1990; Kashimiri, 1992). In the first instance (Gillies and Wesolowski, 1990) the CH2 domain was deleted from a cB72.3($\gamma 1$) gene by utilization of suitable intronic restriction sites. After expression of the cB72.3($\gamma 1 \triangle$CH2) in Sp2/0 cells it was shown that tetrameric (H2L2) material was produced but that a majority of the material was not covalently linked by the hinge cysteine residues (see also 14.18 below). In the second example the CH2 domain was deleted from a cDNA form of the cB72.3($\gamma 1$) gene by PCR directed mutagenesis and replaced by a small linker region ((glycine)$_4$ serine). In this case the protein was cross bridged at the hinge (Kashimiri, 1992).

The Fv of B72.3 has been constructed by placing a translation stop codon at the 3' of the variable domain sequences. The V_L and V_H genes have been introduced by sequential transfection into CHO-K1 cells to give stable, non-amplified cell lines which produce about 2 mg l^{-1} of the Fv. Expression of the Fv in the same *E. coli* secretion system as the cFab' has given yields in the culture supernatant of 40 mg l^{-1} in shake flasks and 500 mg l^{-1} in fermentors (A. Mountain *et al.*, unpublished observations). Co-expression

and secretion of the V_L and V_H domains of B72.3 in either CHO cells or *E. coli* results in the formation of the assembled non-covalently linked Fv (Owens *et al.*, 1991a; King *et al.*, 1992b, 1993). At low concentration the Fv dissociates into V_L and V_H (King *et al.*, 1992b, 1993). A single chain Fv (scFv) (Bird *et al.*, 1988; Huston *et al.*, 1988) in which the B72.3 V_L and V_H coding regions were linked by DNA coding for a 15 amino acid peptide linker has therefore also been prepared and expressed in *E. coli* (D. Desplancq *et al.*, unpublished data). Further, a novel form of the scFv has been produced in *E. coli* by adding a DNA sequence coding for an antibody hinge sequence at the C-terminus of the scFv gene and which has been chemically cross-linked to give bivalent and trivalent scFvs (*Figure 2*).

It has been suggested that co-treatment with antibody and cytokines, in particular IL-2, may lead to enhanced cell killing by tumour infiltrating lymphocytes. Gillies *et al.* (1992) have constructed Fab–IL-2 fusions for B72.3 and for the anti-ganglioside antibody 14.18 (see below). The B72.3 Fab–IL-2 fusion was constructed by linking the IgG1 CH1 domain to the mature human IL-2 gene via a short oligonucleotide such that the CH1 and IL-2 sequences were reconstituted. The fusion genes were expressed in Sp2/0 cells and purified by affinity chromatography using anti-light chain antibody. The Fab–IL-2 was expressed as a monomer with no spontaneous hinge disulphide bridge formation.

Pre-clinical studies

Relative antigen binding ability. Chimeric B72.3(γ1–4, γ4P) and cB72.3F(ab')$_2$ bind to antigen and compete with murine B72.3 for antigen extracted from LS174T cells *in vitro* with equal potency (Whittle *et al.*, 1987; Colcher *et al.*, 1989; Hutzell *et al.*, 1991; Angal *et al.*, 1993; King *et al.*, 1992a; A. Phipps *et al.*, unpublished). cB72.3(γ1$\triangle$CH2) showed a higher avidity for antigen than cB72.3(γ1) (Gillies and Wesolowski, 1990). The binding of the cB72.3(γ1$\triangle$CH2), normalized for the amount of light chain in the antibodies, appeared to be approximately 10 times that of the cB72.3(γ1). However a second form in which the CH2 domain was replaced by a pentapeptide linker, and in which the inter-hinge disulphide bridges were formed, showed no improvement in affinity over the cB72.3(γ1) parent (Kashimiri, 1992). Hybridoma-drived B72.3 and CHO cell-drived mB72.3 and cB72.3(γ4) were each shown to bind to a mammary carcinoma metastasis, a colon carcinoma xenograft and to purified TAG72, but not to extracts from several normal tissues, to carcinoembryonic antigen or to melanoma (A375 cell) xenografts (Colcher *et al.*, 1989).

Equipotency of material produced in CHO cells and in *E. coli* has been formally demonstrated for both the cFab' and the Fv of B72.3 (King *et al.*, 1993). The scFv, expressed and secreted from *E. coli*, displays antigen binding activity similar to the Fv but with the advantage of stability at low concentration. The cFab', Fv, scFv and scFv-hinge fragments of B72.3 bind to antigen and compete with a reduced relative potency, due to the reduced

avidity of the monomeric material (Owens *et al.*, 1991a, b; D. Desplancq *et al.* (unpublished); King *et al.*, 1992b, 1993). The bivalent scFv form has been shown to bind antigen as well as IgG or F(ab')$_2$, and the trivalent to bind antigen two- to three-fold better than these bivalent species (Owens *et al.*, 1991a, b).

Xiang and Chen (1992) have very recently derived a higher affinity variant of B72.3 by mutating residue 99 in CDR3 on the heavy chain from histidine to asparagine. The resultant antibody has four-fold higher affinity for antigen (Xiang and Chen, 1992).

ADCC. cB72.3 has largely been regarded as a candidate for tumour imaging and therapy as a MAb–isotope conjugate, but cB72.3(γ1) and cB72.3(γ4) have been compared for ability to mediate ADCC as alternative therapeutic approach (Hutzell *et al.*, 1991; Primus *et al.*, 1990). Because of the poor antigen expression on cells in culture the target cells were NIH:OVCAR ascites tumour cells grown in nude mice. In a 4 h ^{51}Cr release assay using rIL-2-stimulated effector cells the cB72.3(γ1) showed significantly more ADCC than the mB72.3. Without prior rIL-2 treatment there was negligible ADCC with the mB72.3 and lowered but detectable ADCC with the cB72.3(γ1) (Primus *et al.*, 1990). Incubation of un-fractionated PBMC cells for 24 h with the target cells in an ^{111}In release assay had a pronounced effect when the PBMC were activated with rIL-2 and was much less pronounced with unstimulated cells, but was still higher than the 4 h result with stimulated PBMC. After 24 h pre-incubation with rIL-2 PBMC 100% ADCC could be achieved using cB72.3(γ1) while the cB72.3(γ4) gave maximal 20% lysis. With lower amounts of rIL-2 the effect on ADCC in a 24 h ^{111}In release assay was still noticeable. ADCC with cB72.3(γ1) could be more than doubled, and with mB72.3 and cB72.3(γ4) by a factor of 3. The ADCC was antibody concentration dependent, most noticeably for the cB72.3(γ1) (Hutzell *et al.*, 1991). When NK cell-depleted monocytes (IFNγ-matured) were used as effectors and compared to non-adherent PBMC (rIL-2-stimulated) from the same donor it was shown that the adherent cells could not cause ADCC with mB72.3 and only at high E:T ratios for the cB72.3(γ1) and the cB72.3(γ4). However the non-adherent population showed significant ADCC with the mB72.3 and cB72.3(γ4) showing an equivalent profile which was sensitive to the E:T ratio, and the cB72.3(γ1) showing higher levels at all E:T ratios.

Animal studies. The ability of the chimeric antibodies to localize to tumours *in vivo* was examined in nude mice bearing LS174T xenografts. Colcher *et al.* (1989) showed by co-injection i.v. of ^{125}I-labelled cB72.3(γ4) and ^{131}I-mB72.3 that the cB72.3(γ4) cleared faster from the blood with only 28% of the injected dose remaining at 48 h compared to 68% for the mB72.3. There was lower tissue and tumour uptake of the cB72.3(γ4) at early time points compared to the mB72.3. At later time points the mB72.3 still showed higher blood and tumour values but the differences were less noticeable. The radiolocalization indices were similar, however, showing that the cB72.3(γ4) was not being preferentially deposited in any of the major organs. When

^{125}I-labelled cB72.3(γ1) and ^{131}I-mB72.3 were co-injected they showed equivalent tumour loading and tumour:blood ratios over a 168 h period. Blood clearance was compared between the ^{125}I-mB72.3, ^{131}I-cB72.3(γ1) and ^{125}I-cB72.3(γ4) in tumour-bearing nude mice by paired co-injection of the ^{131}I-cB72.3(γ1) with either of the ^{125}I-labelled antibodies (Hutzell *et al.*, 1991). The $t_{1/2}\alpha$ values were 3·1, 2·6 and 4·6 h respectively and the $t_{1/2}\beta$ values were 82·7, 53·7 and 57·4 h.

Gallinger *et al.* (1991) have also examined biodistribution and tumour uptake of the hybrid murine light chain-chimeric(γ1) heavy chain antibody B72.3-1-3 (Xiang, Roder and Hozumi, 1990). In these studies the mB72.3 showed very high tumour uptake compared to the hybrid antibody, reaching 53·5% i.d.g^{-1} at 8 d post-injection compared to 8·7% i.d.g^{-1} for the hybrid. Blood levels remained constant for the mB72.3 but declined throughout the time course for the hybrid. Uptake in other tissues showed no major differences between the two antibodies. The differences in uptake of the mB72.3 between these results and those described earlier were attributed to the size of the xenografts used in the studies. In earlier studies tumour xenografts of 200–1400 mg had been used whereas those used by Gallinger *et al.* (1990) were 100 mg. Tumours of 100 mg were then compared with 2000 mg tumours and it was shown that with the smaller tumour the tumour:blood radiolocalization index was 5 at day 6 compared to 1·5 for the large tumour, and uptake by the smaller tumour was 18·5% i.d.g^{-1} compared to 1·9% i.d.g^{-1} uptake by the larger tumour.

Using the LS174T tumour xenograft model A. Phipps *et al.* (unpublished) have compared the cB72.3(γ1–4) antibodies. After i.v. injection of ^{125}I-labelled cB72.3 the cB72.3(γ1) and cB72.3(γ2) were retained longer in the circulation and with a very similar biodistribution to give higher tumour:blood ratios at 168 h post-injection than the cB72.3(γ3) and cB72.3(γ4) isotypes. At 24 h the cB72.3(γ1) values for % i.d.g^{-1} in the blood and tumour were both approximately double those for the cB72.3(γ4) and this difference increased to four-to five-fold at 168 h. The cB72.3(γ1) and cB72.3(γ4) isotypes were then labelled with ^{111}In. At 24 h slightly more of the ^{111}In–cB72.3(γ1) than the ^{111}In–cB72.3(γ4) was present in the tumour and all of the other tissues with the exception of the kidneys. At 168 h the tumour loading was approximately double for the ^{111}In–cB72.3(γ1), at 22% i.d.g^{-1}, compared to the ^{111}In–cB72.3(γ4). All of the other tissues and the blood had vaues of 1–5% i.d.g^{-1} for both antibodies, with the exception of the kidneys where there was approximately 11% i.d.g^{-1} of ^{111}In-cB72.3 (γ4) compared to 2·7% i.d.g^{-1} for the ^{111}In-cB72.3(γ1).

The cB72.3(γ4) shows considerably higher uptake in the kidney in mouse biodistribution studies when carrying metallic isotopes than the other isotypes. Therefore the cB72.3(γ4) and cB72.3(γ4P) antibodies were each radiolabelled with ^{125}I or ^{111}In and injected i.v. into nude mice bearing LS174T xenografts (Angal *et al.*, 1993). The ^{125}I–cB72.3(γ4P) antibody appeared to be retained in the circulation for longer and the level of antibody in tissues and on the tumour was higher than with the cB72.3(γ4). This was more pronounced at 168 h where 10.1% i.d.g^{-1} was located on the tumour for

the cB72.3(γ4P) antibody compared to 1.4% i.d.g^{-1} for the cB72.3(γ4). As seen for the ^{125}I-labelled antibodies, the ^{111}In-labelled cB72.3(γ4P) results in higher tumour levels. The major difference between the two ^{111}In-labelled antibodies, however, was the level of antibody retained by the kidney. At all of the time points the kidney levels were higher for cB72.3(γ4). This results in a significantly improved tumour:kidney ratio for the ^{111}In–cB72.3(γ4P). The single amino acid change in the γ4P variant therefore confers a considerable advantage in biodistribution in mice when carrying metallic isotopes.

The pharmacokinetics and biodistribution of several of the engineered B72.3 fragments described above has also been studied. ^{125}I-cB72.3(γ4)F(ab′)$_2$ has been tested in the LS174T xenograft model and showed much faster clearance from blood and loss from the animals via the kidney than the whole antibody and consequently lower tumour loading (King *et al.*, 1992b). Very little radioisotope remained after 72 h. However, radiolocalization indices were improved with the F(ab′)$_2$ leading to improved signal-to-noise ratio for tumour imaging. The ^{125}I-cB72.3(γ4)Fab′ and ^{125}I-cB72.3Fv were then compared. Both fragments cleared from the circulation very rapidly, with 5% i.d.g^{-1} of the Fab′ and 0·8% i.d.g^{-1} of the Fv remaining in the blood after 1 h. At 24 h the blood levels had fallen to 0·4 and 0.02% i.d.g^{-1} respectively. However the Fab′ and Fv did localize to the tumour and high radiolocalization indices were obtained. At 24 h the Fv showed the highest tumour:blood ratio (4.1) compared to 1·5 for Fab and F(ab′)$_2$ and 0·97 for whole antibody. The cB72.3(γ4) still gave the highest absolute loading to the tumour.

The scFv behaved *in vivo* in a very similar fashion to the Fv, showing rapid clearance from the blood and consequently relatively low levels of activity accumulated on the tumour (D. Desplancq *et al.*, unpublished). Again high tumour:blood ratios were seen, indicating that these fragments may be particularly useful for tumour imaging.

As a preparation for human studies plasma clearance in cynomolgus monkeys was compared between the ^{125}I-mB72.3, ^{131}I-cB72.3(γ1) and ^{125}I-cB72.3(γ4) using 100 μCi (20 μg) of each antibody and by paired co-injection i.v. of the ^{131}I-cB72.3(γ1) with either of the ^{125}I-labelled antibodies (Hutzell *et al.*, 1991). A different profile was seen from the mouse studies of Hutzell *et al.* (1991) and Meredith *et al.* (1992b). The $t_{1/2}\alpha$ values were 19·3, 14·3 and 20·1 h respectively and the $t_{1/2}\beta$ values were 79·1, 90·3 and 261·7 h, respectively. These values for the chimeric antibodies are similar to those seen recently for human studies where the IgG4 isotype appears to persist longer than an IgG1 isotype (although the chimeric antibodies were not recognizing the same antigen) (LoBuglio *et al.*, 1989; Meredith *et al.*, 1991; Saleh *et al.*, 1992a).

Clinical studies

Two parallel Phase I studies with ^{131}I-cB72.3(γ4) in patients with metastatic or locally recurrent colorectal carcinoma were undertaken and similar results were reported. In the first study six patients for whom conventional therapy

had failed were treated with up to four 10–20 mg doses of ^{131}I-cB72.3(γ4), (20–50 mCi per dose) (Baker *et al.*, 1991; Begent *et al.*, 1990). In the second study 12 patients, initially, were treated with 3·4–6·9 mg ^{131}I-cB72.3(γ4) (34–67 mCi per dose) (Khazaeli *et al.*, 1991) leading to doses of 18 mCi m^2 (n=3), 27 mCi m^2 (n=3) or 36 mCi m^2 (n=6). None of the patients experienced clinical side-effects from infusion of the ^{131}I-cB72.3(γ4). The only toxicity noted was bone marrow suppression when the whole-body irradiation exceeded 60 cGy, leading to a maximum tolerated dose estimate of 60–70 mCi. Both studies revealed two-compartment pharmacokinetics for disappearance of ^{131}I-cB72.3(γ4) from blood, with mean $t_{1/2}\beta$ of 116 h (range 37–166 h) and 224 h (range 102–298 h), respectively. In contrast the mean $t_{1/2}\beta$ for murine B72.3 was reported to be 65 h (range 32–106 h) (Carrasquillo *et al.*, 1988).

The frequency of patients developing a positive HAMA response to cB72.3 was similar between the two studies and was much higher than that seen in the c17-1A trials. In the first study three of six patients developed an immune response, defined as a 10-fold increase over the pre-treatment anti-antibody levels, after a single infusion of cB72.3. In one case IgM which recognized mB72.3 or cB72.3 rose 201- and 193-fold, respectively, over pre-treatment levels, while IgG which recognized mB72.3 or cB72.3 rose 496- and 30-fold, respectively. In this patient peak IgM and IgG titres coincided at 23 days post-infusion. In the second study 7 of 12 patients developed an immune response, defined as a two-fold increase over the pre-treatment anti-antibody levels. Importantly it was established that when a HAMA response was absent or minimal following infusion of chimeric antibody, repeat doses could be given without further side-effects or gross change in pharmacokinetics. In the first study a total of four doses were able to be given to two patients. When HAMA was present however the result was a drastic reduction in serum half-life for subsequently administered doses. For example Khazaeli *et al.* (1991) gave second doses of ^{131}I-cB72.3 to four patients 7–8 weeks after the first infusion. In two cases there was evidence of a HAMA response to the first infusion. In these patients the second dose of antibody was cleared rapidly with whole body half-life falling from around 93 h to around 20 h. In the third patient, with no pre-existing anti-antibody response the kinetics of clearance were similar to the first infusion. In the fourth case the patient had shown a modest antibody response to the first treatment but this had returned to the normal range at the time of the second infusion. This patient showed a slight reduction in whole-body half-life of the ^{131}I-cB72.3, from 117 h to 84 h.

More recently each of the studies has been extended and the HAMA response currently stands at seven instances in 14 patients treated in the first study (R. Begent, personal communication) and in 16 of 24 patients in the second study (A. LoBuglio, personal communication) with a combined HAMA response in 60.5% of patients. This rate is quite similar to that reported for murine B72.3 (Carrasquillo *et al.*, 1988). Recently LoBuglio and co-workers (LoBuglio *et al.*, 1991; Meredith *et al.*, 1992a) have disclosed that five of the patients treated with ^{131}I-cB72.3 in the study of Khazaeli *et al.* (1991) had been previously entered into the c17-1A study described by

Meredith *et al.* (1991). This observation suggests that there may be some feature of the c17-1A antibody structure which, if identified and applied to other antibodies, could lead to further reductions in immunogenicity for chimeric antibodies.

The level of patient response to cB72.3 prompted a more detailed investigation of the specificity of the immune response. Baker *et al.* (1991) showed that the two strongly positive responders in the first study had a high-affinity anti-cB72.3 component of their IgG titre representing 82% and 34% of total specific binding capacity. This binding could be inhibited by unlabelled cB72.3, mB72.3 or B72.3 antibody fragments (F(ab')$_2$, Fab, Fv). This suggested that a major epitope was located within the variable region domains. The remaining binding capacity was a low-affinity component with specificity for the constant regions and also for a possible conformational epitope exposed by certain configurations of murine V regions with human constant regions (e.g. cB72.3(γ3) and cB72.3(γ4) but not cB72.3(γ1) or cB72.3(γ2). This low-affinity component could be mostly accounted for by pre-existing IgG also shown to be present in pre-therapy patient serum. Khazaeli *et al.* (1991), using a different assay technique could not detect binding to cB72.3 in pre-therapy patient serum but found a similar strong anti-variable region response in HAMA-positive patients, accounting for 80–90% of the binding capacity. The remaining binding component in the two patients studied was directed to a neo-epitope shared by antibody molecules containing murine B72.3 variable region and human CH1/κ constant region domains. Subsequently Khazaeli *et al.* (1992) have shown that 62% of patients treated with murine B72.3 developed a HAMA response with V region specificity. The data thus strongly suggest that the B72.3 variable domain contains one or more particularly immunogenic epitopes which mask the potential benefit of reduced immunogenicity for the chimeric antibody. Recently the anti-light chain variable region component of the immune response in the post-treatment sera from one of the patients who developed a strong immune response has been mapped by identifying a number of peptides derived from the B72.3 chimeric light chain sequence which are specifically recognized by the serum (T.S. Baker, unpublished). These peptides have been mapped onto the deduced structure of the Fab' of the chimeric B72.3 (T. Baker and J. Adair, unpublished) which has been obtained by X-ray crystallography (Brady *et al.*, 1991, 1992). The positive regions form two surface patches, one of which overlaps with the antigen binding region.

14.18

MAb 14.18 illustrates some interesting construction methods and expression problems for the various MAb forms, and also some of the alternative strategies for cell killing. Some of the work has been done in parallel with B72.3 studies, affording the opportunity to compare directly various approaches to antibody engineering. 14.18 is a murine IgG3/κ antibody which reacts with the disialoganglioside GD2 expressed on the surface of tumour

cells of neuroectodermal origin (Mujoo *et al.*, 1987). GD2 expression is associated with the invasive phase of melanoma and anti-GD2 MAbs can block attachment of cells to substrate and prevent invasion of melanoma cells through the basement membrane (reviewed in Herlyn, Menrad and Koprowski, 1990). 14.18 may be of benefit as a treatment for neuroblastoma and malignant melanoma.

An IgG2a switch variant of the original IgG3 antibody, 14G2a, has been identified (Mujoo *et al.*, 1989) which is efficient at mediating cytolysis by human ADCC and ADCMC, and against which the engineered forms can be compared. The antigen becomes internalized upon binding of antibody, but quite slowly, and the rate of internalization varies between tumour cell lines (Wargalla and Reisfeld, 1989). Therefore the antigen may be a good target for several MAb-based cell-killing strategies, including direct ADCC or ADCMC, cytokine-enhanced ADCC, immunotoxins, antibody–drug or antibody–isotope conjugates.

Construction and expression of chimeric antibodies and antibody–cytokine fusions

A chimeric mouse–human (IgG1/κ) antibody has been constructed by cloning the heavy and light chain genes as cDNAs (Gillies, Lo and Wesolowski, 1989). Variable region sequences were isolated and cloned into expression vectors containing the human constant region genes using oligonucleotide linkers which extended from a convenient restriction site near the 3′ of V_H and V_L and which recreated the coding sequence. These linkers also included sequences for a splice donor site and intron sequence extending to a convenient restriction site for cloning of the adapted V regions 5′ to the constant region genes. The chimeric genes were cloned into expression vectors with a mutant DHFR gene as the selectable marker, allowing for subsequent gene amplification of the transfected DNA. Transcription was from the mMT promoter for the chimeric heavy chain and a synthetic mouse Ig κ promoter for the light chain. In both cases the murine IgH enhancer was placed upstream of the chimeric genes. The chimeric genes were assembled into a single vector for transfection into Sp2/0–Ag14 cells by protoplast fusion and cell lines resistant to MTX were selected. Initially no antibody-producing clones were found. Although mRNA for the heavy chain could be detected and some H chain protein was secreted, there was no transcription of the light chain. Reconstruction experiments using a sequence from a human hybridoma antibody, anti-J5, which was well expressed and which showed significant sequence homology to 14.18 human light chain V region, demonstrated that the problem was in the 5′ region of the 14.18 cDNA. A hybrid light chain with the leader and framework 1 from the human sequence and the rest of the 14.18 V_L sequence also led to good expression of antibody (Hu-mouse2 antibody), as did a light chain with the human leader fused to the 14.18 V region (ch14.18a). It was concluded that either the murine leader sequence itself or the sequence created in the cDNA after fusion by splicing together of the leader and V region exons was having a negative effect on the expression

of the light chain gene. Cell lines were established after transfection of vectors containing the Hu-mouse2 chimeric light chain. Using 10 mM MTX selection a cell line was established which accumulated 35–45 mg l^{-1} of Hu-mouse2 chimeric antibody in the medium. Subsequently further cell lines were developed in which the synthetic mouse Ig κ promoter was replaced by the mMT promoter and the expression of the DHFR gene was mutated to reduce translation of the mRNA (pdHL2 vectors). These cell lines produced 120–180 mg l^{-1} of antibody at 5 μM MTX selection. The chimeric 14.18 with the Hu-mouse2 light chain, subsequently referred to as ch14.18, therefore contains more human-derived sequence than a normal mouse–human chimeric as the framework 1 region of the light chain is also derived from a human antibody sequence.

An IgG4 form of the antibody has also been produced to act as controls for some of the ADCC and ADCMC assays (Dorai *et al.*, 1991). Several variant forms of ch14.18 have been generated by manipulation of the chimeric heavy chain gene. These include an Fd′ heavy chain, an IgG1 in which the CH2 domain has been deleted (IgG1($\triangle$CH2)), an aglycosylated IgG1 formed by mutation of the CH2 N-linked glycosylation site from asparagine to glutamine by oligonucleotide-directed mutagenesis, and an IgG1 heavy chain in which the codons for the cysteines which normally form the inter-heavy chain disulphide bridges have been mutated to serine codons (γ1S) (Gillies and Wesolowski, 1990; Dorai *et al.*, 1991). MAb variants fused to cytokines have also been created with a view to recruiting lymphokine activated killer cells or tumour infiltrating T-lymphocytes, improving vascular permeability or stimulating an inflammatory response in the tumour vicinity through eosinophil recruitment. In the first instance human lymphotoxin (LT) was attached immediately 3′ to the last codon of CH3 (CH3-LT) or immediately 3′ to the last codon of CH2 replacing CH3 (CH2-LT) or was fused immediately 3′ to the last codon of the hinge replacing CH2 and CH3 to create a Fab′-LT (Gillies *et al.*, 1991b). Similar constructs have been made using the human IL-2 gene (Gillies *et al.*, 1992).

All of these variants were created by manipulation of existing restriction sites in the inter-domain intron regions or by using synthetic oligonucleotide fragments to engineer precise exon fusions or by oligonucleotide-directed site-specific mutagenesis (Zoller and Smith, 1983). Each of these variant heavy chain constant regions was then placed in the pdHL2 expression vector containing the chimeric light chain and the 14.18 heavy variable region and stable cell lines were established in Sp2/0 cells after transfection of the plasmids by protoplast fusion.

The ch14.18 Fab′, Fab′-LT and Fab′-IL-2 did not spontaneously form F(ab′)$_2$ during expression and secretion. In the case of the ch14.18$\triangle$CH2 a proportion of the material (approx 50%) did not form the inter-heavy chain disulphide bridges although all of the material was in the form of H2L2 tetramer. The analogous B72.3 construct behaved in the same way (see earlier). The ch14.18(γ1S) also formed tetrameric material which was associated through the CH3 interactions. The aglycosylated ch14.18(γ1) was shown, by reducing SDS-PAGE, to have a heavy chain which co-migrated

with native heavy chain which had been treated extensively with *N*-glycanase to remove the *N*-linked carbohydrate. A proportion of the CH2-LT protein was also found to be a tetramer (L:CH2-LT)$_2$, covalently linked by inter-heavy chain hinge disulphide bridges. This tetramer was assumed to have formed after transient association through the C-terminal domains of the LT. It was not determined whether these associations allowed the L:CH2-LT fraction to form non-covalently bridged tetramer in solution. The CH3-LT protein was all of the tetramer (L:CH3-LT)$_2$ form. The CH2-IL-2 protein was in the form, L:CH2-IL-2, as judged by non-reducing SDS-PAGE. However the antigen binding properties of this fusion (see below) suggest that it is likely that (L:CH2-IL-2)$_2$ was forming to some extent in solution. The CH3-IL-2 protein was in the (L:CH3-IL-2)$_2$ form which was fully disulphide bridged.

The specific activity of the LT when attached in the CH3-LT fusion was found to be similar to that of the LT standard, but was 100 times lower in the CH2-LT fusion. The specific activity was sensitive to the pH used in the purification process for the fusion. The difference in the specific activity was assumed to be a consequence of the association of the CH3 domains in the CH3-LT fusion, which brings the LT molecules together and may allow LT dimerization and therefore more LT receptor binding. The specific activity of the IL-2 in all of the constructs was found to be $5 \cdot 0 – 6 \cdot 5 \times 10^6$ U mg^{-1}, slightly lower than that of rIL-2 made in bacteria but equivalent to that made in yeast.

Pre-clinical studies

Relative antigen binding ability. The murine antibody 14.18, the murine switch variant 14.G2a, and the ch14.18a(γ1) antibody differ from the Hu-mouse 2 antibody (ch14.18) in their ability to compete for antigen against a constant amount of HRP-conjugated Hu-mouse2 antibody (Gillies, Lo and Wesolowski, 1989). At low antibody concentration the Hu-mouse2 antibody was stated to compete better, but at higher antibody concentration the murine 14.18 and the ch14.18 antibodies competed better. Interestingly the two murine 14.18 antibodies, the original 14.18, an IgG3, and 14.G2a, also showed a difference in their competitive abilities against the Hu-mouse2 (ch14.18) antibody. The Hu-mouse2 (ch14.18) antibody was further compared against 14.G2a (Mueller *et al.*, 1990). The two antibodies bound to GD2-positive M21 human melanoma cells with equal affinity (11·9 nM for ch14.18 and 11·2 nM for 14.G2a) and with the same number of binding sites per cell. Indirect immunofluorescence showed similar patterns of binding to M21 and to the LOX and A375 human melanoma lines.

The various truncated and fusion proteins were found to vary in their antigen binding abilities. The Fab′ and the Fab–IL-2 fusion did not appear to bind to antigen, suggesting that bivalency is required to generate detectable binding (Gillies and Wesolowski, 1990; Gillies *et al.*, 1992). The c14.18△CH2 antibody showed an approximately three-fold increase in antigen binding

compared to the parental ch14.18 and was more efficient at competing with the ch14.18 for antigen in a 2 h competition assay. However in an 18 h competition assay the difference was much less obvious, suggesting that the ch14.18$\triangle$CH2 variant was superior in on rate rather than in overall affinity for antigen (Gillies and Wesolowski, 1990). Interestingly the γ1S variant, in which the CH2 domain is present but the inter-heavy chain disulphide bridges do not form, showed slightly lower competitive ability compared to the parental antibody (Gillies and Wesolowski, 1990). Therefore it was concluded that the lack of disulphide bridges in a proportion of the $\triangle$CH2 variant was not an influence on the antigen binding seen. Rather it was suggested that the Fab portion of the antibody is normally sterically restricted by the Fc region and that removal of the CH2 domain allows a greater degree of freedom for initial binding of the antibody to antigen (Gillies and Wesolowski, 1990).

The aglycosylated form of the antibody showed little difference in competitive binding compared to the parental antibody in the 2 h assay, but there was an apparent difference in the binding over 18 h, with the aglycosylated form shwoing reduced competitive ability (two- to three-fold) (Dorai *et al.*, 1991). The LT fusions were stated to have higher direct antigen binding than ch14.18 on membrane extracts from neuroblastoma cells. However, the fusion proteins did not compete more efficiently with ch14.18 for antigen using HRP-ch14.18 as the tracer (Gillies *et al.*, 1991). It was proposed that the membrane extract may contain LT receptor, which may allow higher binding of the fusion proteins than the ch14.18.

ADCC.   The ability of the ch14.18 antibody to cause ADCC was measured using human PBMC as effectors (Mueller *et al.*, 1990) in a 4 h ^{51}Cr release assay. Using M21 cells as targets at low antibody concentrations the ch14.18(γ1) was superior to the 14.G2a for a given E:T ratio. The difference was less marked at higher antibody concentrations as the per cent lysis for the 14.G2a approached the levels achieved at lower antibody concentrations for the ch14.18. However the ch14.18 antibody was 50–100-fold more effective at ADCC on M21 cells than the 14G2a. At the highest antibody concentrations used there was an indication of an inhibitory effect on ADCC against the M21 cells. In one experiment with A375 cells the cells were shown to be more resistant to ADCC at low antibody concentrations, but ADCC was equivalent to that seen for M21 cells at higher antibody concentrations. Again the ch14.18 was superior to the 14.G2a.

In a second study, Barker *et al.* (1991) reported the use of ch14.18 for ADCC of GD2 positive ^{51}Cr-labelled neuroblastoma cells. Again the ch14.18 antibody was superior to the 14.G2a antibody in that less antibody was required to achieve maximal cell lysis of NMB-7, LAN-1 and IMR-32 neuroblastoma cell lines using PBMC from healthy donors. This lysis was probably effected by NK cells. With the ch14.18 the NK cell population was able to cause approximately double the ADCC compared to the same number of the PBMCs and at E:T of 10 the ADCC approached that seen for the ch14.18 with NK cells.

ch14.18 antibody was also superior to the 14.G2a antibody in ADCC using granulocytes as effectors when the antibody concentration was lower than 100 ng ml^{-1} in the assay. Above this concentration the 14.G2a reached the same level of lysis. Granulocytes were also shown to be better effectors than PBMC using ch14.18, when both cell populations were obtained from the same donor. The effectiveness of the granulocytes could be increased using 10 ng ml^{-1} of GM-CSF. GM-CSF had no effect on ADCC using PBMC as effectors. Granulocytes from neuroblastoma patients showed a similar effectiveness which was greater than PBMCs from the same patients. The granulocytes were able to be further stimulated by GM-CSF to cause greater levels of lysis, but only at low E:T ratios. It was postulated that the GM-CSF may be causing up-regulation of Fcγ receptors on the cells. Finally it was mentioned that granulocytes from neuroblastoma patients previously treated with 14.G2a could be removed, stimulated *ex vivo* with GM-CSF, and returned to the patient and would then preferentially migrate to the tumour site.

The ability of the aglycosylated ch14.18 to cause ADCC of M21 cells was measured (Dorai *et al.*, 1991). The aglycosylated IgG1 antibody showed the same, minimal, level of ADCC as an IgG4 version of the ch14.18 antibody. The ch14.18△CH2 and ch14.18(γ1S) antibodies showed little ability to cause ADCC using M21 cells as targets and PBMCs as effectors. The two derivative antibodies were over 100-fold poorer at ADCC compared to the ch14.18 cointrol. The ch14.18–LT fusions were tested for ADCC activity (Gillies *et al.*, 1991) using M21 cells as targets and PBMCs as effectors. In these experiments the CH2-LT fusion showed activity only at the highest antibody concentration used, where it was comparable to the ch14.18 and the CH3-LT fusion. The CH3-LT fusion showed activity comparable to the ch14.18 at all of the antibody concentrations tested. The loss of activity of the CH2–LT fusion at lower antibody concentrations may be a consequence of the structural heterogeneity of the CH2–LT fusion.

ADCMC.   ADCMC was measured using human complement to lyse ^{51}Cr-labelled M21 or A375 cells (Mueller *et al.*, 1990). The ch14.18 and 14.G2a antibodies were equally able to cause ADCMC of M21 cells. However the A375 cells were more resistant and maximal lysis was not obtained even at the highest levels of antibody used. In this case the ch14.18 appeared to give twice as much lysis for a given antibody concentration as the 14.G2a. The aglycosylated ch14.18 was tested in the ADCMC assay and showed demonstrable, although much reduced, activity (Dorai *et al.*, 1991) requiring 10-fold more antibody to achieve the same level of lysis as ch14.18. This contrasts with work by others (Tao and Morrison, 1989), who showed no ADCMC with aglycosylated IgG1 and IgG3 mouse–human chimeric anti-DNS antibodies.

The ch14.18△CH2 and ch14.18(γ1S) antibodies differed in their ability to cause ADCMC of M21 cells with human complement (Gillies and Wesolowski, 1990). The ch14.18(γ1S) antibody showed approximately 10-fold lower ADCMC compared to the ch14.18 control. In these experiments the ch14.18△CH2 was completely inactive. The ch14.18-LT fusions

were tested for ADCMC activity (Gillies *et al.*, 1991). In these experiments the CH2–LT fusion was not active while the CH3–LT fusion showed significant activity requiring approximately two-fold more antibody than the ch14.18 to reach 50% of maximal cell lysis. However, the maximal achievable lysis was lower for the CH3–LT (around 70%) than for the ch14.18 antibody (>90%). The loss of activity of the CH2–LT fusion is consistent with the sensitivity of ADCMC to structural alterations in the CH2 domain.

Cytokine activity in antibody–cytokine fusions.   The cytolytic and cytostatic effects of the lymphotoxin fusions were assessed using murine L929 cells (LT receptor positive, GD2 negative) and human GD2 positive (LT receptor negative) M21 cells (Gillies *et al.*, 1991). It was shown that the CH3–LT possessed activity equivalent to LT in both the cytolytic and cytostatic assays on L929 cells (corrected for the proportion of LT in the fusion). The CH2–LT however showed substantially less activity, but the differences were much less at higher LT levels, being equivalent at 2 nM of LT dose. This difference may be due to the requirement for the LT to form a trimer to be functionally active.

Using M21 cells in the cytolytic and cytostatic assays no direct cell killing was seen with CH3–LT or with ch14.18 over a wide range of LT concentration. CH2–LT was not tested. The assay was performed over 48 h, during which time a substantial amount of the fusion could have become internalized (Wargalla and Reisfeld, 1989; Mueller *et al.*, 1990). It might therefore be expected that the killing effect using this fusion would be by secondary immune recruitment processes in those cases where the tumour cells did not express, or could not be induced to express, the LT receptor.

In the case of the IL-2 fusions the assays used were the antibody-induced proliferation or cytolysis of mouse or human T-cell lines, either a murine cytotoxic T-lymphocytic cell line (CTLL-2), or tumour-infiltrating T-cell line from a metastatic melanoma (600 TIL) (Gillies *et al.*, 1992). The CH3-IL-2 fusion induced dose-dependent proliferation of both CTLL-2 and 660TIL cells over the range 2–2000 ng ml^{-1}. The specific activity of the IL-2 in the fusions was seen to be slightly lower than that of IL-2 derived from *E. coli*, but slightly higher than that of Il-2 from yeast cells.

When 660 TIL cells, freshly stimulated with antigen, were tested for their ability to kill autologous melanoma cells (660 MEL), the CH3-IL-2 fusion was shown to improve cell killing. In general there was a higher level of lysis if the 660 TIL cells had been pre-stimulated with IL-2 for 4 days in culture prior to assay. The effect of the CH3–IL-2 was even more marked if the cells were rested for one week after antigenic stimulation prior to assay.

Animal studies.   ch14.18 and 14.G2a antibodies showed similar kinetics of blood clearance in nude mice after i.v. injection of ^{125}I-labelled antibody and showed similar distribution patterns in nude mice bearing M21 xenografts (Mueller *et al.*, 1990). However tumour:blood ratios remained at about 1 at both 24 and 96 h for both antibodies. The kinetics of clearance from the blood of nude mice was examined for the 14.18△CH2 and aglycosylated antibodies

(Mueller, Reisfeld and Gillies, 1990b; Dorai *et al.*, 1991). For both variants the initial clearance phase was much faster than for ch14.18. However, the β-phase for the aglycosylated antibody was stated to be the same as that for ch14.18 with half-lives of 5 d (Dorai *et al.*, 1991). This observation agrees with that of Tao and Morrison (1989) who found no difference in the $t_{1/2}β$ of normal and aglycosylated mouse-human chimeric IgG1 and showed a $t_{1/2}β$ of 6 d. For the 14.18△CH2 antibody the initial clearance of antibody was faster than for the aglycosylated antibody and the $t_{1/2}β$ was 12 h (Mueller, Reisfeld and Gillies, 1990; Dorai *et al.*, 1991). In the latter study (Mueller, Reisfeld and Gillies, 1990) the $t_{1/2}α$ was given as 1.5 h and the clearance was stated to be equivalent to that of $F(ab')_2$ of human IgG. At 4, 24 and 96 h post-injection tumour:blood ratios for the 14.18△CH2 were 1·29, 3·15 and 1·2 compared to 0·18, 0·56 and 1·08 for the ch14.18 antibody. At 4 h there was three-fold more 14.18△CH2 on the tumour than the ch14.18. However at 24 h the situation was reversed with the ch14.18 showing 3·5-fold more antibody on the tumour.

Clinical studies

Saleh *et al.* (1992a) have reported the results of pharmacokinetic and immunogenicity studies from a Phase I trial using ch14.18 in patients with metastatic melanoma. Nine patients received single doses of ch14.18 by i.v. infusion at doses of either 5 mg (*n*=3), 15 mg (*n*=3), 45 mg (*n*-3), while four patients received 50 mg on two consecutive days. Serum samples were taken regularly for analysis of pharmacokinetics and anti-antibody responses. At the 45 and 100 mg doses seven of the 13 patients registered infusion-related abdominal and pelvic pain, or nausea. The pain subsided soon after the ending of the infusion. The antibody clearance from the blood followed two-compartment kinetics and the mean $t_{1/2}α$ was 24±1 h and $t_{1/2}β$ was 181±73 h. Considerable interpatient variation was seen, similar to that seen for the c17-1A and cB72.3 studies. The $t_{1/2}β$ for the ch14.18 was given as 42±6 h (Saleh *et al.*, 1992b). Eight of the 13 patients showed a significant increase in anti-antibody response over pre-treatment levels. (None of the patients had been previously treated with the murine antibody or any other MAb.) Using an assay similar to that used for the c17-1A and cB72.3 studies (LoBuglio *et al.*, 1989, Khazaeli *et al.*, 1991) five patients showed weak, and three patients showed moderate (>10-fold increase over pre-treatment levels) antibody responses. In general these responses were most noticeable 15 d and longer post-infusion. In all of the cases the serum binding to ch14.18 could be inhibited by both ch14.18 and the murine antibody 14.G2a, suggesting a predominantly anti-variable region response. This weak response was different from that seen for the murine antibody where a 100 to 1000-fold higher response was seen (Saleh *et al.*, 1992b). In those patients receiving 45 mg or more antibody could be detected in tumour biopsies 3–5 days post-infusion. However saturation of GD2 in the tumours was not achieved. Use of the antibody at these doses did not appear to affect clinical outcome in these very early clinical studies.

L6

L6 is an IgG2a/κ murine MAb which reacts with a cell surface carbohydrate antigen found on many human lung, breast, colon and ovary carcinoma cells (Hellstrom, Beaumier and Hellstrom, 1986; Hellstrom *et al.*, 1986). The antigen is found on some normal endothelial cell tissue (DeNardo *et al.*, 1991b) and a putative cDNA clone expressing a 24 kDa protein to which L6 binds antigen has been identified by expression cloning in COS cells (Marken *et al.*, 1992). The antigen is related to other membrane proteins associated with cell growth, including CD63 and CO-029, both of which are over-expressed on tumour cells (Marken *et al.*, 1992).

Construction and expression of chimeric antibodies and fragments

Liu *et al.* (1987b) prepared a chimeric (IgG1/κ) version of the murine antibody, cL6(γ1) using a cDNA approach (Liu *et al.*, 1987a). A cDNA library (Gubler and Hoffman, 1983) was screened using J-specific oligonucleotide probes and light and heavy chain cDNAs identified. These were modified to introduce cloning sites 5′ to the signal sequences, in the J sequence of the light chain, and in the 5′ region of the CH1 exon of the heavy chain for attachment to human constant regions. At the same time the murine CH1 sequence 5′ to the cloning site was modified to the human sequence to ensure that no novel sequence motifs were introduced in the cloning process. The chimeric sequences were isolated and cloned into expression vectors. Expression of the antibody genes was accomplished using the SV40 early promoter, mouse heavy chain enhancer, and transcription termination and polyA signals were SV40. The mouse heavy chain enhancer was placed upstream of the promoter in the plasmids. Finally a single vector expression system was prepared by introducing the entire chimeric light chain expression cassette 5′ to the chimeric heavy chain cassette and in the same transcriptional orientation to give the final vector, pING2114. The selectable marker on all of the plasmids was the *neo* gene. pING2114 was transfected into Sp2/0 cells by electroporation. In all cell lines more heavy chain was produced than light chain. For example, clone 3E3 produced 100 μg l^{-1} of light chain but 700 μg l^{-1} of heavy chain. Subsequently the balance was redressed by re-transfecting a second light chain vector carrying the *gpt* marker and 1 mg l^{-1} of assembled, secreted antibody could be obtained. Material was also produced from ascites fluid after injection of 10^6 cells of the cell line into pristane-treated mice. Material from ascites fluid and cell culture appeared to be equivalent in potency in the *in vitro* tests described. More recently cL6(γ1) has been re-expressed in X63.Ag8.653 cells (Hellstrom *et al.*, 1990). of cL6 was expressed in Sp2/0 cells by reconstructing the chimeric heavy chain gene so that the codon for the first of the two cysteines in the hinge which normally form the inter-heavy chain disulphide bridges was mutated to a termination codon, simultaneously introducing a restriction site for sub-cloning into the heavy chain expression vector. The cFab expression vector and the chimeric light chain expression vector were then transfected into Sp2/0 cells and cell

lines secreting cFab were identified. No yields have been reported (Better *et al.*, 1988). Expression of cL6(γ1) and cL6 (F(ab')$_2$ was attempted in yeast cells (Horwitz *et al.*, 1988; Better and Horwitz, 1989). cL6(γ1) was the first antibody with therapeutic potential to be expressed in yeast cells. Yeasts are attractive potential expression hosts because of their well understood technology for large-scale handling, and because, unlike *E. coli*, yeasts are capable of glycosylation of proteins at the asparagine-X-serine/threonine motif used in higher eukaryotes. However the glycosylation is not identical to that seen in hybridomas and myelomas and the carbohydrate is found as a high mannose form (Kukuruzinska, Bergh and Kackson, 1987). Cassettes for cL6(γ1) expression were assembled which consisted of the yeast PGK promoter, the yeast invertase signal sequence, and the antibody genes in cDNA format followed by the PGK transcriptional terminator. The cassettes were bounded by convenient cloning sites. A two-vector system was used in which the light chain cassette was placed on a 2 μm plasmid with the *leu2* marker, and heavy chain cassettes were placed on a derivative of 2 μm which contained the origin of replication and the Rep3 sequence. The selectable marker was the *ura3* gene. The chimeric Fd gene was also cloned into a *ura3* vector.

The light chain vector was co-transfected with each of the heavy chain vectors into *S. cerevisiae* (*leu2 ura3*). Antibody was shown to be secreted into the medium. For cL6(γ1) around 70% of the secreted light and heavy chains were shown by ELISA to be assembled together. Yields were approximately 100 ng ml^{-1} for the light chain and 50–80 ng ml^{-1} for the heavy chain. One clone was grown at 10-l scale and antibody purified by protein A. The antibody appeared to have two heavy-chain forms on reducing SDS-PAGE, probably glycosylation variants, but the light chain and the heavy chain with the higher apparent molecular weight co-electrophoresed with the Sp2/0-derived material. On non-reducing SDS-PAGE an M_r 150 kDa band was seen. Other products, possibly H$_2$L and HL products, were also observed. Fab was secreted in the same way and accumulated to 100–200 ng ml^{-1}. It was purified on S-Sepharose and resolved into M_r 23 and 25 kDa bands on reducing SDS-PAGE and M_r 47 kDa on non-reducing SDS-PAGE.

L6 was also the first antibody for which expression and secretion of an active Fab in *E. coli* was reported (Better *et al.*, 1988). The light chain and Fd genes were essentially the same as in Horwitz *et al.* (1988, see above) but with the Ig signal sequences replaced by those of *pelB*, and were transcribed from the *Salmonella typhimurium araB* promoter in a bi-cistronic gene format. When introduced into *E. coli* MC1061 90% of the exported Fab could be found in the culture medium. Yield before purification was 2 mg l^{-1}. The material was found to be M_r 48 kDa on non-reducing SDS-PAGE and to consist of 23 and 25 kDa species on reducing SDS-PAGE. Therefore the inter-chain disulphide bridges were formed during export. It was shown that significant amounts of material remained within the cell (Better and Horwitz, 1989), suggesting yield improvement was possible. Better, Weickmann and Lin (1990) reported improvement by fermentation optimization which have improved yields to >500 mg l^{-1} in controlled fermentation conditions.

More recently a Fab-Il-2 fusion has been constructed (Fell *et al.*, 1991) in a

manner similar to that described earlier for 14.18. In this case the light and heavy chain variable regions were cloned from the L6 hybridoma as genomic fragments along with their respective promoters and enhancers. The chimeric light chain was assembled in a *gpt*-based expression vector. The heavy variable region was assembled along with the human IgG1 CH1 exon and relevant splice sites and a synthetic hinge region in which the two codons for the cysteines which normally form the inter-heavy chain disulphide bridges were mutated to proline and serine, respectively, together with a linker region coding for 13 amino acids and followed by a PCR-generated cDNA sequence coding for the mature form of human IL-2. This whole cassette was assembled in a *neo*-based expression vector. The two vectors were co-transfected by electroporation into the X63.Ag8.653 cell line. A cell line was identified producing a fusion protein which gave the same signal in a sandwich ELISA using anti-idiotype V_L and V_H monoclonal antibodies as 8.5 mg l^{-1} cL6(γ1). The fusion protein was shown to have both L6 and IL-2 properties by binding to the human 2981 carcinoma cell line (L6 antigen positive) and to PHA-activated human peripheral blood T-cell blasts (which are IL-2 receptor positive).

Pre-clinical studies

Relative antigen binding ability.   The Sp2/0 derived cL6(γ1) and the mL6 antibodies bound to antigen on the human colon carcinoma cell line C-3347 (also referred to as H-3347 in associated papers; Hellstrom *et al.*, 1986, 1990; Liu *et al.*, 1987c) and were equally capable of inhibiting the binding of FITC-labelled mL6. Neither antibody bound to the human T-cell line HSB-2. Binding by both antibodies to the melanoma cell line M-2669 was low but detectable (Liu *et al.*, 1987b). Similarly the Sp2/0 and yeast-derived cL6(γ1) antibodies bound equally well to antigen on C-3347 cells and were equally capable of inhibiting the binding of FITC-labelled mL6 (Horwitz *et al.*, 1988). cFab derived from cL6(γ1) by papain digestion and murine Fab derived from mL6 by papain digestion also bound to the C-3347 cells and competed for binding sites with mL6 but with lower efficiency due to monovalency, as did the cFab produced from yeast (Horwitz *et al.*, 1988). The *E. coli*-derived Fab was shown to compete with murine L6 for binding to the antigen on the carinoma cell line C-3347 in a manner identical to cL6 Fab produced from Sp2/0, Fab produced by papain digestion of cL6(γ1) from Sp2/0 cells, and mL6 Fab produced by papain digestion of hybridoma-derived material (Better *et al.*, 1988).

ADCC.   The cL6(γ1) was more effective in a 4 h ^{51}Cr release ADCC assay using C-3347 cells as target and lymphocytes (<5% NK activity) as effectors than the mL6, reaching 50% maximal lysis at 100-fold lower antibody concentration than for the murine antibody (Liu *et al.*, 1987b). Significant ADCC by cL6(γ1) was seen at very low E:T ratios. The cL6(γ1) differed from the mL6 in that it was also able to cause low, but significant, ADCC of the

M-2669 melanoma cell line on which the L6 antigen is poorly expressed. In previous studies (Hellstrom, Brankovan and Hellstrom, 1985; Hellstrom, Beaumier and Hellstrom, 1986) the effector cells in these separated lymphocyte preparations used by mL6 to cause ADCC had been shown to be Leu11b+, a marker for NK cells. Separation of the lymphocytes into T-cells and large granular leukocytes showed that the ADCC activity was associated with the latter cells. Removal of adherent cells by overnight incubation of the lymphocytes on plastic increased the ADCC activity of the effector cells. More recently Adams *et al.* (1992) have used mL6 and cL6(γ1) derived from ascites culture of the hybridoma and Sp2/0 cells in pristane-treated mice in an 18 h ^{51}Cr release assay. The target cells were HBT-3477 cells and the effectors were PBMCs depleted of adherent cells. The cL6(γ1) was three times more effective than the mL6 at causing ADCC, with 50–60% maximal lysis occurring. cL6(γ1) derived from yeast cells has been shown to cause ADCC at a higher level than that for the Sp2/0-derived material (Horwitz *et al.*, 1988). From the data presented it appears that, at sub-saturating levels of antibody in the assay, the same level of lysis can be achieved by 10-fold lower amount of the yeast-derived cL6 than the Sp2/0-derived cL6(γ1).

ADCMC. cL6(γ1) achieved approximately two-fold more ADCMC compared to mL6 (Liu *et al.*, 1987b) at all dilutions of complement used. Similarly Adams *et al.* (1992) showed that human complement-mediated lysis of HBT-3477 cells was more effective when the cL6(γ1) was used rather than the mL6. In these assays the ADCMC mediated by the cL6(γ1) was significantly higher at lower antibody concentrations. Yeast-derived cL6(γ1) was unable to cause ADCMC, presumably due to the variation in glycosylation between the material from the mammalian cell and yeast sources (Horwitz *et al.*, 1988).

Cytokine activity in antibody–cytokine fusions. The cL6 Fab-IL-2 fusion induced the same pattern of rapid, post-receptor binding and phosphorylation of specific intra-cellular proteins in PHA-stimulated T-cell blasts as rIL-2, and in the same dose range (Fell *et al.*, 1991). The fusion was shown to be capable of supporting the growth of IL-2-dependent CTLL cells (CTLL-2 cells). However the specific activity of the fusion protein was approximately 200-fold lower than an equivalent molar quantity of rIL-2. When the cL6(γ1) antibody, or the cL6 Fab-IL-2 fusion was added to antigen-positive tumour cells (3347 cells) irradiated prior to assay to reduce their proliferation, and these were then used to stimulate proliferation of the CTLL-2 cells, it was shown that the cL6(γ1) was ineffective but that the fusion, in the amounts used in the assay, could induce a 600-fold stimulation over the control values. The fusion protein was less able to recruit resting PBMC (bearing the low-affinity IL-2 receptor) to lyse L6 antigen-positive 2981 cells, but a 10-fold higher molar concentration of IL-2 equivalents was needed compared to rIL-2 in the assay. However activated blast cells stimulated for 3 d prior to assay with immobilized anti-CD3, and then depleted of NK cells and bearing the high-affinity receptor, showed activity equivalent to the rIL-2 over the range tested. Both rIL-2 and the fusion were much less active with the activated

cells than was the rIL-2 with the resting PBLs. It was concluded that while the specific activity of the fusion was much lower than that of the rIL-2, coupling to the antibody could give lower non-specific systemic toxicity with a higher degree of specific tumour cell killing.

Animal studies.   The cL6(γ1) and the mL6 have been compared for pharmacokinetics and biodistribution in a nude mouse xenograft model (Adams *et al.*, 1992). HBT-3477 cells were established as intra-peritoneal xenografts and when the tumours reached 100–200 mg in size the ^{125}I-labelled antibodies were injected i.v. The observed half-lives for the cL6(γ1) and the mL6 were very similar (approximately 5 d versus 6 d, respectively) with a larger initial loss of the ^{125}I-cL6(γ1) from the blood than that seen for the ^{125}I-mL6. The mL6 accumulated more efficiently at the tumour with approximately double the levels present at 24 h post-injection. All of the organs had higher accumulation of mL6 than cL6(γ1). However the non-specific uptake into tissues was reflected by tissue:blood ratios which remained at around 0.25 for both cL6(γ1) and mL6 over a 120 h period. During this time tumour:blood ratios increased from around 0.5 to 0.75 for the cL6(γ1) and from 0.75 to 1.1 for the mL6. From calculations of blood half-life and the expected tumour perfusion and antibody extraction rate it was concluded that the differences in tumour uptake could be entirely accounted for by the longer blood half-life of the mL6.

Clinical studies

^{131}I-labelled cL6 has been used in a clinical study in patients with metastatic breast cancer (DeNardo *et al.*, 1991b, c). Preliminary studies with the murine antibody showed that normal lung tissue expressed the antigen recognized by L6 but non-specific binding could be blocked by pre-treatment with cold antibody. Therefore cL6 was given i.v. after previous treatment with either cold murine L6 or cL6. Blood clearance ($t_{1/2}\beta$) was stated to be 0·8–1·5 d for the murine L6 and 1·5–2·5 d for cL6, with 0·06–0·12% i.d.g^{-1} tumour uptake and tumour clearance $t_{1/2}$ of 1·8–2·7 d. Three patients, each given two doses of ^{131}I-labelled cL6, showed 50%, 70% and 75% reduction of tumour load, respectively. However grade 2 marrow toxicity was seen at doses of 50 and 60 mCi m^{-2} and grade 4 toxicity at 70 mCi m^{-2}. In a study reported by Goodman (1991) on the immune response to patients treated with cL6 HAMA was measured in four of 17 patients tested two weeks after treatment and the number rose to 50% after 11 weeks of study.

ANTI-CD4 CHIMERIC ANTIBODIES

CD4 is the cell surface antigen which specifies the T_H subset of T-cells. As mentioned above, anti-CD4 MAbs are under evaluation as immunosuppressive agents (initially for allograft rejection, rheumatoid arthritis psoriasis and as a means of overcoming HAMA responses to therapeutic murine MAbs) and for treatment of T-cell lymphoma.

SK3

Knox *et al.* (1991) have briefly reported the construction of a chimeric version of SK3 (a murine MAb), and preliminary evaluation in patients. The initial intention was to produce a non-immunogenic version of the murine anti-CD4 antibody to treat mycosis fungoides (MF), a T-cell lymphoma primarily of CD4+ cells, which infiltrates the skin and eventually other organs. Antibody SK3 had not previously been tested in patients so baseline efficacy and HAMA response data were not available.

Construction and expression of chimeric antibodies

The variable regions of SK3 were cloned using the genomic cloning procedure. The variable sequence DNAs were cloned 5′ to human IgG1 and κ constant region sequences and the expression vectors introduced into mouse myeloma cells by protoplast fusion. Stable cell lines expressing cSK3 were produced and used to produce antibody as ascites in pathogen-free mice. In general this would not be a preferred route for the production of a chimeric antibody because of the possibility of cross-contamination with murine IgG. Antibody was purified and shown to have immunoreactivity similar to that of the murine SK3.

Clinical studies

A limited clinical study with MF patients using cSK3(γ1) antibody has been described (Knox *et al.*, 1991). Seven patients with persistent or progressive CD4+ MF and no history of prior antibody therapy or recent chemo-, radio- or UV-therapy were treated with doses of either 10, 20, 40 or 80 mg of antibody i.v., twice weekly for three weeks, and were followed for 12 weeks post-treatment. The half-life of the antibody ranged from 21 to 42 h and of the seven patients only the one who had the highest absolute dose (1·83 mg kg^{-1}), showed detectable antibody remaining prior to the proceeding infusion. All patients treated showed some modest improvement in one or more of the clinical parameters measured, and some patients showed transient partial or minor disease remission. Patients whose lymphocytes were scored as responders in a mixed lymphocyte (MLR) reaction and which reacted to keyhold limpet haemocyanin (KLH) or tetanus toxoid in T-cell proliferation assays prior to treatment were shown to be immunosuppressed by the anti-CD4 treatment. However the majority of the patients showed little pre-treatment responses in these assays and two patients were shown, by lack of initial response to co-inoculation with KLH, to be immuno-incompetent. Coating of CD4+ cells in peripheral blood was observed but there was no significant depletion of CD4+ cells from the lymphocyte population, although there may have been some modulation of CD4 from the cell surface. It is not clear how the partial clinical responses were brought about, especially in the lower dose patients where cSK3 could not be detected in post-infusion biopsies. Of the five patients capable of mounting an immune

response two demonstrated a low titre (1:64 and 1:256) anti-variable region response to cSK3, which developed between days 10 and 18 of treatment. These patients later developed minor allotypic responses against the chimeric antibody, and also, along with one other patient, developed an anti-murine IgG1 response (possibly due to contaminating murine antibody in the antibody preparations). Further studies are planned to compare cSK3 directly with the murine antibody and to establish maximal tolerated doses for clinical efficacy.

M-T412

The murine MAb M-T412 is an IgG2a/κ antibody which recognizes an epitope involving residues in both the V1 and V2 domains of human CD4 (Ghrayeb, Knight and Looney, 1991).

Construction and expression of chimeric antibodies

The chimeric antibody was constructed by isolating the rearranged variable regions from genomic DNA using J region probes. The heavy and light chain genomic fragments, with the Ig promoters, were transferred into pSV2-derived vectors (Sun *et al.*, 1987, see above) to give two light chain expression vectors, with either the *neo* or *gpt* selectable markers, a heavy chain expression vector consisting of a chimeric IgG1 heavy chain gene with the *gpt* selectable marker, and a single vector with both chimeric genes and *gpt* selectable marker. Suitable combinations of the separate vectors, or the single vector, were transfected into Sp2/0 cells by electroporation. A cell line, JL3A3, was produced which yielded 28 mg l^{-1} post-Protein A-Sepharose purification. It was not stated from which vector combination the cell line was derived (Ghrayeb, Knight and Looney, 1991).

Pre-clinical studies

Relative antigen binding ability. The chimeric antibody was shown to bind to CD4+ CEM cells in a direct binding format assay. The murine antibody bound with a relative affinity of 6.5×10^9 M^{-1} while the chimeric antibody gave values of 2.5 and 8×10^9 M^{-1} in two experiments.

Animal studies. The chimeric antibody was administered i.v. to four chimpanzees at 5 mg^{-1} kg^{-1} d for 7 d. CD4+ cell counts were markedly reduced from the first injection until 2–3 weeks after the dosing regime ended. Cell numbers began to increase after 3–4 weeks post-injections but remained below control levels for 3–4 months. No anti-antibody responses were seen over the course of the experiments and no other adverse reactions were noted.

Clinical studies

Fifteen patients with rheumatoid arthritis were treated with single i.v. doses of 1–200 mg of the cM-T412 antibody prepared from Sp2/0 cells (Ghrayeb, Knight and Looney, 1991). The antibody was well tolerated with only transient flu-like symptoms at the time of injection. CD4+ cells were depleted for up to 14 d post-treatment but had returned almost to normal levels after 35 d. CD8+ cells were transiently depleted but returned to normal levels after 3 d. It was reported that there were modest anti-mouse responses in 8 of the 15 patients but the levels relative to anti-murine HAMA were not stated. Clinical responses were seen with significant reductions in swollen joints at up to 21 days and of tender joints at up to 90 d post-injection.

Subsequently two reports have described the use of the antibody for the treatment of psoriasis (Prinz *et al.*, 1991) and relapsing polychondritis, (Choy *et al.*, 1991). In the first article Prinz *et al.* (1991) report the use of cM-T412 in the treatment of a 63-year-old man with generalized pustular psoriasis. cM-T412 was given i.v. at 10 or 20 mg doses alternately on days 1, 2, 3, 6 and 7. From day 2 the pustules began to dry and by day 11 most of the acute symptoms had disappeared, as had chronic plaques, present for 44 years previously. The improvement was correlated with a marked reduction in CD4/CD8 T-cell ratio which fell from 1.8 to 0.5, a drop in leukocyte count from 19.8 to 5.4×10^9 l^{-1}, a reduction in serum C reactive protein which fell from 164 mg l^{-1} prior to therapy to 16 mg l^{-1} at day 20, and a loss of T-cells from the inflammatory infiltrate in the skin. In a second report Choy *et al.* (1991) report the use of cM-T412 in the treatment of a 27-year-old with relapsing polychondritis. The patient was treated with oral prednisolone and dexamethosone which partially reduced the symptoms. Treatment with 50 mg doses of the cM-T412 every 14 days for six weeks in addition to the other treatments caused a reductgion in symptoms and allowed the steroid treatment to be reduced. No side-effects of treatment were reported, but it was not noted whether HAMA response was measured. CD4+ cells fell from 5×10^8 l^{-1} to 1.56×10^8 l^1 after treatment and then returned to 3.9×10^8 l^{-1} two weeks post-treatment. The patient's lymphocyte proliferative responsiveness to tuberculin PPD, pre-antibody therapy, was abolished after treatment.

Fully humanized antibodies in research and clinical development

Overall the clinical studies with chimeric antibodies suggest that chimerization will in general reduce immunogenicity significantly but not completely, and that the remaining HAMA response has both anti-binding site and anti-framework components. The prospects for greater clinical utility of fully humanized antibodies have been addressed using model studies in mice, by two studies with fully humanized MAbs in primates, and by four small clinical studies with two fully humanized MAbs (CAMPATH-1H and H17E2).

The potential immunogenicity of chimeric and reconstructed syngeneic antibodies has been addressed in an elegant mouse model study (Bruggemann

et al., 1989b). The plasmocytoma J558L secrets a λ light chain which, when combined with a suitable heavy chain, gives rise to antibody which can bind to the hapten NP. A number of different heavy chain genes were constructed for transfection. The first was the humanized heavy chain, HuV_{NP}-Huγ2 (described earlier) from the B1.8 MAb. The second gene used the same variable region as HuV_{NP}-Huγ2, but the constant region was derived from a murine IgG2b gene from the C57BL/6 mouse, to give HuV_{NP}-Moγ2B. Finally the murine variable region from the B1.8 light chain was attached to the murine IgG2b constant region gene to give MoV_{NP}-Moγ2b. Antibody was prepared and injected i.p. into (C57BL/6 × BALB/c)F_1 mice. The strongest immune response was against the HuV_{NP}-Huγ2 antibody, with 10% of it directed against the variable region and 90% of it directed against the constant region. The MoV_{NP}-Moγ2b antibody did not generate a response, unlike the HuV_{NP}-Moγ2b antibody, for which the response was directed at the V region and was of the same order as the anti-V region response seen against the HuV_{NP}-Huγ2 antibody. Some of the anti-V region response generated from the HuV_{NP}-Huγ2 or HuV_{NP}-Moγ2b immunizations was directed against the binding site as there was a response to the mouse V region. When a further variant, MoV_{NP}-Huγ2, was used to immunize mice an anti-variable response was seen. Therefore the mouse was able to make a response to its own V regions but only when there were other foreign determinants present. To mimic the effect of polymorphism in the human population the MoV_{NP}-Moγ2b antibody was injected into C57BL/6 and BALB/c mice. A strong response to both V and C regions was seen in the BALB/c mice. Extrapolated to the human situation these results suggest that fully humanized antibodies may be less immunogenic than chimerics but that attention should be paid in their design to the allotypy of both V and C.

DESIGN AND CONSTRUCTION OF FULLY HUMANIZED ANTIBODIES

The various strategies for full humanization were described earlier, and were exemplified by studies with non-therapeutic MAbs and a few of therapeutic interest (1B4, BMA031, 0.5β and CAMPATH-9). We now survey the humanization and evaluation of the remaining therapeutic antibodies published to date. YTH34.5HL, RSV19, BWA31/26, H17E2 and OKT3 were humanized essentially by the reshaping strategy, while anti-Tac, Fd79, Fd138-80, YTH 12.5, M195, 4D5 and UCTH1 were humanized essentially by the hyperchimerization strategy. 425 was humanized by a combination of these approaches.

YTH34.5HL

The first antibody of clinical interest to be fully humanized was the rat antibody YTH34.5HL, which recognizes the CAMPATH-1 (CDw52) antigen (Riechmann *et al.*, 1988). This antigen is present on most human lymphocytes and monocytes, but not on stem cells or other cells of the haematopoietic system (Hale *et al.*, 1983). Several rat antibodies (IgM and IgG) have been

produced and used to treat lymphoid malignant disorders and to purge bone marrow of T-cells prior to transfusion to prevent a graft-versus-host reaction (Hale *et al.*, 1988). An IgG2b class switch variant of the original IgG2a antibody is able to bind complement efficiently and also to mediate ADCC with human effector cells, though an immune response is generated against the rat antibody (e.g. Friend *et al.*, 1991).

Humanization, expression and antigen binding activity

The variable region DNA sequences were isolated from mRNA using specific CH1 and Cκ primers to direct cDNA synthesis (Riechmann *et al.*, 1988). In the first instance the effectiveness of the human IgG isotypes was assessed. Cell lines derived from a heavy chain loss variant of the YTH34.5 hybridoma were transfected by electroporation with pSV2*gpt* vectors containing rat–human chimeric heavy chains of IgG1–4 isotypes. The genes are capable of expression from the rat Ig promoter and use the murine heavy chain enhancer (Neuberger *et al.*, 1985). This series of hybrid anti-CAMPATH-1 antibodies (clgG1, 2, 3, 4), consisting of rat light chains with rat–human chimeric heavy chains, were examined for their ability to induce ADCC and ADCMC of human lymphocytes to decide which isotype to use for the humanization studies. In the ADCC assay the clgG1 was shown to be more lytic than the rat IgG2b, while the clgG3 was poorly lytic. The clgG2 showed no activity while the clgG4 showed slight activity at the highest antibody concentrations used. The clgG1 and clgG3 isotypes were efficient at ADCMC, the clgG1 being the better of the two and equivalent to the activity of the rat IgG2b. The clgG2 chimera showed 50% of maximal complement lysis at an antibody concentration 10–15 times higher than for the clgG1. The clgG4 was non-lytic in the conditions used. On the basis of these experiments the IgG1 isotype was chosen as the human isotype most similar to the rat IgG2b for the further construction of the humanized antibody.

Initially the variable heavy region was humanized by simultaneous substitution of all the CAMPATH-1 CDRs into the humanized B1.8 variable region gene by oligonucleotide directed mutagenesis, in a similar manner to the D1.3 example described earlier. The humanized variable region sequence was then linked to the rat IgG2b constant region for comparison of the rat and humanized heavy variable region. The antibody was expressed in the heavy chain loss variant of YTH34.5HL after transfection of the heavy chain expression vector by electroporation. The concentration of antibody for 50% binding was 27·3 μg ml^{-1} compared to 0·7 μg ml^{-1} for the rat antibody, giving 2.56% relative potency. No ADCMC could be detected for the hybrid antibody. Analysis of the potential structure of the heavy variable domain suggested that poor packing contacts between residue 27 (serine in the humanized antibody) in the structural loop adjacent to CDR1 and residues 32 and 34 in CDR1 might be the problem. In most antibodies amino acid 27 is a phenylalanine or tyrosine, which are able to pack efficiently against amino acids 32 and 34. When the serine to phenylalanine mutation was made and the humanized V_H (ser27phe)-rat IgG2b expressed in the heavy chain loss variant

of YTH34.5HL was tested, the concentration of antibody for 50% binding was found to be 1·8 µg ml^{-1}, 38.9% of the relative potency of the rat antibody — a substantial improvement. A further substitution of the surface residues at position 30 on the heavy chain, adjacent to CDR1, from threonine to serine, gave no additional improvement. However the doubly substituted heavy chain with the rodent residues at 27 and 30 was used for subsequent *in vitro* and clinical analysis. The rat constant region was then exchanged for the human IgG1 constant region. Finally the light chain was humanized by grafting the light chain CDRs as defined by Kabat *et al.* (1987) onto the REI human κ light chain gene. Substitution of the humanized light chain for the rat light chain improved antigen binding to a relative potency of 75–80% (Clark, 1992). The two humanized genes were co-expressed by electroporation into the non-antibody-producing myeloma line YO (YB2/0 3.0 Ag30; Kilmartin, Wright and Milstein, 1982), to give the fully humanized antibody, CAMPATH-1H. Antibody produced from YO cells accumulated up to 5 mg l^{-1} and was used for initial clinical studies. Expression of the CAMPATH-1H antibody has subsequently been improved by expression of the reshaped light and heavy chain genes as cDNAs in CHO cells under the control of the human β-actin promoter (Page and Sydenham, 1991). The light chain expression cassette was assembled in a vector containing a crippled DHFR gene. The heavy chain expression cassette was assembled in a vector containing the *neo* selection marker. Cell lines were constructed by co-transfection of CHO cells by the calcium phosphate precipitation procedure and selecting for DHFR-positive and G418-resistant cells, followed by further stepwise increases of MTX concentration. The highest producing and most stable cell lines were produced when initial DHFR-positive, G418-resistant colonies were subjected to selection at 10^{-7} M MTX, cloned and then cultured at 10^{-6} M MTX to amplify the DHFR gene and associated DNA sequences, and finally re-cloned. The highest producing clone yielded 200 mg l^{-1} of accumulated product.

Pre-clinical studies

ADCC.   The rat light chain/rat variable-human IgG1 constant region hybrid was shown to be superior to the rat antibody in this respect, with CAMPATH-1H showing somewhat less activity than this hybrid. These results suggest that the ADCC assay is sensitive to the small loss of affinity (to 75% relative potency) in the humanized antibody.

ADCMC.   Complement lysis could not be detected using the initial rat light chain/reshaped variable-rat constant heavy chain hybrid antibody. Alteration of the serine at position 27 to phenylalanine produced detectable activity, with 16·3 µg ml^{-1} required for 50% ADCMC compared to 2·1 µg ml^{-1} for the rat antibody, a relative potency of 13%. Further alteration of the antibody to replace threonine at position 30 with serine gave a 50% ADCMC value of 17·6 µg ml^{-1} (12% relative potency). When the rat constant region was

exchanged for the human constant region, to give a hybrid with rat light chain/reshaped heavy chain, the IgG1 isotype proved to effect the same level of ADCMC as the rat light chain/reshaped heavy variable (with position 27 and 30 substitutions)–rat constant region hybrid. When the rat light chain was exchanged for the humanized light chain to produce CAMPATH-1H, the ADCMC was equivalent to the rat antibody. Therefore the approximately two-fold improvement in affinity caused by the humanization of the light chain (to 75% relative potency) improved the ADCMC of the resultant antibody by approximately eight-fold, to equivalent potency.

Clinical studies

Three small studies have been reported with CAMPATH-1H; Mathieson *et al.*, 1990. In the first (Hale *et al.*, 1988) two patients with non-Hodgkins lymphoma (B-cell lymphoma) were treated for up to 43 d with escalating i.v. doses ranging from 1 to 20 mg d^{-1}. The first patient had five months previously been treated extensively with chemotherapy and also with rat CAMPATH-1G, which had cleared the lymphoma cells but caused adverse reactions (fever, nausea, vomiting and bronchospasm). The first treatment with CAMPATH-1H over 28 d led to clearance of lymphoma cells from the bone marrow. One hundred days later lymphoma cells were again detected, but were removed by a second treatment (lasting 12 d). No anti-CAMPATH-1H response was detected. The second patients received CAMPATH-1H as the primary treatment. Lymphoma and CAMPATH-positive cells were again cleared from the bone marrow, but after 43 d treatment was stopped because recurrent fever and malaise occurred after every infusion. Nevertheless the patient was reported to be well. Again no anti-CAMPATH-1H response was seen, but it must be noted that the patients were already immunosuppressed as a consequence of the disease, and that the MAb itself is immunosuppressive.

In the second study (Mathieson *et al.*, 1990) CAMPATH-1H was used in conjunction with the rat CAMPATH-9 (Gorman *et al.*, 1991) on a single patient with a chronic and intractable systemic vasculitis. Immunohistochemical studies suggested a predominance of CD8+ cells in this vasculitis. Treatment with CAMPATH-1H alone gave short-lived partial remissions. A second strategy was therefore adopted which involved attempts to deplete the lymphocyte population using CAMPATH-1H, then use CAMPATH-9 to remove remaining T-helper cells with the aim of inducing tolerance to the auto-antigen presumed to be responsible for the vasculitis. This strategy led to disease remission that has so far lasted for 3 years (Isaacs *et al.*, 1992). CD4+ cells reappeared after one month but the CD4/CD8 ratio has remained depressed. The patient has suffered no opportunistic infections, and no auto-antibody response has been detected.

In the third study 8 patients with rheumatoid arthritis were treated with CAMPATH-1H in a Phase I open study (Crowe, 1992; Isaacs *et al.*, 1992). Patients received CHO cell-produced CAMPATH-1H, by i.v. infusion, in a course of treatment consisting of 4 mg d^{-1} on 5 consecutive days followed by

8 mg d^{-1} for a further 5 consecutive days. Four of the patients later received a second course of treatment consisting of 40 mg d^{-1} infusion each day for 5 days. All of the patients showed slight side effects associated with the first infusion in each course of treatment.

In 7 of the 8 patients objective clinical improvements were noted which lasted for between 12 weeks and 8 months. There was no correlation between relapse and lymphocyte count which remained suppressed for several months after therapy. Serum antibody levels, however, did not reach the 10 µg ml^{-1} level theoretically required to saturate all of the antigen present, reaching only 2.5 µg ml^{-1} at the end of the first course. A peak level of 11 µg ml^{-1} was seen for one patients during the second course of treatment.

None of the 8 patients developed an anti-globulin response after the first course of treatment, but sera from 3 of the 4 patients who were retreated showed an anti-globulin response and showed an increased level, over pre-treatment values, of an ability to block the binding of CAMPATH-1H to PBMCs. The lack of initial response to CAMPATH-1H compares to that seen with the rat antibody where 11 of 14 patients treated showed a clear anti-antibody response 11–18 d after the first course of treatment (Friend *et al.*, 1991).

RSV19

Murine MAb RSV19 (IgG2a/κ) recognizes an epitope on the fusion (F) protein of respiratory syncitial virus (RSV). This virus is the agent of one of the major childhood diseases and is the major cause of acute respiratory illness in children admitted to hospital. Most infections are resolved in a short period of time but in some cases lung damage occurs leading to susceptibility to subsequent infection. There is currently no effective vaccine, but there are indications from the use of immune sera in humans and with monoclonal anti-RSV antibodies in animals that an antibody-based therapy may be of benefit.

Humanization, expression and antigen binding activity

A humanized anti-RSV19 was constructed (Taylor *et al.*, 1991; Tempest *et al.*, 1991). Variable region sequences for the heavy and light chains were obtained by PCR cloning from cDNA preparations. The humanized variable regions used the human NEWM and REI frameworks for the heavy and light chains respectively. The CDRs (as defined by Kabat *et al.*, 1987) from RSV19 were introduced into previously humanized antibody genes (Riechmann *et al.*, 1988) by simultaneous site-directed mutagenesis. In addition the murine residue, phenylalanine, was used at position 27 instead of the NEWM residue, serine, for the reasons described in the D1.3 and CAMPATH examples. The humanized antibody variable region sequences (HuRSV19VH/VK), along with the Ig heavy chain promoter and signal sequences and splice sites, were inserted into pSV*hyg* for the light chain and pSV2*gpt* for the heavy chain. The human IgG1 and κ constant region

sequences were then inserted. The vectors were co-transfected by electroporation into YB2/0 rat myeloma cells and selected for mycophenolic acid resistance. Cell lines secreting 5 mg l^{-1} of antibody were identified. HuRSV19VH/VK antibody did not bind at levels significantly above background to a cell lysate of calf kidney (CK) cells infected with RSV(A2 strain) in an ELISA format assay.

Analysis of the sequence differences between the murine and human framework residues suggested that residues 91, 93 and 94 might be contributing to the lack of activity of the antibody. In particular residue 94, commonly an arginine, and which is believed to form a salt bridge with aspartate at 102 (numbering as in kabat *et al.*, 1987), is a serine in the NEWM framework. Therefore a second heavy chain gene, HuRSV19VHFNS, was constructed in which the murine residues were used at 91, 93 and 94. Antibody was prepared in the same way as previously and significant binding was observed. The relative potency of the HuRSV19VHFNS/VK antibody was not able to be measured because only ELISA format binding assays were done which require different conjugated antibodies to detect the murine and humanized antibodies. No affinity measurements were disclosed.

Pre-clinical studies

HuRSV19VHFNS/VK antibody recognizes the same range of clinical isolates of RSV as the murine antibody and was effective at inhibiting the formation of fused giant cells giving an IC_{50} of 6.3 μg ml^{-1} compared to 4 μg ml^{-1} for the murine antibody (relative potency of 63.5%). In plaque reduction assays the humanized antibody showed an IC_{50} of 1.7 μg ml^{-1} compared to 0.4 μg ml^{-1} for the murine antibody (relative potency of 23.5%). In animal studies BALB/c mice challenged i.n. with 10^4 pfu of RSV (A2 strain) virus could be protected by i.n. or i.p. treatment at day −1 or +4 with 25 μg of the HuRSV19VHFNS/VK antibody. The HuRSV19VHFNS/VK antibody and the murine RSV19, given i.p. at 4 d post-infection, were equally effective at inhibiting the growth of RSv (A2 strain) in the lungs of BALB/c mice challenged i.n. with 10^5 pfu of virus. In both cases 5 mg kg^{-1} was needed to reduce virus titres in the lung by $>10^5$-fold.

BW431/26

As mentioned earlier, murine anti-CEA MAbs have been used in many clinical studies. Several such MAbs have been chimerized (*see Table 2*), and recently Gussow and co-workers (Gussow and Seemann, 1991; Bosslet *et al.*, 1992) have described the humanization of one of them, BW431/26 (Bosslet *et al.*, 1988). Mab BW431/26 (IgG1/κ) has an affinity for antigen of $3.0–4.7 \times 10^{10} \text{ M}^{-1}$ using purified CEA, and $3.7 \times 10^{10} \text{ M}^{-1}$ for CEA on cells.

Humanization, expression and antigen binding activity

The murine variable region sequences were obtained from mRNA by PCR cloning (Orlandi *et al.*, 1989). Overall homology of the murine variable region sequences to the acceptor sequences was 70% for the heavy chain (NEWM) and 68% for the light chain (REI). For the humanization the CDRs used were as defined by Kabat *et al.* (1987), and in addition the human amino acid was replaced at positions 27 and 29 in the heavy chain by alternative residues. Positions 27 and 29 are both packing residues which interact with heavy chain CDR1. By analogy with the CAMPATH example phenylalanine was used at position 27 rather than the serine found in NEWM, and also rather than the tyrosine found in the BW431/26 antibody. At position 29 the BW431/26 specific residue, isoleucine, was used rather than the NEWM residue, phenylalanine. These sequences were transplanted into the human frame-work of the D1.3 anti-lysozyme antibodies described earlier (Verhoeyen, Milstein and Winter, 1988) by simultaneous site-directed mutagenesis. No changes were made in the light chain framework. The light and heavy chain V-regions were transferred into expression vectors in a genomic format 5' to the Cκ and IgG3 constant regions, respectively. Expression was driven by the SV40 promoter and used the Ig signal sequences. The plasmids were co-expressed into BHK cells by calcium phosphate precipitation along with two further vectors, one supplying a *neo* selectable marker and another supplying a DHFR marker. An antibody-producing cell line producing 15 mg l^{-1} was generated, without amplification, in serum-free medium.

Seventeen of 18 anti-idiotype antibodies, raised against the murine antibody, bound to the humanized BS431/26. One bound with lower affinity, suggesting that the structure of the variable region resembled the murine structure. However the affinity for antigen of the humanized antibody was reduced compared to the murine antibody. The humanized antibody bound to purified CEA with K_a of $1.2–2.2 \times 10^{10}$ M^{-1} and to CEA on cells with K_a of 1.4×10^{10} M^{-1} (Bosslet *et al.*, 1992), giving relative potencies of 41.5% and 37.8% respectively for the humanized antibody from the two methods of analysis.

H17E2

The antibody H17E2 is a murine MAb (IgG1/κ) which was raised against placental membranes (Travers and Bodmer, 1984) and recognizes PLAP but shows no reactivity with intestinal or liver alkaline phosphatases. The MAb has been used clinically as a radioimmunotherapy for ovarian tumours (Epenetos *et al.*, 1987) but a significant HAMA response has limited therapeutic application.

Humanization, expression and antigen binding activity

cDNA was generated from the H17E2 hybridoma and heavy and light chains were identified with antibody-specific cDNA probes. The H17E2 sequence

(not disclosed in full) was described as being highly homologous to D1.3 for which the antibody V sequences are known and for which a structure has been deduced from X-ray crystallography (Amit *et al.*, 1986; Boulot *et al.*, 1990). These data were used to build a model structure to search for possible contacts between the Kabat CDRs and neighbouring framework residues. For the humanization the framework sequences were as described by Verhoeyen, Milstein and Winter (1988), Reichmann, Foote and Winter (1988) and Riechmann *et al.* (1988). The H17E2 CDRs were substituted into these frameworks by simultaneous SDM (Verhoeyen *et al.*, 1991). For the light chain no framework changes were made. For the heavy chain residues 27 and 30 in the loop region adjacent to CDR1 were considered to be close to the CDRs and potentially to be involved in CDR positioning and/or direct antigen contact. Therefore at these positions the murine residues were used. The heavy chain was assembled as a human IgG1 with a view to recruiting ADCC and ADCMC and so improve upon the parent murine IgG1 antibody. The humanized genes were finally assembled on pSV2gpt and *neo*-based vectors (Riechmann *et al.*, 1988) and co-transfected by electroporation into NS0 cells. A cell line secreting 10 mg l^{-1} of humanized antibody using a serum-free medium was generated. The humanized antibody was compared to mH17E2 by binding to PLAP in a direct binding assay format. At equivalent dilutions of antibody in the assay the antibodies showed similar binding curves, with the humanized form appearing to be only slightly less active than the murine antibody.

Clinical studies

Data are available for humanized H17E2 (renamed hu2PLAP) from seven patients with ovarian, stomach and breast carcinomas (Hird *et al.*, 1991b). Two patients had HAMA antibodies before treatment, and one of these had received previous treatment with the murine antibody HMFG1 conjugated to ^{90}Y via the macrocycle DTPA. The patients received imaging doses (220–833 µg) of hu2PLAP carrying ^{111}In via the macrocycle DOTA (Moi, Meares and DeNardo, 1988). The patients were monitored at 24 h and 96 h. In patients with no pre-existing HAMA the $t_{1/2}\beta$ was 73 h, compared to 27 h for the murine MAb. In patients with pre-existing HAMA the $t_{1/2}\beta$s were 39 and 47 h. No patients developed anti-hu2PLAP responses over the 96 h period, although three patients apparently showed anti-DOTA antibodies. One of these patients had previously been treated with murine hu2PLAP carrying DOTA-^{111}In.

OKT3

OKT3 is a murine IgG2a/κ MAb which recognizes an epitope on the ε-subunit within the human CD3 complex. *In vitro* studies have demonstrated that OKT3 possesses potent T-cell activating and suppressive properties. Binding of OKT3 to the TCR results in coating of the TcR and/or modulation, thus mediating TCR blockade, and inhibiting alloantigen recognition

and cell-mediated cytotoxicity. Fc receptor-mediated cross-linking of TCR-bound anti-CD3 MAb results in T-cell activation with Ca^{2+} mobilization, phosphoinositidyl hydrolysis, lymphokine production, activation marker expression, and proliferation. Administration of OKT3 results in both T-cell activation and suppression of immune responses in animals. Repeated daily administration of OKT3 results in profound immunosuppression in humans, and provides effective treatment of rejection following renal transplantation (summarized in Alegre *et al.*, 1992; Woodle *et al.*, 1992). The clinical utility of OKT3 is limited, however, by the problems of 'first-dose' side-effects, ranging from mild flu-like symptoms to severe toxicity, which are believed to be caused by lymphokine production stimulated by OKT3 and by a HAMA response (Ortho Multi-Center Transplant Study Group, 1985), a proportion of the response being directed to the variable region of the antibody (Jaffers *et al.*, 1986). While low titre HAMA may present no significant problem, some patients develop high titre anti-isotope and/or anti-idiotype responses.

Humanization, expression and antigen binding activity

OKT3 variable region sequences were obtained by cDNA cloning (Jolliffe *et al.*, 1991). The IgG4 constant region was used initially for the development of humanized forms of the antibody since the ultimate aim is to design a humanized OKT3 IgG antibody which can efficiently bind to CD3 while retaining useful pharmacokinetics and having no first-dose side-effects. For the design of the humanized OKT3 variable region sequences, REI and KOL were chosen as the human light and heavy chain frameworks respectively, as in both cases structures have been determined by X-ray crystallography so that a detailed examination of individual residues in the human variable region frameworks could be made. In the initial humanized sequences the CDR choices were as suggested by Kabat *et al.* (1987). In the light chain no other non-CDR murine residues were used. In the heavy chain design the murine residues at positions 27, 28 and 30, which are normally found in a loop region adjacent to CDR1, were also used. The importance of residue 27 for antigen binding was shown by Riechmann *et al.* (1988) in the reconstitution of binding activity of the CAMPATH-1 antibody. Residues 28 and 30 are predicted to be at the surface of the antibody and near to CDR1.

The DNA sequences coding for the humanized variable regions were constructed by simultaneous replacement (site-directed mutagenesis) of humanized B72.3 light and heavy chain DNA sequences (J.S. Emtage *et al.*, unpublished). The DNA sequences coding for the humanized variable region DNA sequences were attached to the human γ4 and κ constant region sequences, inserted into expression vectors with the hCMV promoter (Whittle *et al.*, 1987) and co-expressed in COS cells to give gOKT3-1. This antibody binds poorly to CD3-positive HPB-ALL cells and does not block the binding of mOKT3 to the cells. Therefore other residues from the framework regions were substituted into the humanized antibody to reconstitute antigen binding. These residues were identified by analysis of those residues within murine and human variable regions which would be predicted to have an influence on the

correct positioning of the CDRs (Adair, Athwal and Emtage, 1991). For the light chain these positions are at 1 and 3, which are usually surface residues located near to the CDRs, residue 46, which is usually at the domain interface, and the packing residue at 47. For the heavy chain positions 23 and 76, which are believed to be solvent-exposed residues near the CDRs, positions 6, 24, 48, 49, 71, 73, 78 and 88, which are residues believed to be involved in positioning of the CDRs and/or in domain packing, and the domain interface residue 91 were identified. A further alteration to the heavy chain sequence was made in CDR2 at residue 63, which is usually an intra-domain packing residue. At this position the residue found in KOL was used so that potentially unfavourable contacts with other packing residues from the human framework could be avoided. Several light and heavy chain variants were made and tested to examine the effect of having the murine or human residue at these locations. Including the murine residue at all of these positions led to an antibody (gOKT3-5) with full binding affinity (relative affinity of $1 \cdot 1 \times 10^9$ M^{-1} compared to $1 \cdot 2 \times 10^9$ M^{-1} for murine OKT3). However, the murine amino acid was found not to be required at all of the locations to retain full binding affinity, and at the solvent-accessible locations V_L1, V_L3 and V_H76 in the domain interface (V_H91) and at V_H71, V_H73 and V_H88 the human residue could be used (gOKT3-7) with no loss of binding affinity (relative affinity of $1 \cdot 4 \times 10^9$ M^{-1}). The binding affinity did not appear to be markedly affected by choosing either the human or murine amino acid at position 71 on the heavy chain. This position has been suggested to be a determinant of the canonical form of CDR2 (Chothia et al., 1989; Tramontano, Chothia and Lesk, 1990). The predicted canonical loop structure for CDR2 of OKT3 would be structure 2 while KOL has a structure 1 CDR2 loop and the arginine found in KOL at V_H71 might be expected to cause a different positioning of CDR2 compared to the threonine found in OKT3. However, a more detailed analysis needs to be undertaken, for example as described by Kelley et al. (1992, see below) for the anti-p185[HER2] antibody, to determine whether more subtle effects can be distinguished.

Pre-clinical studies

Woodle et al. (1992) have compared a number of the biological properties of the gOKT3-5 antibody. The antibody was able to bind to CD3 and modulate the surface expression of CD3. The amounts of antibody required to achieve a given level of antigen coating or modulation differed between the murine antibody, gOKT3-5, and a chimeric OKT3 (cOKT3) control, with cOKT3 showing slightly lower activity. Both gOKT3-5 and cOKT3 were able to stimulate [³H]thymidine incorporation in human PBMC, but required approximately 10-fold more antibody to achieve a given level of incorporation compared to the murine OKT3. This is consistent with a lower interaction with human Fc receptors of the IgG4 constant region of the humanizekd antibodies compared to the IgG2a constant region of the murine antibody (Burton and Woof, 1992). The three antibodies were equally able to induce IL-2R and Leu23 expression on human PBMC, with expression by CD4+

cells being greater than that on CD8+ cells. Similarly the three antibodies showed equivalent stimulation of TNF-α and IFN-γ by PBMC. These latter experiments were done with antibody in the range 10^{-2}–10^2 ng ml^{-1}, quantities sufficient to coat and/or modulate 90–100% of CD3 from PBMC with the murine OKT3 and gOKT3-5 and over 60% with the cOKT3. Finally, all three antibodies were equally able to inhibit cytotoxic T-lymphocyte (CTL) lytic activity.

The ability of the humanized IgG4 versions of OKT3 to induce the same proliferative and lymphokine release activities as the murine OKT3 suggested that the reduction in Fc binding caused by the constant region switch was not sufficient to prevent effective cell–cell cross-linking by the antibody between CD3-positive T-lymphocytes and FcR-bearing cells. Therefore the FcRI binding site in the CH2 domain of gOKT3-5 was altered by a single point mutation (Alegre *et al.*, 1992) to the sequence found in murine IgG2b, which does not bind efficiently to FcRI. The mutation did not affect antigen binding activity, but FcR binding was reduced by approximately 100-fold. The mutation also caused a delay in the coating and modulation of CD3 from PBMC. When PBMC were incubated for 12 h with the mutant antibody approximately 1000-fold more mutant antibody was required to achieve half-maximal coating and modulation compared to the murine OKT3 and gOKT3-5, which showed equivalent activity. However after 48 h incubation all three antibodies showed the same degree of coating and modulation. No stimulation of [^{3}H]thymidine incorporation by PBMCs was seen with the mutated gOKT3-5 even when 10^4-fold more antibody was used than was required to cause 50% of maximal uptake using gOKT3-5 or murine OKT3. The ability to up-regulate Leu23 and IL-2 receptor expression, and TNF-α, IFN-γ and GM-CSF production by PBMCs was also reduced by the same degree. CTL inhibitory activity was measured by incubation of effector cells for 30 min with antibody followed by 18 h co-incubation of effectors with allogeneic targets. No difference in CTL inhibitory activity was seen between the mutated gOKT3-5, gOKT3-5 and murine OKT3, with antibody concentrations in the range where a maximum of 20% of coating and modulation occurred over 12 h for the mutated gOKT3-5.

The single point mutation appears to be capable of abolishing the proliferative and lymphokine release properties of gOKT3-5 without affecting its immunosuppressive functions. Recently similar results have been obtained by a similar modification to the gOKT3-7 antibody (Zivin *et al.*, 1992). Whether the point mutation in the CH2 domain will introduce an immunogenic epitope has yet to be determined.

ANTI-TAC

The murine MAb anti-Tac (anti-Tac-M, IgG2a/κ) recognizes and binds to an epitope on the p55 subunit of the interleukin-2 receptor (IL-2R, CD25) and inhibits the proliferation of activated T-cells by blocking the binding of IL-2 to the IL-2R. This antibody may have an advantage over OKT3 for use as an

immunosuppressant as it binds only to activated T-cells. The affinity of anti-Tac-M for p55 has been measured as 9×10^9 M^{-1}.

Humanization, expression and antigen binding activity

The variable region sequences were identified from a cDNA library using constant region oligonucleotide probes. The antibody EU was chosen as the human acceptor for both light and heavy chains because of its high sequence homology to the heavy chain of anti-Tac. From a molecular model of the Fv and by reference to Kabat *et al.* (1987) the CDRs and key non-CDR residues were identified. These were of two kinds, residues thought to be interacting with the CDRs, and residues at locations where the mouse antibody had an amino acid which was the same as the most commonly found human amino acid and the EU antibody had a different amino acid. In total three non-CDR residues in the light chain (at positions 47, 59 and 62) and 12 in the heavy chain (at positions 27, 30, 48, 67, 69, 89, 91, 93, 103, 104, 105, and 107) were identified. The variable region genes were constructed from long, overlapping oligonucleotides and gap-fill by T4 DNA polymerase. The variable regions were cloned into expression vectors under the control of Ig promoters and the antibody genes were essentially genomic in organization. The vectors were electroporated sequentially into Sp2/0 cells with *neo* selection for the light chain and *gpt* selection for the heavy chain. Antibody yields of 3 μg per 10^6 cells were reported. Chimeric forms of the antibody using IgG1 and IgG3 constant regions were prepared for comparison. Humanized antibody, anti-Tac-H(γ1) was purified and the affinity measured as 3×10^9 M^{-1} in competition experiments with the anti-Tac-M antibody (relative potency of 33%) (Queen *et al.*, 1989).

Pre-clinical studies

ADCC. Anti-Tac-M was not able cause ADCC using human PBMC, although lysis was observed if the murine antibody was cross-linked with rabbit anti-murine IgG. The chimeric anti-Tac(γ1) was, however, able to cause ADCC which was sensitive to the E:T ratios used, or if the cells were activated using IL-2. The chimeric anti-Tac(γ3) was unable to cause ADCC with or without IL-2 and at all E:T ratios used. The anti-Tac-H(γ1) was less able than the chimeric anti-Tac(γ1) to cause ADCC over a range of E:T ratios but this difference was less apparent if the cells were activated with IL-2. At an E:T ratio of 100, and with IL-2 activation, the difference in ADCC of the anti-Tac-H(γ1) antibody and the chimeric (IgG1) over a range of antibody concentrations correlates very closely with the measured affinity of the humanized antibodies. The three-fold reduction in affinity translates in this case into a 10-fold reduction in ADCC under the conditions used (Junghans *et al.*, 1991).

ADCMC. The murine, chimeric and humanized forms of the antibody were

all unable to cause ADCMC of ^{51}Cr-labelled Hut-102 cells using human complement.

Suppression of T-cell proliferation. The anti-Tac-H and both chimeric antibodies were as potent as the anti-Tac-M in suppressing T-cell proliferation in response to tetanus toxoid or influenza virus (Junghans *et al.*, 1991).

Animal studies. The anti-Tac-H(γ1) antibody was examined for the development of an immune response and for pharmacokinetics in cynomolgus monkeys (Hakimi *et al.*, 1991). Antibody was administered to eight groups each of four cynomolgus monkeys. The groups were given either anti-Tac-H(γ1) or anti-Tac-M i.v. in doses of 0, 0·05, 0·5 or 5 mg ml^{-1} each day for 14 d followed by challenge with the same antibody on day 42 if no anti-antibody response was seen. The response to the humanized antibody was seen to be lower in absolute amount and delayed in onset compared to the response to the murine antibody. In the anti-Tac-M groups response in 9 of 12 animals was seen during the first course of treatment, while in those animals given the humanized antibody response was generally seen 5–10 d after the last injection. For both antibodies the level of response appeared in general to be inversely proportional to the dosage. In all cases where a second dose at day 42 could be given a large, >10-fold, increase in specific titre was observed. The response was both anti-idiotypic and anti-isotypic. The majority of the response against the humanized antibody could be inhibited by the presence of humanized or murine anti-Tac or soluble IL-2R, suggesting that the response was predominantly anti-binding site.

The pharmacokinetics of the antibodies was also measured. It was shown that serum levels increased throughout the 14 d dosing period, suggesting that equilibrium had not been achieved between the receptor sites or the extra-vascular spaces. However at the end of the dosing period the decay in the serum values could be measured to provide approximate $t_{1/2}\beta$ values. These values were found to be very variable for the humanized antibody, ranging from 47 to 432 h, and were independent of dosing regime. For the murine antibody $t_{1/2}\beta$ values could not be calculated because the levels were declining even during the dosing period. Some of the animals were given single doses of 5 mg ml^{-1} of either humanized or murine antibody. From these animals $t_{1/2}\beta$ values of 213.6±58.8 h and 47.8±9 h were obtained for the humanized and murine antibody, respectively. These results are similar to those seen earlier for the murine and chimeric B72.3 antibody in cynomolgus monkeys and also to those values seen for murine and chimeric antibodies in humans (see earlier). It seems from these and other studies that the cynomolgus monkey is a more accurate predictor of pharmacokinetics in humans than are normal or nude mice. This may also prove to be the case for immunogenicity.

The anti-Tac-H(γ1) has also been used to study efficacy in animals by measuring the survival of primate cardiac allografts (Brown *et al.*, 1991). ^{131}I-labelled anti-Tac-M and ^{125}I-labelled anti-Tac-H(γ1) were co-injected into cynomolgus monkeys either along with 0.5 mg ml^{-1} of each of the

unlabelled antibodies or 24 h after the injection of 2 mg ml^{-1} of unlabelled anti-Tac-H(γ1). The unlabelled antibody was injected to attempt to saturate soluble Tac and T-cell-presented Tac. The pharmacokinetics of the antibodies were measured. After an initial rapid loss of radioactivity from the blood for both antibodies, representing transfer to the extra-vascular compartments, the anti-Tac-M showed a $t_{1/2}\beta$ of 38 ± 3 h. The anti-Tac-H(γ1) showed a $t_{1/2}\beta$ of 103 ± 9 h. Cardiac allografts were performed on groups of the cynomolgus monkeys (five per group). One group received 1 mg ml^{-1} of anti-Tac-H(γ1) on day 1 and then on alternate days until graft rejection, one group received the same treatment but with anti-Tac-M and one group were untreated. Mean graft survival and anti-antibody response were measured. For the untreated group mean graft survival was $9\cdot2\pm0\cdot48$ d, for the anti-Tac-M group 14 ± 1.98 d, and for the anti-Tac-H(γ1) treated group $20\pm0\cdot55$ d. For the anti-Tac-M group an anti-antibody response became measurable between 6 and 15 d after commencement of treatment and 1–10 d prior to rejection and remained high for 17 months post-treatment where measured. For the anti-Tac-H(γ1) group an anti-antibody response only became measurable for three of the animals, at days 33, 33 and 42. Further, this anti-anti-Tac-H(γ1) response was approximately 1000-fold lower than the anti-Tac-M response.

Significant sero-conversion was also manifested in a rapid reduction in the $t_{1/2}\beta$ of subsequently injected radiolabelled anti-Tac-M when this was later studied, falling from the mean of 38 h to 9 h and <10 min in two animals examined. Circulating anti-Tac-M and anti-Tac-H(γ1) antibody was present at all times during the course of the allograft study but dropped markedly in the 2 d prior to observable sero-conversion in the anti-Tac-M group. Mean time of sero-conversion was 3 d prior to mean time of rejection in this group. In the anti-Tac-H(γ1) group rejection occurred while there was still significant (>12 μg ml^{-1}) amounts of circulating anti-Tac-H(γ1) while the sero-conversion occurred >12 d post-rejection. While significant improvements in graft survival occurred with both antibodies compared to the untreated group, and the survival of the anti-Tac-H(γ1) group was significantly improved over the murine antibody-treated group, all of the grafts were eventually rejected, suggesting that the anti-Tac treatment alone is not sufficient as a therapy for the treatment of graft rejection.

FD79 AND FD138–80

The antibodies Fd79 and Fd138–80 recognize type common epitopes on the glycoproteins B (gB) and D (gD) respectively of *Herpes simplex* virus (HSV) (Koga, Chatterjee and Whitley, 1986). HSV infections range from asymptomatic to life-threatening and recurrent infections after reactivation of latent virus in the trigeminal ganglia are a major problem. Both Fd79 and Fd138–80 have shown efficacy in mouse models, reducing the severity of viral infections.

Humanization, expression and antigen binding activity

The variable regions were isolated from cDNA libraries by PCR (Co *et al.*, 1991). For the humanization of Fd79 the human antibody POM was used as the acceptor framework. A model-built structure suggested the potential interaction of residues 49 in the light chain and 93 in the heavy chain with CDR residues and therefore they were left as the murine residues in the design of the humanized variable regions. Also at positions 9, 41, 42, 77 and 81 in the light chain and 81 and 103 in the heavy chain the consensus human residue was used because at these positions POM has residues rarely found in human antibodies. For six of the seven locations the murine residue is the same as the consensus. The exception is at L77, a residue located in a turn region of light chain structures where the consensus residue is arginine, the murine residue is proline and POM has a serine.

For antibody Fd138–80 the human antibody EU was used as in the anti-Tac example described earlier. Model building suggested that in the light chain residues 36 and 87, and in the heavy chain residues 27, 30, 37, 48, 66 and 67, were potentially interacting with CDR residues or residues adjacent to the CDRs. The murine residues were therefore used at these positions. At a number of other positions, 48 and 63 in the light chain and 89, 93, 103, 104, 105 and 107 in the heavy chain, the murine residues (which were the same as the human consensus residue) were used because at these positions EU has rarely found residues. The variable region sequences were built as described in Queen *et al.* (1989) and were assembled into full length antibody genes (IgG1/κ) and expressed in Sp2/0 cells. Chimeric antibodies were constructed in parallel to act as controls. The humanized and chimeric antibodies showed essentially similar patterns of binding to HSV-1-infected Vero cells in a fluorocytometric assay. The humanized Fd79 showed a two-fold drop in affinity (K_a of 5.3×10^7 M^{-1}) compared to the murine Fd79 (K_a of 1.1×10^8 M^{-1}) while the Fd138–80 antibodies had a similar affinity with a K_a of 4.8×10^7 M^{-1} for the humanized antibody compared to a K_a of 5.2×10^7 M^{-1} for the murine antibody. It should be noted that in the case of the Fd79 antibody the use of the POM heavy chain introduces a potential site for N-linked glycosylation in the loop region adjacent to CDR2 at sequences 72–74 inclusive, which if glycosylated may influence affinity.

Pre-clinical studies

The humanized and murine antibodies were equally capable of inhibiting viral infection when antibody and virus were pre-incubated together prior to incubation with Vero cells and subsequently scored as a plaque reduction assay. The humanized and murine Fd79, but not the humanized or murine Fd138–80, were able to inhibit the spread of virus when virus was allowed to incubate with Vero cells for 1 h prior to titration of antibody in to the cultures. This suggests that while the anti-gD antibodies can neutralize antibody infectivity, they cannot prevent the cell-to-cell spread of the virus as it appears that the anti-gB antibodies can.

YTH12.5

The rat MAb YTH12.5.14.2 (IgG2b/λ) recognizes an epitope on the human CD3 complex involved in T-cell activation. Anti-CD3 antibodies are being evaluated as immunosuppressive agents, for which purpose they have two major drawbacks. The first is that despite their immunosuppressive nature a specific immune response to the antibody still arises in patients, and the second is cytokine release as a consequence of binding to the T-cells (see earlier, OKT3). Routledge *et al.* (1991) have attempted to address both issues.

Humanization, expression and antigen binding activity

The variable region sequences were obtained from mRNA by PCR cloning as described by Orlandi *et al.* (1989). For the light chain primers a rat λ sequence was used and the PCR product was cloned between a murine κ signal sequence and the human λ (Kern Oz) constant region (Rabbitts, Forster and Mathews, 1983) in M13mp18. Human regions were obtained from VH26-D-J, and sequences with 83.2% and 69.6% homology to the heavy and light chain, respectively, were used as the frameworks for humanization. Only 15 of 87 and 25 of 81 substitutions were required to humanize the heavy and light variable regions, respectively. The humanized heavy variable region was cloned between the human β-actin promoter and the human IgG1 constant region sequence in a vector based on pHβApr-1-*gpt* (Page and Sydenham, 1991). This vector also contained the murine DHFR gene for subsequent gene amplification after transfection. The humanized light chain gene was introduced into a similar vector which did not contain either selectable marker. The humanized antibody was produced after co-transfection of these vectors into CHO cells. The murine and humanized antibodies competed equally for binding to CD3 on HPB-ALL cells. In three experiments the average relative potency of the humanized antibody compared to the rat antibody was 86.7%.

A monovalent form of the antibody antibody was also prepared to examine the role of bivalency in T-cell activation after antibody binding. A truncated heavy chain consisting of the signal sequence and first three N-terminal amino acids of the humanized D1.3 heavy variable region sequence linked to the hinge, CH2 and CH3 domains was prepared and co-expressed with the heavy and light chains. The signal sequence was shown to be removed efficiently during secretion. The CHO cell line was shown to secrete a mixture of antibody and antibody fragments including the monovalent form composed of one light chain, one full length heavy chain and one truncated heavy chain. The monomeric form bound to CD3 on HPB-ALL cells with a lowered avidity of around six-fold compared to the bivalent form.

Pre-clinical studies

ADCC. The humanized bivalent and monovalent antibodies were able to direct killing of FcRI-positive ^{51}Cr-labelled U937 cells by T-cell blasts previously activated by mitogenic anti-CD3 and IL-2, at E:T of 1. Each was able to effect slightly more killing than the rat antibody at low antibody concentrations (<1 ng ml^{-1}), but the three antibodies were equivalent at concentration >10 ng ml^{-1}. Therefore the six-fold difference in avidity between the monovalent and bivalent forms did not affect the ability to direct T-cells to kill targets.

ADCMC. The rat antibody was able to effect low level ADCMC using the T-cell blasts and human serum. The bivalent humanized antibody was inactive in the assay, but the monovalent antibody showed increasing activity above 10 μg ml^{-1}, at 100 μg ml^{-1} showing higher activity than the rat antibody. The reason for this phenomenon is not clear but has also been observed for monovalent derivatives of the rat antibody.

M195

M195 is a murine (IgG2a/κ) MAb which recognizes an epitope on the CD33 antigen present on cells of the early myeloid lineage and on some monocytes, but not on the earliest haematopoietic stem cells. The antigen is also present on many myeloid leukaemic cells, and anti-CD33 MAbs are being evaluated for treatment of AML (Co *et al.*, 1992). Murine M195 has been used in two Phase I studies. In the first low doses of ^{131}I-labelled antibody were used and shown to localize rapidly to CD33-positive leukaemic cells in the blood and marrow, but there was no evidence for cell killing. In the second study much higher doses of the conjugate were administered (Scheinberg *et al.*, 1991) and shown to kill >99% of leukaemic cells (Co *et al.*, 1992). HAMA responses to the murine MAb were observed even though AML patients are seriously immunocompromised.

Humanization, expression and antigen binding activity

The variable regions were isolated by anchored PCR cloning from cDNA. Chimeric antibodies were constructed by placing the light chain variable region 3' to the hCMV-MIE promoter/enhancer (Boshart *et al.*, 1985) and 5' to the human κ constant region exon in a genomic format. The expression vector also had the *gpt* selectable marker. The heavy chain variable region was inserted between the hCMV-MIE promoter/enhancer and either the IgG1 or IgG3 constant region genomic DNA. The heavy chain expression vectors contained the DHFR amplifiable, selectable marker. The light and heavy chain expression vectors were co-transfected by electroporation into Sp2/0 cells. Cell lines producing 2·5 and 6 pg per cell per 24 h were produced for the chimeric IgG1 and IgG3 antibodies respectively.
    EU was chosen to act as the human acceptor framework for both light and

heavy chains because of its good homology to both murine variable region sequences (54 and 51% homology in the light and heavy chain variable sequences) and because of the desire to use a matched human heavy and light chain pair. A computer-generated model of the M195 Fv was produced and non-CDR sequences which might be interacting with the antigen binding site were identified. In particular residues 36, 48 and 70 in the light chain and 27, 30, 48, 66, 67, 94 and 103 in the heavy chain were identified (numbering as Kabat *et al.*, 1987). At these positions the murine residue was used. Also at a number of other positions, 10, 63 and 107 on the light chain and 89, 91, 104, 105 and 107 on the heavy chain EU displays idiosyncratic residues. At these positions the consensus human amino acid was used. The resultant human-ized variable regions differ from the murine sequences at 24 of 111 and 22 of 116 positions in the light and heavy variable regions, respectively. The humanized variable regions were attached to the human κ and IgG1 or IgG3 constant regions as described for the chimeric antibodies and expressed in Sp2/0 cells. Cell lines producing 8 and 3 pg per cell per 24 h were produced for the humanized IgG1 and IgG3 antibodies, respectively. The murine antibody bound to CD33 on HL60 cells with K_a of $2 \cdot 2 \times 10^9 \ M^{-1}$, determined by Scatchard analysis in the presence of human serum. The cM195(γ1) showed a similar affinity of $2 \cdot 28 \times 10^9 \ M^{-1}$ whereas the cM195(γ3) showed a lower affinity of $0 \cdot 69 \times 10^9 \ M^{-1}$. This reduced affinity was ascribed to instability in the IgG3 protein. The humanized antibodies differed in affinity from the chimeric antibodies. The hM195(γ1) showed a higher affinity than the murine or cM195(γ1), at $5 \cdot 9 \times 10^9 \ M^{-1}$, while the hM193(γ3) had an affinity of $3 \cdot 33 \times 10^9 \ M^{-1}$. Again the IgG3 was stated to show protein instability which may account for the reduced affinity compared to the IgG1 version. Interestingly the Scatchard analysis suggests that there were more binding sites available for the murine antibody than for the hM195(γ1) with the x-axis intercept at 17 pM for the murine antibody and at 10 pM for the hM195(γ1) per 10^5 cells.

The improved affinity for the hM195(γ1) was unexpected. Examination of the reducing SDS-PAGE analysis of the antibodies shows that the heavy chain in the murine and both of the cM195 antibodies is heterogeneous compared to the hM195 antibodies. In particular the majority of the heavy chain migrates in electrophoresis with a higher apparent molecular weight, while a small fraction co-migrates with the hM195(γ1). This suggests that there may be glycosylation differences between the two antibodies. In fact in the murine and chimeric variable region sequences there is a potential N-linked glycosylation site at residues 72–74 inclusive (Kabat numbering) which is not present in the humanized antibodies. This sequence motif occurs in a loop adjacent to the CDRs and in an orientation suitable for glycosylation by the glycotransferase. Glycosylation may partially occlude the binding site for the chimeric and murine forms.

4D5

This MAb recognizes an epitope on the extra-cellular domain of hEGFR-2 (also known as erbB2 and p185^{HER2}), an antigen that is over-expressed in 20–30% of breast cancers and a lower percentage of several other solid tumours. For breast cancer over-expression correlates with severity of the disease and with patient prognosis. Full humanization of 4D5 has recently been reported (Carter *et al.*, 1992a, b; Kelley *et al.*, 1992; Shalaby *et al.*, 1992), and a Phase I study of advanced breast cancer with a naked humanized variant has just begun.

Humanization, expression and antigen binding activity

The variable regions were isolated by PCR amplification of mRNA from the 4D5 hybridoma. The primers were designed based on N-terminal sequencing of the 4D5 antibody light and heavy chains. Computer generated models were built for both the murine Fv and the potential human acceptor, which was based on the consensus sequences of the human $V_L \kappa I$ and $V_H III$ subgroups, and finally also for a model of the first humanized form of the Fv. Differences between the murine and human frameworks were examined to investigate influences on CDR conformation or antigen binding. From these studies a number of locations for possible framework alterations were identified. A glycosylation site in V_L was shown not to be required for binding activity and was not included in the design for humanization. The humanization was accomplished by cloning the murine V_H and V_L sequences, linked to the human $C\kappa$ and CH1 constant region DNA sequences respectively, into the same plasmid vector. Then the V_H and V_L sequences were mutated simultaneously using six oligonucleotides which together spanned the murine V_H and V_L domains. In total 24 and 32 amino acid changes were made to the murine light and heavy variable regions, respectively, to transform the murine frameworks into the human consensus sequences. The sequences in the CDR regions in the first of the humanized antibodies, humAb4D5-1, were residues 25–34, 50–54 and 92–96 for the light chain and 27–35, 50–58 and 95–101 for the heavy chain. These choices differ, particularly for both CDR2 motifs, from other humanization experiments described to date and reflect the emphasis on the model building and the likely contribution to binding and conformation of the sequences at 54–56 and 60–65 in the light and heavy variable regions respectively, which are normally included in the CDR2 definition (Kabat *et al.*, 1987). Variant sequences altering one or more of the human framework residues to the murine equivalent were then generated by site-directed mutagenesis. The humanized variable region sequences were then substituted for the murine variable regions in expression vectors containing chimeric 4D5 antibody genes with human $C\kappa$ and IgG1 (non-A allotype) constant region sequences. Chimeric and humanized antibody genes were expressed in 293 cells from the hCMV promoter/enhancer. The expression vectors were co-transfected and antibody was accumulated over a 5 d period at levels of 7–15 µg ml^{-1} yielding 0·2–0·5 mg per transfection.

The humAb4D5-1 antibody contains only CDRs as defined above. The affinity of this antibody for the soluble extra-cellular domains of p185^{HER2}, measured at equilibrium in solution phase, was 25 nM compared to 0·3 nM for the murine antibody (relative potency of 1·2%). The affinity of the antibody was improved to 4·7 nM (6·4% relative potency) in humAb4D5-2, by substituting the murine residue alanine, for the human residue arginine, at position 71 on the heavy chain. This residue has been noted earlier as possibly being involved in the correct positioning of the CDR2 loop (Chothia and Lesk, 1987; Chothia et al., 1989; Tramontano, Chothia and Lesk, 1990). A number of other locations were examined for their influence on binding affinity. Replacing the human residue by that from 4D5 at positions 73, 78 and 93 on the heavy chain in humAb4D5-3 did not significantly affect binding affinity (K_d 4·4 nM, relative potency 6.8%), but did affect the anti-proliferative activity of the antibody (see below). A significant improvement in antigen binding was seen when the 4D5 specific residue arginine 66 on the light chain was introduced into humAb4D5-3 to give humAb4D5-5. The affinity improved to 1·1 nM (relative potency 27.3%). Residue 66 probably interacts with both CDR1 and CDR2 on the light chain. Normally a glycine is found at this position in both murine and human antibodies. A small improvement in affinity was seen if the alanine at H78 in humAb4D5-5 was returned to the human residue, leucine, to give humAb4D5-4. The resultant binding affinity was 0·82 nM (relative potency 36.6%). The binding affinity of humAb4D5-5 could be further improved by alterations which may affect interactions at the domain interface. Single substitutions at residue 55 on the light chain, glutamate to tyrosine, to give humAb4D5-6, or at residue 102 on the heavy chain, valine to tyrosine, to give humAb4D5-7, improved affinity to 0·22 nM (relative potency 136.4%) and 0·62 nM (relative potency 48.4%) respectively, while the double substitution to give humAb4D5-8 resulted in an affinity for antigen of 0.1 nM (relative potency 300%).

Fab' fragments of the humAb4D5-8 antibody have been prepared in an E. coli secretion system (Carter et al., 1992a) using the phoA promoter and stII signal sequence. The Fd sequence was extended at the 3' end by addition of coding sequence for a modified hinge region with only one cysteine available for inter-heavy chain cross-linking (as also performed for cB72.3 Fab'). Very high yields of functional Fab' (1–2 g l^{-1}) were achieved for this system in fermentors with induction at high culture ODs when the cells were disrupted by sonication. Only 100 mg l^{-1} of this material was found to be exported into the culture medium but it was all correctly processed, suggesting periplasmic localization. Using lysozyme to partially digest the bacterial cell wall >50% of the Fab' could be recovered. The material was almost all in the form of Fab' and 75–90% of the hinge cysteines were in the reactive free thiol form. Bivalent Fabs were prepared by cross-linking the hinge thiols with a maleimide-based linker. The very high levels of expression seen were related to the human variable region framework sequences, because expression of the murine Fab' and the chimeric 4D5 Fab' under the same conditions produced 100- and 10-fold lower yields respectively, and the same framework

sequences were reported to give very high yields for other humanized antibodies.

The $F(ab')_2$ was shown to bind two molecules of the extra-cellular domains of $p185^{HER2}$. The affinity of the bacterially produced $F(ab')_2$ was found to be 0·29 nM compared to a value of 0·2 nM for proteolytically derived $F(ab')_2$ from the hum Ab4D5-8 antibody expressed in 293 cells. A direct comparison of affinity with the whole antibody was not done in this experiment so the significance of the apparent difference in affinity of 0.1 nM for the whole antibody (Carter *et al.*, 1992b, see above) compared to 0·29–0·3 nM for the $F(ab')_2$ is not clear.

Recently a more detailed analysis of the antigen binding characteristics of several of the humanized 4D5 Fabs has been published (Kelley *et al.*, 1992). Fab, lacking the hinge cysteines, was prepared in *E. coli* as described by Carter *et al.* (1992a) and purified. K_d measurements for the Fabs gave different absolute values to those determined for the whole antibody (Carter *et al.*, 1992b) and the $F(ab')_2$ (Carter *et al.*, 1992a) in the earlier studies, but the relative rank order of the variants was generally consistent. The relative binding affinity of the variants was analysed by examining $\triangle G°$, $\triangle H$ and $\triangle S°$ values. In the case of humAb4D5-8, which shows higher affinity of binding than the chimeric Fab but with equivalent free energy of binding, a $\triangle H$ of $-12·9$ kcal mol^{-1} compared to -10 kcal mol^{-1} for humAb4D5-5 and $-17·2$ kcal mol^{-1} for chimeric Fab is countered by a $\triangle S°$ value of 3 kcal mol^{-1} K^{-1} compared to 9 kcal mol^{-1} K^{-1} for humAb4D5-5 and -12 kcal mol^{-1} L^{-1} for the chimeric Fab. In several instances the detailed effect of single changes can be discerned. For example at position V_H71, believed to be involved in the positioning of CDR2 on the heavy chain (Chothia *et al.*, 1989; Tramontano, Chothia and Lesk, 1990), the presence of the human residue leads to an unfavourable $\triangle G°$ of 1·2 kcal mol^{-1} which is composed of a large unfavourable enthalpy change with a small favourable entropy effect. Residue changes at a number of sites appear to be additive in terms of their energy contributions, but the energetics of antigen binding among variants which differ only in the choice of residue at the spatially adjacent residues V_L55 and V_H102 suggests that these residues are interacting. For example, introducing the murine residue at V_L55 has a more dramatic effect on free energy and affinity if the residue at V_H102 is also the murine residue.

Pre-clinical studies

ADCC. HumAb4D5-8 was able to effect ADCC in a 4 h ^{51}Cr release assay using IL-2 activated PBMC against the mammary adenocarcinoma cell line SK-BR-3 which over-expresses $p185^{HER2}$, but showed reduced activity against the cell line WI-38, which expresses the antigen at 100-fold lower levels. ADCC of SK-BR-3 cells was sensitive both to a range of E:T from 3 to 25 and antibody concentrations of 10 or 100 μg ml^{-1} of antibody in the assay and ranged from 13% at E:T of 3 with 10 μg ml^{-1} antibody to 40% at E:T 25

and 100 μg ml^{-1} antibody. In contrast the murine antibody showed lysis in the 1 to 7% range which was less sensitive to antibody concentration and E:T ratio. The level of response with the murine antibody on the SK-BR-3 cells was similar to that seen for the humanized antibody on the low antigen expressing line, WI-38. On the WI-38 cells the humanized antibody showed 1 to 3% lysis at 10 μg ml^{-1} and 8.5 to 11% lysis at E:T of 3 to 25. The murine antibody was inactive on the WI-38 cells with all combinations tested.

Anti-proliferative activity.   The murine antibody was able to reduce the proliferation of the mammary adenocarcinoma cell line, SK-BR-3, which over-expresses p185^{HER2}, to below 40% of control values with maximal inhibition occurring at levels of antibody of 1 μg ml^{-1} or higher in the assay. The anti-proliferative activities of the humanized antibodies did not correlate with binding affinities. The humAb4D5-1 and humAb4D5-2 antibodies showed no anti-proliferative activity at up to 16 μg ml^{-1} in the assay despite a five-fold improvement in binding affinity. HumAb4D5-3 however, showed significant anti-proliferative activity, with only minor improvement in binding affinity over humAb4D5-2. HumAb4D5-5, with binding affinity of 1·1 nM, showed anti-proliferative activity (48% of control values). The remaining series of variants, humAb4D5-4, 6, 7 and 8, all showed similar slightly poorer anti-proliferative activities to humAb4D5-5. The F(ab′)$_2$ of humAb4D5-8 showed 50% relative cell proliferation (compared to whole IgG) when produced in *E. coli* or by proteolytic digestion of whole antibody (Carter *et al.*, 1992a). Chimeric Fab was able to inhibit the proliferation of SK-BR-3 cells with maximal inhibition occurring at the same concentration as the murine IgG, but with half of the absolute level of growth inhibition. The humAb4D5-8 Fab was not able to inhibit the growth of the SK-BR-3 cells. These results suggest that some feature of the mechanics of antigen binding, not indicated by the net affinity measurement, leads to the loss of the ability of the humanized Fab to trigger the anti-proliferative signal.

UCHT1

Shalaby *et al.* (1992) have humanized the anti-human CD3 antibody UCHT1 with a view to developing a UCHT1-4D5 bispecific F(ab′)$_2$ which targets immune effector cells efficiently to solid tumours. UCHT1 is an IgG2a/κ murine MAb (Beverley and Callard, 1981).

Humanization, expression and antigen binding activity

The variable region sequences from UCHT1 were obtained as for 4D5. N-terminal sequencing of the murine antibody enabled specific framework 1 primers to be designed for PCR cloning of the V_H and V_L sequences from first-strand cDNA. The differences in sequence between the UCHT1 sequence and human consensus framework sequences were examined on molecular models of the UCHT1 and human consensus Fv regions. A number of different humanized antibodies were built. The first humAb, anti-CD3 v1, was built by replacing the CDRs and a number of the framework residues

from HumAb4D5-8, described earlier, with equivalent residues from the UCHT1 sequence. The introduced sequences were residues 26–34, 50–53 and 92–96 inclusive in the light chain, and 27–35, 50–58 and 95–101 inclusive in the heavy chain. Outside of these CDR regions other residues were also substituted into the human framework from the murine sequence. These substitutions were at residues 71 on the light chain and 71, 73 and 78 on the heavy chain. The murine and humanized variable regions were assembled as full length humanized light chain and humanized Fd' as described for the 4D5 antibody and were co-expressed in *E. coli* to provide chimeric and humanized Fab' molecules for derivatization to Fab'-TNB as described above, and for subsequent cross-linking to humAb4D5-8 Fab'-SH to give a bispecific $F(ab')_2$. A bispecific $F(ab')_2$ containing chimeric UCHT1 Fab' bound to both NR6/10 cells or SK-BR-3 cells (which over-express p185^{HER2}), and to human CTL cells. HumAb4D5-8 Fab'-SH linked to the v1 anti-CD3 Fab' bound to the NR6/10 cells and SK-BR-3 in the same manner as the bispecific $F(ab')_2$ containing the chimeric Fab', but showed reduced binding to the CTL cells, with approximately 10-fold reduction in fluorescence intensity of binding as detected by flow cytometry analysis. Further variants were constructed to test the contribution to binding of two of the murine residues in the humanized UCHT1 antibody. The murine residue lysine, at 73 on the heavy chain, was returned to the human residue, aspartate, to give humanized anti-CD3 v2. The murine residue at 53 on the light chain, arginine, was returned to the human residue, serine, to give humanized anti-CD3 v3. Both mutations were combined to give humanized anti-CD3 v4. When Fab'-TNB was produced and combined with the humAb4D5-8 Fab'-SH all of the bispecific $F(ab')_2$ variants were able to bind to NR6/10 or SK-BR-3 cells as well as the bispecific $F(ab')_2$ containing the chimeric anti-CD3 Fab'. The v3 Fab'-derived bispecific $F(ab')_2$ showed a slightly reduced binding to CTL cells compared to the binding of the v1-containing bispecific $F(ab')_2$, suggesting that the arginine to serine substitution was having no further major deleterious effect on binding to CD3. However, replacement of the murine residue at 73 on the heavy chain from lysine to aspartate in the v2 and v4 variants reduced binding to CTL cells by the bispecific $F(ab')_2$ by a further order of magnitude, with the v4 double mutant being slightly worse in binding affinity than the v2 mutant.

Pre-clinical studies

The chimeric and humanized $F(ab')_2$ antibodies were tested for their ability to recruit CTL cells to kill SK-BR-3 cells, which over-express p185^{HER2}, or MDA-MB-175 cells, which express low levels of p185^{HER2}. The chimeric, v1- and v3-containing bispecific $F(ab')_2$ fragments showed equivalent targeted cell killing. The v2- and v4-based $F(ab')_2$ fragments stimulated no extra lysis over the no-antibody control. These data show that the loww of affinity seen in the v1 and v3 humanized Fab' compared to the chimeric Fab' does not affect the ability to activate the T-cells, but that the further loss of affinity in the v2- and v4-based bispecific $F(ab')_2$s caused by the lysine 73 to aspartate mutation leads to an abolition of antibody-stimulated CTL killing.

MAb 425, is a murine (IgG2a/κ) monoclonal antibody raised against the carcinoma cell line A431 and which binds to an epitope on the extracellular domain of the hEGFR-1. This antigen is over-expressed in several solid tumour types, for which treatment with anti-hEGFRI MAbs is under evaluation. MAb 425 has been shown to suppress tumour growth of epidermal and colorectal carcinoma derived cell lines *in vitro* (Rodeck *et al.*, 1987) and to bind to human xenografts of malignant glioma in mice (Takahashi *et al.*, 1987).

Humanization, expression and antigen binding activity

The variable region sequences were isolated from the hybridoma W425–15 which expresses MAb425 by cDNA cloning using constant region primers for first strand synthesis. The V regions were adapted for insertion into expression vectors (Maeda *et al.*, 1991) by anchor PCR. The light chain was humanized by reshaping and the heavy chain by hyperchimerization. A molecular model of the 425 Fv was built based on the Hy-HEL5 (Sheriff *et al.*, 1987) and Hy-HEL10 (Padlan *et al.*, 1989) structures to examine non-CDR residues for their potential contribution to affinity. For the light chain the humanized variable regions were constructed by simultaneous mutagenesis of the humanized light variable region of D1.3 to introduce the 425 CDRs, defined as in Kabat *et al.* (1987). Two sequences were generated which vary at residue 71 where the first, RVLa, has the human residue (phenylalanine) from the modified REI framework (Verhoeyen, Milstein and Winter, 1988) and the second, RVLb, has the murine residue (tyrosine). Both light chains in combination with the chimeric heavy chain were able to bind hEGFR, but the RLb/cH antibody showed superior binding.

Nine variants of the humanized heavy variable region were made to examine the role of a small number of framework positions. The framework used was the consensus sequence for the human subgroup I (Kabat *et al.*, 1987) rather than any individual high homology sequence. Subgroup I shares 73% homology with 425 within the framework regions. At positions in the consensus sequence where there was no overall preferred residue the 425 residue was used. In all of the variants residue 94 (Kabat numbering) was the murine residue, serine. The initial V_H region was built by oligonucleotide assembly and subsequent variants were generated by site-directed mutagenesis. By examination of direct-binding ELISA studies it appears that certain of the residue changes in the heavy chain sequence had more of an effect than others. Significant improvements were seen whenever residue 30 was the murine residue, threonine, rather than the human residue, serine. Having residues 66 or 67 or both as the murine residues (lysine–alanine in the murine sequence and arginine–valine in the human sequence) also caused a significant improvement, as does having the murine residue, valine, at 71 rather than the human residue, leucine. The presence of the murine residues at 30, 66, 67 and 71 led to significant direct binding, but still not at the level of the

chimeric antibody. The presence of the murine residue, valine, at position 48 appeared to give no advantage in direct binding assays. However when tested by competition assay there appeared to be an advantage (approximately two-fold) in having the valine at 48 instead of the isoleucine found in the human sequence. The authors suggested a value of 60% relative potency for the best humanized antibody which has murine residues at 71 in the light chain and at 30, 48, 66, 67, 71 and 94 in the heavy chain.

Summary and conclusions

For most MAb-based therapies single doses of MAbs or MAb conjugates will not be curative. Rodent MAbs are highly immonogenic in almost all patients. The HAMA response abrogates efficacy and can cause toxicity in organs of clearance, especially for MAb–cytotoxic agent conjugates. Humanization is the most promising generally applicable approach to overcoming the immunogenicity of rodent MAbs. Chimerization reduces immunogenicity in patients significantly, but not completely. Full humanization of rodent antibodies with retention of most of their antigen binding activity is now a routine procedure. The studies with 4D5 (Kelley *et al.*, 1992), however, illustrate that even when antigen binding activity is retained, humanization may affect the overall conformation of the antibody in ways which influence its interaction with cells (for example when the antigen is internalized or involved in signal transduction) and hence its *in vivo* properties. As yet there are not sufficient data to judge whether full humanization will (in practical terms) completely overcome the immunogenicity problem in patients, but these data will be available within a year.

Antibody fragments are the most promising general approach to manipulating the pharmacokinetics and biodistribution of therapeutic MAbs. Such fragments are clearly superior to whole IgGs for tumour detection and will very likely prove superior for tumour therapy also. MAb targeting of highly potent cytotoxic agents to tumours represents a much-needed approach to improving therapeutic ratios in cancer treatment. Radioisotopes and highly potent low molecular weight drugs are the most promising cell-killing agents for MAb targeting, and conjugation technology suitable for clinical use of some of the best of these agents has now been developed. Very encouraging data have already been obtained in clinical studies of haematopoietic malignancies with MAb–isotope conjugates. Tumour loading data from clinical studies suggest that killing of solid tumours in patients will be achieveable in the near future with repeated administration of humanized antibody fragments carrying the superior isotopes or highly potent drugs which are now available.

Future developments: beyond humanized MAbs

Although humanization is the most promising general approach in the short term to overcoming the immunogenicity of rodent MAbs, many other approaches are being taken which may prove superior in the long term. The

most interesting of these are PEGylation, human antibodies and low molecular weight antibody mimetics. PEGylation has been discussed above. The isolation of a limited range of therapeutically useful (i.e. high affinity) human antibodies from patients immunized (either deliberately or through infection) has now become practicable. This is because the recently developed 'repertoire cloning' techniques allow the isolation of antibody genes from very small numbers of cells without the need to immortalize the relevant human lymphocytes (reviewed by Burton, 1991). The human MAbs of therapeutic interest which have been isolated with this procedure include: anti-tetanus toxin (Gillies *et al.*, 1989); anti-PE (Nakatani *et al.*, 1989); anti-Hepatitis B (Zebedee *et al.*, 1992); anti-hCMV (Azuma *et al.*, 1991); and anti-HIV (Burton *et al.*, 1991). The approach may also be practicable for some TAAs, since these often elicit anti-TAA antibodies in patients (Haisma *et al.*, 1990; Goldstein *et al.*, 1990; Koda, Glassy and Chang, 1990; Posner *et al.*, 1991) and for isolation of some immunosuppressive antibodies. It is important to emphasize that immunization is required in order to isolate high affinity antibodies (or their genes) from either humans or animals. The approach of immortalizing human lymphocytes making high affinity antibodies (i.e. from immunized patients) has made some progress in the last few years (James and Bell, 1987; reviewed by Boyd and James, 1989; James, 1990), but is still far from a routine, generally applicable procedure. Significant progress has also been made towards isolating human lymphocytes making high affinity antibodies by *in vitro* immunization. The first prerequisite for this approach, long-term B-cell propagation *in vitro*, has now been accomplished by several different means (Wallen and Borrebaeck, 1991). Primary immune responses (i.e. clonal expansion) have been achieved following such *in vitro* immunization, but there is no evidence yet that secondary immune responses (i.e. involving affinity maturation to give high affinity IgG antibodies) can be achieved. Similarly clonal expansion has been achieved in response to immunization after repopulation of immunocompromized mice with human lymphocytes (Abedi *et al.*, 1992; Duchosal *et al.*, 1992), but affinity maturation has not yet been observed. Since a large proportion of therapeutically interesting MAbs will be directed against human proteins, tolerance to self-antigens may be a major limitation for all of these approaches to isolating high affinity human antibodies. This problem does not apply to raising rodent antibodies against human proteins. In the longer term the development of transgenic mice expressing part or all of the human Ig repertoire may represent a general approach to isolating high affinity human antibodies (Bruggeman *et al.*, 1989a, 1991; Neuberger *et al.*, 1989; Bruggeman, Surani and Neuberger, 1990; Sharpe *et al.*, 1991).

There has been a great deal of media interest recently in the idea of isolating human MAbs from large antibody gene repertoires made from non-immunized human peripheral blood lymphocytes or spleen cells (Marks *et al.*, 1991, 1992; Chiswell and McCafferty, 1992; Winter and Milstein, 1991). The highest affinity antibodies isolated from such 'naive' repertoires have affinities in the 1 to 5×10^{-7} M range. To be useful for most therapies affinities in the 10^{-9} to 10^{-10} M range are very likely to be required. Although

repertoire cloning, fragment expression and phage presentation technology in *E. coli* lend themselves to mutagenesis and screening approaches to affinity improvement, such approaches will be technically difficult and multiple amino acid changes will certainly be required. The resulting mutated human antibody may be no less immunogenic than a fully humanized rodent antibody.

In the longer term an alternative approach to overcoming immunogenicity would involve non-peptidic antibody mimetics, which would potentially also offer superior pharmacokinetics, biodistribution (especially tissue penetration), and administration route. Peptide analogues have been identified for several antibodies, notably ones blocking attachment of viruses to cells (Williams *et al.*, 1989a, 1989a, b; Saragovi *et al.*, 1991, 1992; Taub and Greene, 1992). These analogues were identified by semi-rational approaches, and gave peptides with binding activity 100- to 10000-fold less than that of the antibodies themselves. In one case a non-peptidic mimetic was designed and synthesized. Technology has recently been developed which will allow random screening approaches to antibody peptide analogues to be attempted. These approaches involve screening libraries of random peptides on the surface of bacteriophages (Scott, 1992), on beads (Houghten *et al.*, 1991; Lam *et al.*, 1991; Geisow, 1992) or free in solution for antigen binders. Deriving therapeutically useful mimetics from such peptides will be a major medicinal chemistry challenge, and it is by no means clear whether a low molecular weight compound can ever truly mimic the affinity and specificity of an antibody for its antigen.

Acknowledgements

References

ABEDI, M.R., CHRISTENSSON, B., ISLAM, K.B., HAMMARSTROM, L. AND SMITH, C.I.E. (1992). Immunoglobulin production in severe combined immunodeficient (SCID) mice reconstituted with human peripheral blood mononuclear cells. *Eur. J. Immunol.* **22**, 823–828.

ADAIR, J.R., ATHWAL, D.S. AND EMTAGE, J.S. (1991). *Humanised antibodies*. WO91/09967.

ADAIR, J.R., BAKER, T.S., BEBBINGTON, C.R., BEELEY, N.R.A., EMTAGE, J.S., KING, D.J., LAWSON, A.D.G., MILLICAN, T.A., MOUNTAIN, A., OWENS, R.J. AND YARRANTON, G.T. (1993). Engineering monoclonal antibody B72.3 for cancer therapy. In *Protein Engineering of Antibody Molecules for Prophylactic and Therapeutic Applications in Man* (M.R. Clark, Ed.). Academic Titles, Nottingham, in press.

ADAMS, G.P., DENARDO, S.J., AMIN, A., KROGER, L.A., DENARDO, G.L., HELLSTROM, I. AND HELLSTROM, K.E. (1992). Comparison of the pharmacokinetics in mice and the biological activity of murine L6 and human–mouse chimeric Ch-L6 antibody. *Antibod. Immunoconj. Radiopharm.* **5**, 81–95.

AHMAD, A. AND LAW, K. (1990). Recombinant targeted proteins for biotherapy. *Mol. Biother.* **2**, 67–73.

AKBAR, A.N., AMLOT, P.L., HAWKINS, C., NEWSHOLME. W., DELANEY, S., BORTHWICK, N. AND JANOSSY, G. (1991). The effect of a chimeric mouse-human CD7 antibody on human T, natural killer, and lymphokine activated killer cell activity *in vitro. Transplantation* **52**, 325–330.

ALBERINI, C.M., BET, P., MILSTEIN, C. AND SITIIA, R. (1990). Secretion of immunoglobulin M assembly intermediates in the presence of reducing agents. *Nature.* **347**, 485–487.

ALEGRE, M.-L. COLLINS, A.M., PULITO, V.L., BROSIUS, R.A., OLSON, W.C., ZIVIN, R.A., KNOWLES, R., THISTLETHWAITE, J.R., JOLLIFFE, L.K. AND BLUESTONE, J.A. (1992). Effect of a single amino acid mutation on the activating and immunosuppresive properties of a 'humanized' OKT3 monoclonal antibody. *J. Immunol.* **148**, 3461–3468.

ALT, F.W., BLACKWELL, T.K. AND YANCOPOLOUS, G.D. (1987). Development of the primary antibody repertoire. *Science* **238**, 1079–1087.

ALZARI, P.M., LASCOMBE, M.-B. AND POLJAK, R.J. (1987). Three-dimensional structure of antibodies. *Ann. Rev. Immunol.* **6**, 555–580.

AMANN, E., BROSIUS, J. AND PTASHNE, M. (1983). Vectors bearing a hybrid trp–lac promoter useful for regulated expression of cloned genes in *Escherichia coli. Gene* **25**, 167–178.

AMIT, A.G., MARIUZZA, R.A., PHILLIPS, S.E.V. AND POLJAK, R.J. (1986). Three-dimensional structure of an antigen–antibody complex at 2.8 Å resolution. *Science* **233**, 747–753.

ANGAL, S., KING, D.J., BODMER, M.W., TURNER, A., LAWSON, A.D.G., ROBERTS, G.PEDLEY, B. AND ADAIR, J.R. (1993). A single amino acid substitution abolishes the heterogeneity of a chimeric mouse/human (IgG4) antibody. *Mol. Immunol.* **30**, 105–108.

AZUMA, J., KURIMOTO, T., TSUJI, S., MOCHIZUKI, N., FUJINAGA, S., MATSUMOTO, Y. AND MASUHO, Y. (1991). Phase I study on human monoclonal antibody against Cytomegalovirus: pharmacokinetics and immunogenicity. *J. Immunother.* **10**, 278–285.

BACKMANN, K., PTASHNE, M. AND GILBERT, W. (1976). Construction of plasmids carrying the cl gene of bacteriophage λ. *Proc. Natl. Acad. Sci. USA* **73**, 4174–4178.

BAGSHAWE, K.D. (1989). Towards generating cytotoxic agents at cancer sites. *Br. J. Cancer* **60**, 275–281.

BAGSHAWE, K.D., SHARMA, S.K., SPRINGER, C.J., ANTONIW, P., ROGERS, G.T., BURKE, P., BODEN, J.A., SHERWOOD, R.F. AND MELTON, R.G. (1992). Antibody directed enzyme prodrug therapy (ADEPT) — report on a pilot clinical trial in progress. *Antibod. Immunoconj. Radiopharm.* **5**, 133.

BAKER, T.S., BEGENT, R.H.J., DEWJI, M.R., CONLAN, J. AND SECHER, D.S. (1991). Characterization of the antibody response in patients undergoing radioimmuntherapy with chimeric B72.3. *Antibod. Immunoconj. Radiopharm.* **4**, 799–809.

BARKER, E., MUELLER, B.M., HANDGRETINGER, R., HERTER., M. YU, A.L. AND REISFELD, R.A. (1991). Effect of a chimeric anti-ganglioside G_{D2} antibody on cell-mediated lysis of human neuroblastoma cells. *Cancer Res.* **51**, 144–149.

BEBBINGTON, C.R. (1991). Expression of antibody genes in nonlymphoid mammalian cells. *Methods* **2**, 136–145.

BEBBINGTON, C.R. AND HENTSCHEL, C.C.G. (1987). The use of vectors based on gene amplification for the expression of cloned genes in mammalian cells. In *DNA Cloning*, volume 3 (D. Glover, Ed.), pp. 163–188. Academic Press, New York.

BEBBINGTON, C.R., RENNER, G., THOMSON, S., KING, D., ABRAMS, D. AND YARRANTON, G.T. (1992). High-level expression of a recombinant antibody from

myeloma cells using a glutamine synthetase gene as an amplifiable selectable marker. *Bio/Technology* **10**, 169–175.

BEATTY, J.D., DUDA, R.B., WILLIAMS, L.E., SHEIBANI, K., PAXTON, R.J., BEATTY, B.J., PHILBEN, V.J., WERNER, J.L., SHIVELY, J.E., VLAHOS, W.G., KOHAL, W., RIIHIMAKI, D., TERZ, J.J. AND WAGMAN, L.D. (1986). Preoperative imaging of colorectal carcinomas with ^{111}In-labelled anti-carcinoembryonic antigen monoclonal antibody. *Cancer Res.* **46**, 6494–6502.

BEELEY, N.R.A. AND ANSELL, P.R.J. (1992). Macrocyclic chelators as anticancer agents in radioimmunotherapy. *Curr. Opin. Therapeutic Patents* **2**, 1539–1553.

BEGENT, R.H.J., BAGSHAWE, K.D., PEDLEY, R.B., SEARLE, F., LEDERMANN, J.A., GREEN, A.J., KEEP, P.A., CHESTER, K.A., GLASER, M.G. AND DALE, R.G. (1987). Use of second antibody in radioimmunotherapy. *NCI-Monogr.* **3**, 59–61.

BEGENT, R.H.J., LEDERMANN, J.A., BAGSHAWE, K.D., GREEN, A.J., KELLY, A.M.B., LANE, D., SECHER, D.S., DEWJI, M.R. AND BAKER, T.S. (1990). Phase I/II study of chimeric B72.3 antibody in radioimmunotherapy of colorectal carcinoma. *Br. J. Cancer* **62**, 487.

BEIDLER, C.B., LUDWIG, J.R., CARDENAS, J., PHELPS, J., PAPWORTH, C.G., MELCHER, E., SIERZEGA, M., MYERS, L.J., UNGER, B.W., FISHER, M., DAVID, G.S. AND JOHNSON, M.J. (1988). Cloning and high level expression of a carcinoembryonic antibody with specificity for human carcinoembryonic antigen. *J. Immunol* **141**, 4053–4060.

BENTLEY, G.A., BOULOT, G., RIOTTOT, M.M. AND POLJAK, R.J. (1990). The three-dimensional structure of an idiotype–anti–idiotype complex. *Nature* **347**, 1050–1053.

BERNSTEIN, I.D., PRESS, O.W., EARY, J.F., APPELBAUM, F.R., BADGER, C.C., MATTHEWS, D.C., FISHER, D.R., MARTIN, P.J., DURACK, L., LEVY, R., MILLER, R., KROHN, K., NELP, W.B. AND THOMAS, E.D. (1991). Treatment of leukemia and lymphoma using antibody labelled with high doses of [131]I. *Antibod. Immunoconj. Radiopharm.* **4**, 771–776.

BETTER, M. AND HORWITZ, A.H. (1989). Expression of engineered antibodies and antibody fragments in microorganisms. *Methods Enzymol* **178**, 476–496.

BETTER, M.D., HORWITZ, A.D. AND ROBINSON, R.R. (1990). *Chimeric mouse–human KM10 antibody with specificity to a human tumour cell antigen.* EP 0 404 003.

BETTER, M., WEICKMANN, J. AND LIN, Y.-L. (1990). Production and scaleup of a chimeric Fab from bacteria. *Proceedings of the 1990 Miami Bio/Technology Winter Symposium*, p. 105.

BETTER, M., CHANG, C.P., ROBINSON, R.R. AND HORWITZ, A.H. (1988). *Escherichia coli* secretion of an active chimeric antibody fragment. *Science* **240**, 1041–1043.

BEVERLEY, P.C.L. AND CALLARD, R.E. (1981). Distinctive functional characteristics of human 'T' lymphocytes defined by E rosetting or a monoclonal anti-T cell antibody. *Eur. J. Immunol* **11**, 329–334.

BHAT, T.N., BENTLEY, G.A., FISCHMANN, T.O., BOULOT, G. AND POLJAK, R.J. (1990). Small rearrangements in structures of Fv and Fab fragments of antibody D1.3 on antigen binding. *Nature* **347**, 483–485.

BINDON, C.I., HALE, G. AND WALDMANN, H. (1990). Complement activation by immunoglobulin does not depend solely on C1q binding. *Eur. J. Immunol.* **20**, 277–281.

BINDON, C.I., HALE, G., BRUGGEMANN, M. AND WALDMANN, H. (1988). Human monoclonal IgG isotypes differ in complement activating function at the level of C4 as well as C1q. *J. Exp. Med.* **168**, 127–142.

BIRD, R.E., HARDMAN, K.D., JACOBSON, J.W., JOHNSON, S., KAUFMAN, B.M., LEE, S.-M. LEE, T., POPE, S.H., RIORDAN, G.S. AND WHITLOW, M. (1988). Single chain antigen binding proteins. *Science* **242**, 423–426.

BISCHOFF-DELALOYE, A., DELALOYE, B., BUCHEGGER, F., GILGIEN, W. STUDER,

A., CURCHOD, S., GIVEL, J.C., MOSIMANN, F., PETTAVEL, J. AND MACH, J.-P. (1989a). Clinical value of immunoscintigraphy in colorectal carcinoma patients: a prospective study. *J. Nucl. Med.* **30**, 1646–1656.

BISCHOFF-DELALOYE, A., DELALOYE, B., BUCHEGGER, F., MACH, J.-P., HEUSSER, C.H. AND HARDMAN, N. (1989b). Chimeric mouse–human anti-CEA antibody of IgG4 isotype used in a pilot immunoscintigraphy study of patients with colorectal carcinomas. Proceedings of the 36th Annual Meeting of the Society for Nuclear Medicine. *J. Nucl. Med.* **30**, 809.

BLOTTIERE, H.M., STEPLEWSKI, Z., HERLYN, D. AND DOUILLARD, J.-Y. (1991). Human anti-murine immunoglobulin responses and immune functions in cancer patients receiving murine monoclonal antibody therapy. *Hum. Antibod. Hybridomas* **2**, 16–25.

BODMER, M.W., ADAIR, J.R. AND WHITTLE, N.R. (1989). *Recombinant antibody*. WO89/01974.

BORREBAECK, C.A.K., DANIELSSON, L. AND MOLLER, S.A. (1988). Human monoclonal antibodies produced by primary *in vitro* immunization of peripheral blood lymphocytes. *Proc. Natl. Acad. Sci. USA* **85**, 3995–3999.

BOSHART, M., WEBER, F., JAHN, G., DORSCH-HASLER, K., FLECKENSTEIN, B. AND SCHAFFNER, W. (1985). A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. *Cell* **41**, 521–530.

BOSSLET, K., STEINSTRAESSER, A., SCHWARZ, A., HARTHUS, H.P., LUBER, G., KUHLMANN, L. AND SEDLACEK, H.H. (1988). Quantitative considerations supporting the irrelevance of circulating serum CEA for the immunoscintigraphic visualization of CEA expressing carcinomas. *Eur. J. Nucl. Med.* **14**, 523–527.

BOSSLET, K., CZECH, J., LORENZ, P., SEDLACEK, H.H., SCHUERMANN, M. AND SEEMANN, G. (1992). Molecular and functional characterization of a fusion protein suited for tumour specific pro-drugs activation. *Br. J. Cancer* **65**, 234–238.

BOULIANNE, G.L., HOZUMI, N. AND SHULMAN, M.J. (1984). Production of a functional chimaeric mouse/human antibody. *Nature* **312**, 643–646.

BOULIANNE, G.L., ISENMAN, D.E., HOZUMI, N. AND SHULMAN, M.J. (1987). Biological properties of chimeric antibodies. Interaction with complement. *Mol. Biol. Med.* **4**, 37–49.

BOULOT, G., ROJAS, C., BENTLEY, G.A., POLJAK, R.J., BARBIER, E., LE GUERN, C. AND CAZENAVE, P.A. (1987). Preliminary crystallographic study of a complex between the Fab fragment of a monoclonal anti-lysozyme antibody (D1.3) and the Fab fragment from an anti-idiotypic antibody against D1.3. *J. Mol. Biol.* **194**, 577–579.

BOULOT, G., EISELE, J.-L., BENTLEY, G.A., BHAT, T.N., WARD, E.S., WINTER, G. AND POLJAK, R.J. (1990). Crystallisation and preliminary X-ray diffraction study of the bacterially expressed Fv from the monoclonal anti-lysozyme antibody D1.3 and of its complex with the antigen lysozyme. *J. Mol. Biol.* **213**, 617–619.

BOXER, G.M., BEGENT, R.H.J., KELLY, A.M.B., SOUTHALL, P.J., BLAIR, S.B., THEODOROU, N.A., DAWSON, P.M. AND LEDERMANN, J.A. (1992). Factors influencing variability of localisation of antibodies to carcinoembryonic antigen (CEA) in patients with colorectal carcinoma — implications for radioimmunotherapy. *Br. J. Cancer* **65**, 825–831.

BOYD, J.E. AND JAMES, K. (1989). Human monoclonal antibodies: their potential, problems and prospects. *Adv. Biotechnol. Processes* **11**, 1–43.

BOYER, C.M., LIDOR, Y., LOTTICH, S.C. AND BAST, R.C., JR (1988). Antigenic cell surface markers in human solid tumours. *Antibody, Immunoconj. Radiopharm.* **1**, 105–162.

BRADWELL, A.R., VAUGHN, A., FAIRWEATHER, D.S. AND DYKES, P.W. (1983). Improved radioimmunodetection of tumours using second antibody. *Lancet* **i**, 247.

BRADY, R.L., HUBBARD, R.E., KING, D.J., LOW, D.C., ROBERTS, S.M. AND TODD, R.J. (1991). Crystallisation and preliminary X-ray diffraction study of a chimeric

Fab' fragment of an antibody binding tumour cells. *J. Mol. Biol.* **219**, 603–604.

BRADY, R.L., EDWARDS, D.J., HUBBARD, R.E., JIANG, J.-J., LANGE, G., ROBERTS, S.M., TODD, R.J., ADAIR, J.R., EMTAGE, J.S., KING, D.J. AND LOW, D.C. (1992). Crystal structure of a chimeric Fab' fragment of an antibody binding tumour cells. *J. Mol. Biol.* **227**, 253–264.

BREMER, E.G., LEVERY, S.B., SONNINO, S., GHIDNI, R., CANEVARI, S., KANAGI, R. AND HAKOMORI, S. (1984). Characterization of a glycosphingolipid antigen defined by the monoclonal antibody MBr1 expressed in normal and neoplastic epithelial cells of human mammary gland. *J. Biol. Chem.* **259**, 14 773–14 777.

BRIGGS, M.S. AND GIERASCH, L.M. (1986). Molecular mechanisms of protein secretion: the role of the signal sequence. *Adv. Protein Chem.* **38**, 109–180.

BRIGHT, S., ADAIR, J. AND SECHER, D. (1991). From the laboratory to the clinic: the development of an immunological reagent. *Immunol. Today* **12**, 130–134.

BROOIMANS, R.A., VAN DER ARK, A.A., TOMITA, M., VAN ES, L.A. AND DAHA, M.R. (1992). CD59 expressed by human endothelial cells functions as a protective against complement mediated lysis. *Eur. J. Immunol.* **22**, 791–797.

BROWN, B.A., DAVIS, G.L., SALTZGABER-MULLER, J., SIMON, P., HO, M.-K., SHAW, P.S., STONE, B.A., SANDS, H. AND MOORE, G.P. (1987). Tumor-specific genetically engineered murine/human chimeric monoclonal antibody. *Cancer Res.* **47**, 3577–3583.

BROWN, B.A., DEARBORN, C.B., DROZYNSKI, C.A. AND SANDS, H. (1990). Pharmacokinetics of ^{99m}Tc-metallothionein-B72.3 and its F(ab')$_2$ fragment. *Cancer Res. Suppl.* **50**, 835s–839s.

BROWN, J. (1990). Therapeutic monoclonal antibodies: European regulatory issues. In *Therapeutic Monoclonal Antibodies* (C.A.K. Borrebaeck and J.W. Larrick, Eds), pp. 321–361. Stockton Press, New York.

BROWN, P.S. JR, PARENTEAU, G.L., DIRBAS, F.M., GARSIA, R.J., GOLDMAN, C.K., BUKOWSKI, M.A., JUNGHANS, R.P., QUEEN, C., HAKIMI, J., BENJAMIN, W.R., CLARK, R.E. AND WALDMANN, T.A. (1991). Anti-Tac-H, a humanized antibody to the interleukin 2 receptor, prolongs primate cardiac allograft survival. *Proc. Natl. Acad. Sci. USA* **88**, 263–2667.

BRUGGEMANN, M., SURANI, A.M. AND NEUBERGER, M.S. (1990). *Production of antibodies from transgenic animals.* WO 90/04036.

BRUGGEMANN, M., WILLIAMS, G.T., BINDON, C.I., CLARK, M.R., WALKER, M.R., JEFFERIS, R., WALDMANN, H. AND NEUBERGER, M.S. (1987). Comparison of the effector functions of human immunoglobulins using a matched set of chimeric antibodies. *J. Exp. Med.* **166**, 1351–1361.

BRUGGEMANN, M., CASKEY, H.M., TEALE, E., WALDMANN, H., WILLIAMS, G.T., SURANI, M.A. AND NEUBERGER, M.S. (1989a). A repertoire of monoclonal antibodies with human heavy chains from transgenic mice. *Proc. Natl. Acad. Sci. USA* **86**, 6709–6713.

BRUGGEMANN, M., WINTER, G., WALDMANN, H. AND NEUBERGER, M.S. (1989b). The immunogenicity of chimeric antibodies. *J. Exp. Med.* **170**, 2153–2157.

BRUGGEMANN, M., SPICER, C., BULUWELA, L., ROSEWELL, I., BARTON, S., SURANI, M.A. AND RABBITTS, T.H. (1991). Human antibody production in transgenic mice: expression from 100kb of the human IgH locus. *Eur. J. Immunol.* **21**, 1323–1326.

BUCHEGGER, F., MACH, J.-P., LEONNARD, P. AND CARREL, S. (1986). Selective tumour localisation of radiolabelled anti-human melanoma monoclonal antibody fragments demonstrated in the nude mouse model. *Cancer* **58**, 655–662.

BUCHEGGER, F., VACCA, A., CARREL, S., SCHREYER, M. AND MACH, J.-P. (1988). Radioimmunotherapy of human colon carcinoma by ^{131}I-labelled monoclonal anti-CEA antibodies in a nude mouse model. *Int. J. Cancer* **41**, 127–134.

BUCHEGGER, F., PELEGRIN, A., DELALOYE, B., BISCHOFF-DELALOYE, A. AND MACH, J.-P. (1990). Iodine-131-labeled MAb F(ab')$_2$ fragments are more efficient and less toxic than intact anti-CEA antibodies in radioimmunotherapy of

large human colon carcinoma grafted in nude mice. *J. Nucl. Med.* **31**, 1035–1044.

BUCHNER, J. AND RUDOLPH, R. (1991). Renaturation, purification and characterization of recombinant Fab fragments produced in *Escherichia coli*. *Bio/Technology* **9**, 157–162.

BUCHSBAUM, D.J., BRUBAKER, P.G., HANNA, D.E., GLATFELTER, A.A., TERRY, V.H., GUIBAULT, D.M. AND STEPLEWSKI, Z. (1990). Comparative binding and pre-clinical localization and therapy studies with radiolabeled human chimeric and murine 17-1A monoclonal antibodies. *Cancer Res (Suppl.)* **50**, 993s–999s.

BULENS, F., VANDAMME, A.-M., BERNAR, H., NELLES, L., LIJNEN, R.H. AND COLLEN, D. (1991). Construction and characterization of a functional chimeric mouse–human antibody directed against human fibrin fragment-D dimer. *Eur. J. Biochem.* **195**, 235–242.

BURTON, D.R. (1990). The conformation of antibodies. In *Fc Receptors and the Action of Antibodies* (H. Metzger, Ed.), pp. 31–54. American Society for Microbiology, Washington, DC.

BURTON, D.R. (1991). Human and mouse monoclonal antibodies by repertoire cloning. *Trends Biotechnol.* **9**, 169–175.

BURTON, D.R. AND WOOF, J.M. (1992). Human antibody effector function. *Adv. Immunol.* **51**, 1–84.

BURTON, D.R., BARBAS III, C.F., PERSSON, M.A.A., KOENIG, S., CHANOCK, R.M. AND LERNER, R.A. (1991). A large array of human monoclonal antibodies to type I human immunodeficiency virus from combinatorial libraries of asymptomatic seropositive individuals. *Proc. Natl. Acad. Sci. USA* **88**, 10 134–10 137.

BYERS, V.S., HENSLEE, P.J., KERNAN, N.A., BLAZAR, B.R., GINGRICH, R., PHILLIPS, G.L., LeMAISTRE, C.F., GILLILAND, G., ANTIN, J.H., MARTIN, P., TUTSCHA, P.J., TROWN, P., ACKERMAN, S.K., O'REILLY, R.J. AND SCANNON, P.J. (1990). Use of an anti-pan T-lymphocyte ricin A chain immunotoxin in steroid-resistant acute graft-versus-host disease. *Blood* **75**, 1426–1432.

CABILLY, S. AND RIGGS, A.D. (1985). Immunoglobulin transcripts and molecular history of a hybridoma that produces antibody to carcinoembryonic antigen. *Gene* **40**, 157–161.

CABILLY, S., RIGGS, A.D., PANDE, H., SHIVELY, J.E., HOLMES, W.E., REY, M., PERRY, L.J., WETZEL, R. AND HEYNECKER, H.L. (1984). Generation of antibody activity from immunoglobulin polypeptide chains produced in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **81**, 5369–5373.

CANFIELD, S.M. AND MORRISON, S.L. (1991). The binding affinity of human IgG for its high affinity Fc receptor is determined by multiple amino acids in the C_H2 domain and is modulated by the hinge region. *J. Exp. Med.* **173**, 1483–1491.

CARRASQUILLO, J., SUGARBAKER, P., COLCHER, D., REYNOLDS, J., EESTEBAN, J., BEGENT, G., KEENAN, A., PERENTESIS, P., YOKOYAMA, K., SIMPSON, D., FERRONI, P., FARKAS, R., SCHLOM, J. AND LARSON, S. (1988). Radioimmunoscintigraphy of colon cancer with iodine 131-labeled B72.3 monoclonal antibody. *J. Nucl. Med.* **29**, 1022–1030.

CARTER, P., KELLEY, R.F., RODRIGUES, M.L., SNEDCOR, B., COVARRUBIAS, M., VELLIGAN, M.D., WONG, W.L.T., ROWLAND, A.M., KOTTS, C.E., CARVER, M.E., YANG, M., BOURELL, J.H., SHEPARD, H.M. AND HENNER, D. (1992a). High level *Escherichia coli* expression and production of a bivalent humanized antibody fragment. *Bio/Technology* **10**, 163–167.

CARTER, P., PRESTA, L., GORMAN, C.M., RIDGWAY, J.B.B., HENNER, D., WONG, W.L.T., ROWLAND, A.M., KOTTS, C., CARVER, M.E. AND SHEPARD, H.M. (1992b). Humanization of an anti-p185[HER2] antibody for human cancer therapy. *Proc. Natl. Acad. Sci. USA* **89**, 4285–4289.

CATANE, R. AND LONGO, D.L. (1988). Monoclonal antibodies for cancer therapy. *Isr. J. Med. Sci.* **24**, 471–476.

CHARI, R.V., MARTELL, B.A., GROSS, J.L., COOK, S.B., SHAH, S.A., BLATTLER, W.A., McKENZIE, S.J. AND GOLDMACHER, V.S. (1992). Immunoconjugates

containing novel maytansinoids: promising anti-cancer drugs. *Cancer Res.* **52**, 127–131.

CHARNAY, P., PERRICAUDET, M., GALIBERT, F. AND TIOLLAIS, P. (1978). Bacteriophage lambda and plasmid vectors allowing fusion of cloned genes in each of the three translational phases. *Nucl. Acids Res.* **5**, 4479–4494.

CHATENOUD, L. (1986). The immune response against therapeutic monoclonal antibodies. *Immunol. Today* **7**, 367–368.

CHATENOUD, L., BAUDRIHAYE, M.F., CHKOFF, N., KREIS, H., GOLDSTEIN, G. AND BACH, J.F. (1986). Restriction of the human *in vivo* response against the mouse monoclonal antibody OKT3. *J. Immunol.* **137**, 830–838.

CHISWELL, D.J. AND MCAFFERTY, J. (1992). Phage antibodies: will new 'coliclonal' antibodies replace monoclonal antibodies? *Trends Biotechnol.* **10**, 80–84.

CHOTHIA, C. AND LESK, A.M. (1987). Canonical structures for the hypervariable regions of immunoglobulins *J. Mol. Biol.* **196**, 901–917.

CHOTHIA, C., LESK, A.M., TRAMONTANO, A., LEVITT, M., SMITH-GILL, S.J., AIR, G., SHERIFF, S., PADLAN, E.A., DAVIES, D., TULIP, W.R., COLMAN, P.M., SPINELLI, S., ALZARI, P.M. AND POLJAK, R.J. (1989). Conformations of immunoglobulin hypervariable regions. *Nature* **342**, 877–883.

CHOY, E.H.S., CHIKANZA, I.C., KINGSLEY, G.H. AND PANAYI, G.S. (1991). Chimaeric anti-CD4 monoclonal antibody for relapsing polychondritis. *Lancet* **338**, 450.

CLARK, M.R. (1992). Antibody engineering to date. Proceedings of the conference "*Monoclonal Antibodies. State-of-the-Art in Research and Potential Clinical Applications*". London (abstract).

CO, M.S., DESCHAMPS, M., WHITLEY, R.J. AND QUEEN, C. (1991). Humanized antibodies for antiviral therapy. *Proc. Natl. Acad. Sci. USA* **88**, 2869–2873.

CO, M.S., AVDALOVIC, N.M., CARON, P.C., AVDALOVIC, M.V., SCHEINBERG, D.A. AND QUEEN, C. (1992). Chimeric and humanized antibodies with specificity for the CD33 antigen. *J. Immunol.* **148**, 1149–1154.

COCKETT, M.I.C., BEBBINGTON, C.R. AND YARRANTON, G.T. (1990). High-level expression of tissue inhibitor of metalloproteinases in Chinese hamster ovary cells using Glutamine synthetase gene amplification. *Bio/Technology* **8**, 662–667.

COCKETT, M.I.C., BEBBINGTON, C.R. AND YARRANTON, G.T. (1991). The use of engineered E1A genes to transactivate the hCMV-MIE promoter in permanent CHO cell lines. *Nucl. Acids Res.* **19**, 319–325.

COLAPINTO, E.V., HUMPHREY, P.A., ZALUTSKY, M.R., GROOTHUIS, D.R., FRIEDMAN, H.S., DE TRIBOLET, N., CARREL, S. AND BIGNER, D.D. (1988). Comparative localization of murine monoclonal antibody MEL-14 F(ab′)2 fragment and whole IgG2a in human glioma xenografts. *Cancer Res.* **48**, 5701–5707.

COLCHER, D., HORAN HAND, P., NUTI, M. AND SCHLOM, J. (1981). A spectrum of monoclonal antibodies reactive with human mammary tumor cells. *Proc. Natl. Acad. Sci. USA* **78**, 3199–3203.

COLCHER, D., MILENIC, D., ROSELLI, M., RAUBITSHEK, A., YARRANTON, G., KING, D., ADAIR, J., WHITTLE, N., BODMER, M. AND SCHLOM, J. (1989). Characterization and biodistribution of recombinant and recombinant/chimeric constructs of the monoclonal antibody B72.3. *Cancer Res.* **49**, 1738–1745.

COLCHER, D., MILENIC, D.E., FERRONI, P., ROSELLI, M. AND SCHLOM, J. (1991). *In vivo* and *in vitro* clinical applications of monoclonal antibodies against TAG-72. *Nucl. Med. Biol.* **18**, 395–401.

COLMAN, P.M., LAVER, W.G., VARGHESE, J.N., BAKER, A.T., TULLOCH, P.A., AIR, G.M. AND WEBSTER, R.G. (1987). Three-dimensional structure of a complex of antibody with influenza virus neuraminidase. *Nature* **326**, 358–363.

COURTENAY-LUCK, N.S., EPENETOS, A.A., WINEARLS, C.G. AND RITTER, M.A. (1987). Pre-existing human anti-murine immunoglobulin reactivity due to polyclonal rheumatoid factors. *Cancer Res.* **47**, 4520–4525.

COVELL, D.G., BARBET, J., HOLTON, O.D., BLACK, C.D.V., PARKER, R.J. AND

WEINSTEIN, J.N. (1986). Pharmacokinetics of monoclonal immunoglobulin G_1, $F(ab')_2$, and F(ab') in mice. *Cancer Res.* **46**, 3969–3978.

CROWE, S. (1992). The CAMPATH study: a review. Proceedings of the conference *"Monoclonal Antibodies. State-of-the-Art in Research and Potential Clinical Applications"*. London (abstract).

CZUCZMAN, M.S., DIVGI, C.R., STRAUS, D.J., LOVETT, D.R., GARIN-CHESA, P., YEH, S., FEIRT, N., GRAHAM, M., LEIBEL, S., GEE, T.S., MYERS, J., PENTLOW, K., FINN, R., SCHWARTZ, M., OETTGEN, H.F., LARSON, S., OLD, L.J. AND SCHEINBERG, D.A. (1991). 131 Iodine-labeled monoclonal antibody therapy of acute myelogenous leukemia and B-cell lymphoma. *Antibod. Immunoconj. Radiopharm.* **4**, 787–795.

DANGL, J.L., WENSEL, T.G., MORRISON, S.L., STRYER, L., HERZENBERG, L.A. AND OI, V.T. (1988). Segmental flexibility and complement fixation of genetically engineered chimeric human, rabbit and mouse antibodies. *EMBO J.* **7**, 1989–1994.

DAUGHERTY, B.L., DEMARTINO, J.A., LAW, M.-F., KAWKA, D.W., SINGER, I.I. AND MARK, G.E. (1991). Polymerase chain reaction facilitates the cloning, CDR-grafting and rapid expression of a murine monoclonal antibody directed against the CD18 component of leukocyte integrins. *Nucl. Acids Res.* **19**, 2471–2476.

DAVIDSON, B.R., BABICH, J., YOUNG, H., WADDINGTON, W., CLARKE, G., SHORT, M., BOULOS, P., STYLES, J. AND DEAN, C. (1991). The effect of circulating antigen and radiolabel stability on the biodistribution of an indium labelled antibody. *Br. J. Cancer* **64**, 850–856.

DAVIES, D.R., PADLAN, E.A. AND SHERIFF, S. (1990). Antibody–antigen complexes. *Ann. Rev. Biochem.* **59**, 439–473.

DAVIES, D.R., SHERIFF, S. AND PADLAN, E.A. (1988). Antibody–antigen complexes. *J. Biol. Chem.* **263**, 10541–10544.

DAVIES, D.R., SHERIFF, S. AND PADLAN, E.A. (1989). Comparative study of two Fab-lysozyme crystal structures. *Cold Spring Harbor Symp. Quant. Biol.* **54**, 233–238.

DAVIES, J. AND JIMINEZ, A. (1980). A new selective agent for eukaryotic cloning vectors. *Am. J. Trop. Med. Hyg.* **29** (Suppl.), 1089–1092.

DELALOYE, B., BISCHOFF-DELALOYE, A., BUCHEGGER, F., VON FLIEDNER, V., GROB, V.P., VOLANT, J.C., PETTAVEL, J. AND MACH, J.-P. (1986). Detection of colorectal carcinoma by emission computerised tomography after injection of ^{123}I-labelled Fab' or F(ab')2 fragments from anti-CEA antibodies. *J. Clin. Invest.* **77**, 301–311.

DELEIJ, L. DEJONGE, M., TERHAAR, A., SPAKMAN, H., THE, H., DEVRIES, L., MULDER, N., BERENDSEN, H., ELIAS, M. AND SMIT-SIBINGA, C. (1991). In *Proceedings of the Second International Conference on Bispecific Antibodies and Targeted Cellular Cytotoxicity*, Seillac, France (J. Romet-Lemonne, M. Fanger, and D.M. Segal, Eds), pp. 249–253.

DEMANET, C., BRISSINCK, J., VAN MECHELEN, M., LEO, O. AND THIELMANS, K. (1991). Treatment of murine B cell lymphoma with bispecific monoclonal antibodies (anti-idiotype x anti-CD3). *J. Immunol.* **147**, 1091–1097.

DEMARTINO, J.A., DAUGHERTY, B.L., LAW, M.-F., CUCA, G.C., ALVES, K., SILBERKLANG, M. AND MARK, G.E. (1991). Rapid humanization and expression of murine monoclonal antibodies. *Antibod. Immunoconj. Radiopharm.* **4**, 829–835.

DENARDO, S.J., DENARDO, G.L., O'GRADY, L.F., LEVY, N.B., MILLS, S.L., MACEY, D.J., MCGAHAN, J.P., MILLER, C.H. AND EPSTEIN, A.L. (1988). Pilot studies of radioimmunotherapy of B-cell lymphoma and leukemia using 131-I Lym 1 monoclonal antibody. *Antibod. Immunoconj. Radiopharm.* **1**, 17–34.

DENARDO, S.J., O'GRADY, L.F., MACEY, D.J., KROGER, L.A., DENARDO, G.L., LAMBORN, K.R., LEVY, N.B., MILLS, S.L., HELLSTROM, I. AND HELLSTROM,

K.E. (1991a). Quantitative imaging of mouse L-6 monoclonal antibody in breast cancer patients to develop a therapeutic strategy. *Nucl. Med. Biol.* **18**, 621–631.

DeNardo, S.J., Warhoe, K.A., O'Grady, L.F., DeNardo, G.L., Hellstrom, I., Hellstrom, K.E. and Mills, S.L. (1991b). Radioimmunotherapy with I-131 chimeric L-6 in advanced breast cancer. In *Breast Epithelial Antigens* (R.L. Ceriani, Ed.), pp. 227–232. Plenum Press, New York.

DeNardo, S.J., Warhoe, K.A., O'Grady, L.F., Gobuty, A.H., Macey, D.J., Hellstrom, I., Hellstrom, K.E., Kroger, L.E. and DeNardo, G.L. (1991c) I-131 Human–chimeric MoAB L6: bionetics and radioimmunotherapy in patients with metastatic breast cancer. *Antibod. Immunoconj. Immunother.* **4**, 34.

Divgi, C.R., Kemeny, N., Kelsen, D., Cordon-Cardo, C., Finn, R.D., Schlom, J., Houghton, A. and Larson, S. (1992). Radioimmunotherapy with 131 I-labelled monoclonal antibody CC49 in patients with colorectal carcinoma. *Antibod. Immunoconj. Radiopharm.* **5**, 135.

Dorai, H., Mueller, B.M., Reisfeld, R.A. and Gillies, S.D. (1991). Aglycosylated chimeric mouse/human IgG1 antibody retains some effector function. *Hybridoma* **10**, 211–217.

Dorner, A.J., Bole, D.G. and Kaufman, R.J. (1987). The relationship of N-linked glycosylation and heavy chain binding protein association with the secretion of glycoproteins. *J. Cell. Biol.* **105**, 2665–2674.

Drak, J., Iwasawa, N., Danishefsky, S. and Crothers, D.M. (1991). The carbohydrate domain of calicheamicin $\gamma 1^1$ determines its sequence specificity for DNA cleavage. *Proc. Natl. Acad. Sci. USA* **88**, 7464–7468.

Duchosal, M.A., Eming, S.A., Fischer, P., Leturo, D., Barbas III, C.F., McConahey, P.J., Caothien, R.H., Thornton, G.B., Dixon, F.J. and Burton, D.R. (1992). Immunization of hu-PBL-SCID mice and the rescue of human monoclonal Fab fragments through combinatorial libraries. *Nature* **355**, 258–262.

Duncan, A.R. and Winter, G. (1988). The binding site for C1q on IgG. *Nature* **332**, 738–740.

Duncan, A.R., Woof, J.M., Partridge, L.J., Burton, D.R. and Winter, G. (1988). Localization of the binding site for the human high affinity Fc receptor on IgG. *Nature* **332**, 563–564.

Eary, J.F., Press, O.W., Badger, C.C., Durack, L.D., Richter, K.Y., Addison, S.J., Krohn, K.A., Fisher, D.R., Porter, B.A., Williams, D.L., Martin, P.J., Appelbaum, F.R., Levy, R., Brown, S.L., Miller, R.A., Nelp, W.B. and Bernstein, I.E. (1990). Imaging and treatment of B-cell lymphoma. *J. Nucl. Med.* **31**, 1257–1268.

Endo, K., Kamma, H. and Ogata, T. (1988). Radiolabelled monoclonal antibody 15 and its fragments for localisation and imaging of xenografts of human lung cancer. *J. Natl. Cancer. Inst.* **80**, 835–842.

Epenetos, A.A., Munro, A.J., Stewart, S., Rampling, R., Lambert, H.E., McKenzie, C.G., Soutter, P., Rahemtulle, A., Hooker, G. and Sivolapenko, G.B. (1987). Antibody-guided irradiation of advanced ovarian cancer with intra-peritoneally administered monoclonal antibodies. *J. Clin. Oncol.* **5**, 1890–1899.

Fanger, M.W. and Guyre, P.M. (1991). Bispecific antibodies for targeted cellular cytotoxicity. *Trends Biotechnol.* **9**, 375–380.

Farnsworth, A., Turner, A. Rhind, S.K., Millican, A., Millar, K., Boyce, B., Beeley, N., Boden, J., Boden, R., Pedley, B. and Parker, D. (1991). Comparative biodistributioins of Y-90 and In-111-macrocycle chimeric B72.3 conjugates in a human colon carcinoma xenograft model. *J. Nucl. Med.* **32**, 1842.

Fasler, S., Skarvill, F. and Lutz, H.U. (1983). Electrophoretic properties of human IgG and its subclasses on sodium dodecyl-sulfate-polyacrylamide gel electrophoresis and immunoblots. *Anal. Biochem.* **174**, 593–600.

Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M.,

NORTHROP, J.P., RINGOLD, G.M. AND DANIELSEN, M. (1987). Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. USA* **84**, 7413–7417.

FELL, H.P., YARNOLD, S., HELLSTROM, I., HELLSTROM, K.E. AND FOLGER, K.R. (1989). Homologous recombination in hybridoma cells: Heavy chain chimeric antibody produced by gene targeting. *Proc. Natl. Acad. Sci. USA* **86**, 8507–8511.

FELL, H.P., GAYLE, M.A., GROSMAIRE, L. AND LEDBETTER, J.A. (1991). Genetic construction and characterization of a fusion protein consisting of a chimeric F(ab') with specificity for carcinomas and human IL-2. *J. Immunol.* **146**, 2446–2452.

FIELD, H., YARRANTON, G.T. AND REES, A.R. (1989). Expression of mouse immunoglobulin light and heavy chain variable regions in *Escherichia coli* and reconstitution of antigen-binding activity. *Prot. Eng.* **3**, 641–647.

FOGLER, W.E., SUN, L.K., KLINGER, M.R., GHRAYEB, J. AND DADDONA, P.E. (1989). Biological characterization of a mouse–human IgM antibody directed against the 17-1A antigen. *Cancer Immunol. Immunother.* **30**, 43–50.

FOOTE, J. AND WINTER, G. (1992). Antibody framework residues affecting the conformation of the hypervariable loops. *J. Mol. Biol.* **224**, 487–499.

FRADD, R.B. (1992). Biopharmaceutical firms put emphasis on biology to cure human diseases. *Genetic Eng. News*, January, 26–28.

FRIEND, P.J., WALDMANN, H., HALE, G., COBBOLD, S., REBELLO, P., THIRU, S., JAMIESON, N.V., JOHNSTON, P.S. AND CAINE, R.Y. (1991). Reversal of allograft rejection using the monoclonal antibody CAMPATH-1G. *Transplant. Proc.* **23**, 2253–2254.

FRODIN, J.-E., LEFVERT, A.-K. AND MELLSTEDT, H. (1990). Pharmacokinetics of the mouse monoclonal antibody 17-1A in cancer patients receiving various treatment schedules. *Cancer Res.* **50**, 4866–4871.

FUJII, S., NISHIURA, T., NISHIKAWA, A., MIURA, R. AND TANIGUCHI, N. (1990). Structural heterogeneity of sugar chains in immunoglobulin G. Conformation of immunoglobulin G molecule and substrate specificities of glycotransferases. *J. Biol. Chem.* **265**, 6009–6018.

GALFRE, G. AND MILSTEIN, C. (1981). Preparation of monoclonal antibodies: strategies and procedures. *Methods Enzymol.* **73**, 3–46.

GALFRE, G., MILSTEIN, C. AND WRIGHT, B. (1979). Rat x rat hybrid myelomas and a monoclonal anti-Fd portion of mouse IgG. *Nature* **277**, 131–134.

GALLINGER, S., PAPA, M.Z., REILLY, R.M., XIANG, J., KIRSH, J.C., MULLEN, B.M., STERN, H.S., HOZUMI, N. AND RODER, J.C. (1991). Comparative biodistribution and antibody-dependent cellular cytotoxicity of native and heavy chain chimeric antibody. *Mol. Biother.* **3**, 197–203.

GARRED, P., MICHAELSEN, T.E. AND AASE, A. (1989). The IgG subclass pattern of complement activation depends on epitope density and antibody and complement concentration. *Scand. J. Immunol.* **30**, 379–382.

GARRED, P., MICHAELSEN, T.E., AASE, A. AND MOLLNES, T.E. (1990). C3, C4 and the terminal complement complex differ from C1q by binding predominantly to the antigenic part of solid phase immune complexes. *J. Immunol.* **144**, 198–203.

GARRIDO, M.A., VALDAO, M.J., WINKLER, D.F., TITUS, J.A., HECHT, T.T., PEREZ, P. AND SEGAL, D.M. (1990). Targeting human T-lymphocytes with bispecific antibodies to react against human ovarian carcinoma cells growing in *nu/nu* mice. *Cancer Res.* **50**, 4227–4232.

GAVEL, Y. AND VON HEIJNE, G. (1990). Sequence differences between glycosylated and non-glycosylated Asn-X-Thr/Ser acceptor sites: implications for protein engineering. *Prot. Eng.* **3**, 433–442.

GAVILONDO-COWLEY, J.V., COLOMA, M.J., VAZQUEZ, J., AYALA, M., MACIAS, A., FRY, K.E. AND LARRICK, J.W. (1990). Specific amplification of rearranged immunoglobulin variable region genes from mouse hybridoma cells. *Hybridoma* **9**, 407–417.

GEISOW, M.J. (1992). Bioactive peptides from 'alphabet soup'. *Trends Biotechnol.* **10**, 110–111.

GEISSLER, F., ANDERSEN, S. AND PRESS, O.W. (1991). Intracellular catabolism of radiolabelled anti-CD33 in leukemia cells. *Cell. Immunol.* **137**, 96–101.

GERRETSEN, M., QUAK, J.J., SUH, J.S., VAN WALSUM, M., MEIJER, C.J.L.M., SNOW, G.B. AND VAN DONGEN, G.A.M.S. (1991). Superior localisation and imaging of radiolabelled monoclonal antibody E48 F(ab')2 fragment in xenografts of human squamous cell carcinoma of the head and neck and of the vulva as compared to monoclonal antibody E48 IgG. *Br. J. Cancer* **63**, 37–44.

GHRAYEB, J., KNIGHT, D.M. AND LOONEY, J.E. (1991). *Chimeric immunoglobulin for CD4 receptors.* WO 91/10722.

GILLIES, S.D. AND WESOLOWSKI, J. (1990). Antigen binding and biological activities of engineered mutant chimeric antibodies with human tumour specificities. *Hum. Antibod. Hybridomas* **1**, 47–54.

GILLIES, S.D., LO, K.-M. AND WESOLOWSKI, J. (1989). High-level expression of chimeric antibodies using adapted cDNA variable region cassettes. *J. Immunol. Methods* **125**, 191–202.

GILLIES, S.D., DORAI, H., WESOLOWSKI, J., MAJEAU, G., YOUNG, D., BOYD, J., GARDNER, J. AND JAMES, K. (1989). Expression of human anti-tetanus toxoid antibody in transfected murine myeloma cells. *Bio/Technology* **7**, 799–804.

GILLIES, S.D., YOUNG, D., LO, K.-M. FOLEY, S.F. AND REISFELD, R.A. (1991). Expression of genetically engineered immunoconjugates of lymphotoxin and chimeric anti-ganglioside GD2 antibody. *Hybridoma* **10**, 347–356.

GILLIES, S.D., REILLY, E.B., LO, K.-M. AND REISFELD, R.A. (1992). Antibody-targeted interleukin 2 stimulates T-cell killing of autologous tumor cells. *Proc. Natl. Acad. Sci. USA* **89**, 1428–1432.

GOLD, L. (1990). Expression of heterologous proteins in *Escherichia coli*. *Methods Enzymol.* **185**, 11–14.

GOLD, P. AND FREEMAN, S.L. (1965). Demonstration of tumour-specific antigens in human colonic carcinomata by immunological tolerance and absorption techniques. *J. Exp. Med.* **121**, 439–462.

GOLD, D.V. AND MATTES, M.J. (1988). Monoclonal antibody B72.3 reacts with a core region structure of O-linked carbohydrates. *Tumor Biol.* **9**, 137–144.

GOLDENBERG, D.M., GOLDENBERG, H. AND PRIMUS, F. (1987). In *Immunoconjugates. Antibody Conjugates in Radioimaging and Therapy of Cancer* (C.W. Vogel, Ed.). Oxford University Press, Oxford.

GOLDENBERG, D.M., SHARKEY, R.M. AND FORD, E. (1987). Anti-antibody enhancement of iodine-131 anti-CEA radioimmunodetection in experimental and clinical studies. *J. Nucl. Med.* **28**, 1604–1610.

GOLDENBERG, D.M., HOROWITZ, J.A., SHARKEY, R.M., HALL, T.C., MURTHEY, S., GOLDENBERG, H., LEE, R.E., STEIN, R., SIEGEL, J.A., IZON, D., *et al.* (1991a). Targeting, dosimetry and radioimmunotherapy of B-cell lymphomas with iodine-131-labelled monoclonal antibody LL2. *J. Clin. Oncol.* **9**, 548–564.

GOLDENBERG, D.M., SHARKEY, R.M., HALL, T.C., MURTHY, S., SIEGEL, J.A., IZON, D.O., SWAYNE, L.C., LAKE, D., HANSEN, H.J. AND PINSKY, C.M. (1991b). Radioimmunotherapy of B-cell lymphomas with ^{131}I-labelled LL2 (EPB-2) monoclonal antibody. *Antibod. Immunoconj. Radiopharm.* **4**, 763–769.

GOLDSTEIN, N.I., NAGLE, R., VILLAR, H., HERSH, E. AND FISHER, P.B. (1990). Isolation and characterization of a human monoclonal antibody which reacts with breast and colorectal carcinoma. *Anticancer Res.* **10**, 1491–1500.

GOODMAN, G. (1991). Phase I trial of chimeric monoclonal antibody L6: comparisons to murine L6. *6th Annual Conference on Antibody Immunoconjugates and Radiopharmaceuticals.* San Diego, USA.

GORMAN, C. (1990). Mammalian cell expression. *Curr. Opin. Biotechnol.* **1**, 36–47.

GORMAN, S.D. AND CLARK, M.R. (1990). Humanisation of monoclonal antibodies for therapy. *Semin. Immunol.* **2**, 457–466.

GORMAN, S.D., CLARK, M.R., ROUTLEDGE, E.G., COBBOLD, S.P. AND WALD-MANN, H. (1991). Reshaping a therapeutic CD4 antibody. *Proc. Natl. Acad. Sci. USA.* **88**, 4181–4185.

GOTTLINGER, H.G., FUNKE, I., JOHNSON, J.P., GOKEL, J.M. AND RIETHMULLER, G. (1986). The epithelial cell surface antigen 17-1A, a target for antibody-mediated therapy: its biochemical nature, tissue distribution and recognition by different monoclonal antibodies. *Int. J. Cancer* **38**, 47–53.

GOULD, B.J., BOROWITZ, M.J., GROVES, E.S., CARTER, P.W., ANTHONY, D., WEINER, L.M. AND FRANKEL, A.E. (1989). Phase I study of an anti-breast cancer immunotoxin by continuous infusion: report of a targeted toxic effect not predicted by animal studies. *J. Natl. Cancer Inst.* **81**, 775–781.

GRAHAM, F.L. AND VAN DER EB, A.J. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52**, 456–467.

GRANTHAM, R. AND PERRIN, P. (1986). Codon usage in the immune system. *Immunol. Today* **7**, 160–162.

GRITZ, L. AND DAVIES, J. (1983). Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression in *Escherichia coli* and *Saccharomyces cerevisiae*. *Gene* **25**, 179–188.

GROSSBARD, M.L., GRIBBEN, J.G., FREEDMAN, A.S., KINSELLA, J.M., EPSTEIN, C.L., BLATTLER, W.A. AND NADLER, L.M. (1992). Immunotherapy with anti-B4-blocked ricin for B-cell NHL: immunotoxin therapy of minimal residual disease. *Antibod. Immunoconj. Radiopharm.* **5**, 152.

GUBLER, U. AND HOFFMAN, B.J. (1983). A simple and very efficient method for generating cDNA libraries. *Gene* **25**, 263–269.

GUSSOW, D. AND SEEMANN, G. (1991). Humanization of monoclonal antibodies. *Methods Enzymol.* **203**, 99–121.

HAISMA, H.J., KESSEL, M.A.P., SILVA, C., VAN MUIJEN, M., ROOS, J.C., BRIL, H., MARTENS, H.J.M., McCABE, R. AND BOVEN, E. (1990). Human IgM monoclonal antibody 16.88: pharmacokinetics and distribution in mouse and man. *Br. J. Cancer* **62** (Suppl. X), 40–43.

HAKIMI, J., CHIZZONITE, R., LUKE, D.R., FAMILLETTI, P.C., BAILON, P., KONDAS, J.A., PILSON, R.S., LIN, P., WEBER, D.V., SPENCE, C., MONDINI, L.J., TSIEN, W.-H., LEVIN, J.E., GALLATI, V.H., KORN, L., WALDMANN, T.A., QUEEN, C. AND BENJAMIN, W.R. (1991). Reduced immunogenicity and improved pharmacokinetics of humanized anti-Tac in cynomolgus monkeys. *J. Immunol.* **147**, 1352–1359.

HAKOMORI, S. (1991a). New directions in cancer therapy based on aberrant expression of glycosphingolipids. *Cancer Cells* **3**, 461–470.

HAKOMORI, S. (199ab). Possible functions of tumour-associated carbohydrate antigens. *Curr. Opin. Immunol.* **3**, 646–653.

HALE, G., BRIGHT, S., CHUMBLY, G., HOANG, T., METCALF, D., MUNRO, A.J. AND WALDMANN, H. (1983). Removal of T cells from bone marrow for transplantation: a monoclonal anti-lymphocyte antibody that fixes human complement. *Blood* **62**, 873–882.

HALE, G., COBBOLD, S.P., WALDMANN, H., EASTER, G., MATEJTSCHUK, P. AND COOMBS, R.R.A. (1987). Isolation of low frequency class-switch variants from rat hybrid myelomas. *J. Immunol. Methods* **103**, 59–67.

HALE, G., DYER, M.J., CLARK, M.R., PHILLIPS, J.M., MARCUS, R., RIECHMANN, L., WINTER, G. AND WALDMANN, H. (1988). Remission induction in non-Hodgkin lymphoma with reshaped human monoclonal antibody CAMPATH-1H. *Lancet* **ii**, 1394–1399.

HALLER, D., CANNON, L., ALAVI, A. AND GULFO, J. (1992). A Phase 1 trial of ^{90}Y-labelled monoclonal antibody B72.3 (CYT-103-^{90}Y) administered with concurrent EDTA in refractory adenocarcinoma. *Antibod. Immunoconj. Radiopharm.* **5**, 147.

HAMADA, H., MIURA, K., ARIYOSHI, K., HEIKE, Y., SATO, S., KAMAYAMA, K.,

YOSHIKAZU, K. AND TSURUO, T. (1990). Mouse–human chimeric antibody against the multidrug transporter P-glycoprotein. *Cancer Res.* **50**, 3167–3171.

HAMAGUCHI, M., TAKAHASI, I., TAKEHARS, T., TAKAMATSU, J. AND SAITO, H. (1989). Comparison of recombinant tissue-type plasminogen activator (rt-PA) expressed in mouse C127 cells and human vascular plasminogen activator (HV-PA). *Biochim. Biophys. Acta.* **1009**, 143–150.

HAMMARSTROM, S., SHIVELY, J.E., PAXTON, R.J., BEATTY, B.G., LARSSON, A., GHOSH, R., BORMER, O., BUCHEGGER, F., MACH, J.-P., BURTIN, P., SEGUIN, P., DARBOURET, B., DEGORCE, F., SERTOUR, J., JOLU, J.P., FUKS, A., KALTHOFF, H., SCHMIEGEL, W., ARNDT, R., KLOPPEL, K., VON KLEIST, S., GRUNERT, F., SCHWARTZ, K., MATSUOKA, Y., KUROKI, M., WAGENER, C., WEBER, T., YACHI, A., IMAI, K., HISHIKAWA, N. AND TSUJISAKI, M. (1989). Antigenic sites in carcinoembryonic antigen. *Cancer Res.* **49**, 4852–4858.

HANK, J.A., ROBINSON, R.R., SURFUS, J., MUELLER, B.M., REISFELD, R.A., CHEUNG, N.-K. AND SONDEL, P.M. (1990). Augmentation of antibody dependent cellular cytotoxicity following *in vivo* therapy with recombinant interleukin 2. *Cancer Res.* **50**, 5234–5239.

HARDMAN, N. GILL, L.L., DE WINTER, R.F.J., WAGNER, K., HOLLIS, M., BUSINGER, F., AMMATURO, D., BUCHEGGER, F., MACH, J.-P. AND HUESSER, C. (1989). Generation of a recombinant mouse-human chimaeric monoclonal antibody directed against human carcinoembryonic antigen. *Int. J. Cancer* **44**, 424–433.

HASEMANN, C.A. AND CAPRA, J.D. (1990). High-level expression of a functional immunoglobulin heterodimer in a baculovirus expression system. *Proc. Natl. Acad. Sci. USA* **87**, 3942–3946.

HAY, B.N., SORGE, J.A. AND SHOPES, B. (1992). Bacteriophage cloning and *Escherichia coli* expression of a human IgM Fab. *Hum. Antibod. Hybridomas* **3**, 81–85.

HEIN, M. (1990). Antibody production in plants. *Second International Conference on Antibody Engineering*. San Diego, IBC Conferences.

HEINRICH, G., GRAM, H., KOCHER, H.P., SCHREIER, M.H., RYFFEL, B., AKBAR, A., AMLOT, P.L. AND JANOSSY, G. (1989). Characterization of a human T cell specific chimeric antibody (CD7) with human constant and mouse variable regions. *J. Immunol.* **143**, 3589–3597.

HELLSTROM, I., BEAUMIER, P.L. AND HELLSTROM, K.E. (1986). Antitumor effects of L6, an IgG2a antibody that reacts with most human carcinomas. *Proc. Natl. Acad. Sci. USA* **83**, 7059–7063.

HELLSTROM, I., BRANKOVAN, V. AND HELLSTROM, I.E. (1985). Strong antitumor activities of IgG3 antibodies to a human melanoma-associated ganglioside. *Proc. Natl. Acad. Sci. USA* **82**, 1499–1502.

HELLSTROM, I., HORN, D., LINSLEY, P., BROWN, J.P., BRANKOVAN, V. AND HELLSTROM, K.E. (1986). Monoclonal mouse antibodies raised against human lung carcinoma. *Cancer Res.* **46**, 3917–3923.

HELLSTROM, K.E., YELTON, D.E., FELL, H.P., BEATON, D., GAYLE, M., MacLEAN, M., KAHN, M. AND HELLSTROM, I. (1990). Epitope mapping and use of anti-idiotypic antibodies to the L6 monoclonal anticarcinoma antibody. *Cancer Res.* **50**, 2449–2454.

HENDERSHOT, L.M., TING, J. AND LEE, A.S. (1988). Identity of the immunoglobulin heavy-chain binding protein with the 78,000 dalton glucose-regulated protein and the role of posttranslational modifications in its binding function. *Mol. Cell. Biol.* **8**, 4250–4256.

HENDERSHOT, L., BOLE, D., KOHLER, G. AND KEARNEY, J. (1987). Assembly and secretion of heavy chains that do not associate post-translationally with immunoglobulin heavy chain binding protein. *J. Cell. Biol.* **104**, 761–767.

HERLYN, D. AND KOPROWSKI, H. (1982). IgG2a monoclonal antibodies inhibit

tumour growth through interaction with effector cells. *Proc. Natl. Acad. Sci. USA* **79**, 4761–4765.

HERLYN, D., STEPLEWSKI, Z., HERLYN, M. AND KOPROWSKI, H. (1980). Inhibition of growth of colorectal carcinoma in nude mice by monoclonal antibody. *Cancer Res.* **40**, 717–721.

HERLYN, D., POWE, H., ALAVI, A., MATTIS, H., HERLYN, M., ERNST, C., VAUM, R. AND KOPROWSKI, H. (1983). Radioimmunodetection of human tumour xenografts by monoclonal antibodies. *Cancer Res.* **43**, 2731–2735.

HERLYN, D., LUBECK, M., SEARS, H.F. AND KOPROWSKI, H. (1985). Specific detection of anti-idiotypic immune responses in cancer patients treated with mouse monoclonal antibody. *J. Immunol. Methods* **85**, 27–38.

HERLYN, M., MENRAD, A. AND KOPROWSKI, H. (1990). Structure, function, and clinical significance of human tumour antigens. *J. Natl. Cancer Inst.* **82**, 1883–1889.

HERLYN, M., STEPLEWSKI, Z., HERLYN, D. AND KOPROWSKI, H. (1986). CO71-1A and related monoclonal antibodies: their production and characterization. *Hybridoma* **5** (Suppl. 1), S3–S10.

HINMAN, L., ADAIR, J.R. BAKER, T.S., HAMANN , P.R., BEYER, C.F., WALLACE, R. AND UPESLACIS, J. (1993). Lysine-linked conjugates of N-acetyl calicheamicin $\gamma1^1$ with a fully humanized anti-polymorphic epithelial mucin antibody show potent antitumour effects in ovarian tumour xenografts. American Association of Cancer Research, Orlando, FL, USA.

HIRD, V., SNOOK, D., KOSMAS, C., DHOKIA, B., STEWART, S., MASON, P., LAMBERT, J., MEARES, C. AND EPENETOS, A. (1991a). Intraperitoneal radioimmunotherapy with stable immunoconjugates of yttrium-90. In *Advances in the applications of monoclonal antibodies in clinical oncology* (A.A. Epenetos, Ed.), pp. 267–272. Chapman and Hall, London.

HIRD, V., VERHOEYEN, M., BADLEY, R.A., PRICE, D., SNOOK, D., KOSMAS, C., GOODEN, C., BAMIAS, A., MEARES, C., LAVENDER, J.P. AND EPENETOS, A.A. (1991b). Tumour localisation with a radioactively labelled reshaped human monoclonal antibody. *Br. J. Cancer* **64**, 911–914.

HOLZSCHU, D.L. AND DAISS, J.L. (1989). *Generation of chimeric antibodies in vivo by site-specific recombination.* EPO 345 466.

HOOGENBOOM, H.R., RAUS, J.C.M. AND VOLCKAERT, G. (1990). Cloning and expression of a chimeric antibody directed against the human transferrin receptor. *J. Immunol* **144**, 3211–3217.

HORGAN, C., BROWN, K. AND PINCUS, S.H. (1990). Alteration in H chain V region affects complement activation by chimeric antibodies. *J. Immunol.* **145**, 2527–2532.

HORWITZ, A.H., CHANG, C.P., BETTER, M. HELLSTROM, K.E. AND ROBINSON, R.R. (1988). Secretion of functional antibody and Fab fragment from yeast cells. *Proc. Natl. Acad. Sci. USA* **85**, 8678–8682.

HOUGHTEN, R.A., PINILLA, C., BLONDELLE, S.E., APPEL, J.R., DOOLEY, C.T. AND CUERVO, J.H. (1991). Generation and use of synthetic peptide combinatorial libraries for basic research and drug discovery. *Nature* **354**, 84–86.

HUSTON, J.S., LEVINSON, D., MUDGETT-HUNTER, M., TAI, M., NOVOTNY, J., MARGOLIES, M.N., RIDGE, R.J., BRUCCOLERI, R.E., HABER, E., CREA, R. AND OPPENHEIM, H. (1988). Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli. Proc. Natl. Acad. Sci. USA* **85**, 5879–5883.

HUTZELL, P., KASHMIRI, S., COLCHER, D., PRIMUS, F.J., HORAN HAND, P., ROSELLI, M., FINCH, M., YARRANTON, G., BODMER, M., WHITTLE, N., KING, D., LOULLIS, C.C., McCOY, D.W., CALLAHAN, R. AND SCHLOM, J. (1991). Generation and characterization of a recombinant/chimeric B72.3 (human $\gamma1$). *Cancer Res.* **51**, 181–189.

ISAACS, J.D., WATTS, R.A., HAZLEMAN, B.L. HALE, G., KEOGAN, M.T. COBBOLD,

S.P. AND WALDMANN, H. (1992). Humanised monoclonal antibody therapy for rheumatoid arthritis. *Lancet* **340**, 748–752.

JAFFERS, G.J., FULLER, T.C., COSIMI, A.B., RUSELL, P.S., WINN, H.J. AND COLVIN, R.B.T. (1986). Monoclonal antibody therapy. Anti-idiotypic and non-anti-idiotypic antibodies to OKT3 arising despite intense immunosuppression. *Transplantation* **41**, 572–578.

JAIN, R.K. (1990). Physiological barrier to delivery of monoclonal antibodies and other macromolecules in tumours. *Cancer Res.* **50** (Suppl.), 814s–819s.

JAMES, K. (1990). Human monoclonal antibodies and engineered antibodies in the management of cancer. *Semin. Cancer Biol.* **1**, 243–253.

JAMES, K. AND BELL, G.T. (1987). Human monoclonal antibody production. Current status and future prospects. *J. Immunol. Methods* **100**, 5–40.

JEFFERIS, R., LUND, J. AND POUND, J. (1990). Molecular definition of interaction sites on human IgG for Fc receptors (huFcγR). *Mol. Immunol.* **27**, 1237–1240.

JOHNSON, T.K., MADDOCK, S., KSALIWAL, R., BLOEDOW, D., HARTMANN, C., FEYERABEND, A., DIENHART, D.G., THICKMAN, D., GLENN, S., GONZALEZ, R., LEAR, J. AND BUNN, P. JR. (1991). Radioimmunoadsorption of KC-4G3 antibody in peripheral blood: implications for radioimmunotherapy. *Antibod. Immunoconj. Radiopharm.* **4**, 885–893.

JOLLIFFE, L.J., ZIVIN, R.A., ADAIR, J.R. AND ATHWAL, D.S. (1991). *CD3 specific recombinant antibody.* WO91/09968.

JONES, P.T., DEAR, P.H., FOOTE, J., NEUBERGER, M.S. AND WINTER, G. (1986). Replacing the complementarity-determining regions in a human antibody with those from a mouse. *Nature* **321**, 522–525.

JONES, S.T. AND BENDIG, M.M. (1991). Rapid PCR-cloning of full length mouse immunoglobulin variable regions. *Bio/Technology* **9**, 88–89.

JONKER, M. AND DEN BROK, J.H.A.M. (1987). Idiotype switching of CD4-specific monoclonal antibodies can prolong the therapeutic effectiveness in spite of host anti-mouse IgG antibodies. *Eur. J. Immunol.* **17**, 1547–1553.

JUNGHANS, R.P., WALDMANN, T.A., LANDOLFI, N.F., AVDALOVIC, N.M., SCHNEIDER, W.P. AND QUEEN, C. (1991). Anti-Tac-H, a humanized antibody to the interleukin 2 receptor with new features for immunotherapy in malignant and immune disorders. *Cancer Res.* **50**, 1495–1502.

KABAT, E.A., WU, T.T., REID-MILLER, M., PERRY, H.M. AND GOTTESMAN, K.S. (1987). *Sequences of Proteins of Immunological Interest*, 4th edition. United States Department of Health and Human Services, Washington, DC.

KASHIMIRI, S.V.A. (1992). Genetically engineered MAbs for cancer diagnosis and therapy. Proceedings of the conference "*Monoclonal Antibodies. State-of-the-Art in Research and Potential Clinical Applications*". London (abstract).

KATRE, N.V. (1990). Immunogenicity of recombinant IL-2 modified by covalent attachment of polyethylene glycol. *J. Immunol.* **144**, 209–213.

KELLEY, R.F., O'CONNELL, M.P., CARTER, P., PRESTA, L., EIGENBROT, C., COVARRUBIAS, M., SNEDCOR, B., BOURELL, J.H. AND VETTERLEIN, D. (1992). Antigen binding thermodynamics and anti-proliferative effects of chimeric and humanized anti-p185^{HER2} antibody Fab fragments. *Biochemistry* **31**, 5434–5441.

KETTLEBOROUGH, C.A., SALDANHA, J., HEATH, V.J., MORRISON, C.J. AND BENDIG, M.M. (1991). Humanisation of a mouse monoclonal antibody by CDR grafting: the importance of framework residues on loop conformation. *Prot. Eng.* **4**, 773–783.

KHAZAELI, M.B., SALEH, M.N., WHEELER, R.H., HUSTER, W.J., HOLDEN, H., CARRANO, R. AND LoBUGLIO, A.F. (1988). Phase I trial of multiple large doses of murine monoclonal antibody CO17-1A. II Pharmacokinetics and immune response. *J. Natl. Cancer Inst.* **80**, 937–942.

KHAZAELI, M.B., WHEELER, R., ROGERS, K., TENG, N., ZIEGLER, E., HAYNES, A., SALEH, M.N., HARDIN, J.M., BOEMER, S. AND CORNETT, J. (1990). Initial

evaluation of a human IgM monoclonal antibody (HA-1A) in man. *J. Biol. Resp. Modif.* **9**, 178–183.

KHAZAELI, M.B., SALEH, M.N., LIU, T.P., MEREDITH, R.F., WHEELER, R.H., BAKER, T.S., KING, D., SECHER, D., ALLEN, L., ROGERS, K., COLCHER, D., SCHLOM, J., SHOCHAT, T.D. AND LoBUGLIO, A.F. (1991). Pharmacokinetics and immune response of ^{131}I-chimeric mouse/human B72.3 (human gamma 4) monoclonal antibody in man. *Cancer Res.* **51**, 5461–5466.

KHAZAELI, M.B., SALEH, M.N., LIU, T.P., KALADAS, P.M., GILMAN, S. AND LoBUGLIO, A.F. (1992). Frequent anti-V region immune response to mouse B72.3 monoclonal antibody. *J. Clin. Immunology* **12**, 116–121.

KILMARTIN, J.V., WRIGHT, B. AND MILSTEIN, C. (1982). Rat monoclonal antibodies derived by using a non-secreting rat cell line. *J. Cell. Biol.* **93**, 576–582.

KING, D.J., ADAIR, J.R., ANGAL, S., LOW, D.C., PROUDFOOT, K.A., LLOYD, J.C., BODMER, M. AND YARRANTON, G.T. (1992a). Expression, purification and characterisation of a mouse: human chimeric antibody and chimeric Fab' fragment. *Biochem. J.* **281**, 317–323.

KING, D.J., MOUNTAIN, A., ADAIR, J.R., OWENS, R.J., HARVEY, A., WEIR, N., PROUDFOOT, K.A., PHIPPS, A., LAWSON, A., RHIND, S.K., PEDLEY, B., BODEN, J., BVEGENT, R.H.J. AND YARRANTON, G.T. (1992b). Tumour localisation of engineered antibody fragments. *Antibod. Immunoconj. Radiopharm.* **5**, 159–170.

KING, D.J., BYRON, O.D., MOUNTAIN, A., WEIR, N., HARVEY, A., LAWSON, A.D.G., PROUDFOOT, K.A., BALDOCK, D., HARDING, S.E., YARRANTON, G.T. AND OWENS, R.J. (1963). Expression, purification and characterisation of B72.3 Fv fragments. *Biochem J.*, in press.

KITAMURA, K., TAKAHASHI, T., TAKASHINA, K., YAMAGUCHI, T., NOGUCHI, A., TSURUMI, H., TOYOKUNI, T. AND HAKOMORI, S. (1989). Polyethylene glycol modification of the antibody A7 enhances its tumor localization. *Biochem. Biophys. Res. Commun.* **171**, 1387–1394.

KITAMURA, K., TAKAHASHI, T., YAMAGUCHI, T., NOGUCHI, A., NOGUCHI, A., TAKASHINA, K., TSURUMI, H., INAGAKE, M., TOYOKUNI, T. AND HAKOMORI, S. (1991). Chemical engineering of the monoclonal antibody A7 by polyethylene glycol for targeting cancer chemotherapy. *Cancer Res.* **51**, 4310–4315.

KLAUSNER, R.D. AND SITIA, R. (1990). Protein degradation in the endoplasmic reticulum. *Cell* **62**, 611–614.

KLIBANOV, A.L., MARTYNOV, A.V., SLINKIN, M.A., SAKHAROV, I., YU, SMIRNOV, M.D., MUZYKANTOV, V.R., DANILOV, S.M. AND TORCHILIN, V.P. (1988). Blood clearance of radiolabelled antibody: enhancement by lactosamination and treatment with biotin-avidin or anti-mouse IgG antibodies. *J. Nucl. Med.* **29**, 1951–1956.

KNOX, S.J., LEVY, R., HODGKINSON, S., BELL, R., BROWN, S., WOOD, G.S., HOPPE, R., ABEL, E.A., STEINMAN, L., BERGER, R.G., GAISER, C., YOUNG, G., BINDL, J., HANHAM, A. AND REICHERT, T. (1991). Observations on the effect of chimeric anti-CD4 monoclonal antibody in patients with Mycosis Fungoides. *Blood* **77**, 20–30.

KODA, K. AND GLASSY, M.C. (1990). *In vitro* immunization for the production of human monoclonal antibody. *Hum. Antibod. Hybridomas* **1**, 15–22.

KODA, K., GLASSY, M.C. AND CHANG, H.R. (1990). Generation of human monoclonal antibodies against colon cancer. *Arch. Surg.* **125**, 1591–1597.

KOGA, J., CHATTERJEE, S. AND WHITLEY, R.J. (1986). Studies on Herpes Simplex Virus Type 1 Glycoproteins using monoclonal antibodies. *Virology* **151**, 385–389.

KOGA, H., KANDA, H., NAKASHIMA, M., WATANABE, Y., ENDO, K. AND WATANABE, T. (1990). Mouse–human chimeric monoclonal antibody to carcinoembryonic antigen (CEA): *in vitro* and *in vivo* activities. *Hybridoma* **9**, 43–56.

KOHLER, G. (1980). Immunoglobulin chain loss in hybridoma lines. *Proc. Natl. Acad. Sci. USA* **77**, 2197–2199.

KOHLER, G. AND MILSTEIN, C. (1975). Continuous culture of fused cells secreting antibody of predefined specificity. *Nature* **265**, 495–497.

KOPROWSKI, H., HERLYN, D., LUBECK, M., DeFREITAS, E. AND SEARS, H.F. (1984). Human anti-idiotype antibodies in cancer patients: is the modification of the immune response beneficial for the patient? *Proc. Natl. Acad. Sci. USA* **81**, 216–219.

KOZAK, M. (1987). At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. *J. Mol. Biol.* **196**, 947–950.

KUKURUZINSKA, M.A., BERGH, M.L.E. AND KACKSON, B.J. (1987). Protein glycosylation in yeast. *Ann. Rev. Biochem.* **56**, 915–944.

KURRLE, R., SHEARMAN, C.W., MOORE, G.P. AND SEILER, F. (1990). *Improved monoclonal antibodies against the human alpha/beta T-cell receptor, their production and use.* EP 0403156.

LAI, E., WILSON, R.K. AND HOOD, L.E. (1989). Physical maps of the mouse and human immunoglobulin-like loci. *Adv. Immunol.* **46**, 1–59.

LAM, K.S., SALMON, S.E., HERSH, E.M., HRUBY, V.J., KAZMIERSKI, W.M. AND KNAPP, R.J. (1991). A new type of synthetic peptide library for identifying ligand-binding activity. *Nature* **354**, 82–84.

LANGMUIR, V.K. (1992). Radioimmunotherapy: clinical results and dosimetric considerations. *Nucl. Med. Biol.* **19**, 213–225.

LARRICK, J.W. AND FRY, K.E. (1991). Recombinant antibodies. *Hum. Antibod. Hybridomas* **2**, 172–189.

LARSON, S.L. (1991). Overview of radioimmunodetection: technical advances. *Antibod. Immunoconj. Radiopharm.* **4**, 525–531.

LAW, M.-F., MARK, G.E., III AND WILLIAMSON, A.R. (1991). *Method for producing recombinant immunoglobulins.* EP 0438310.

LEDERMANN, J.A., BEGENT, R.H.J., BAGSHAWE, K.D., RIGGS, S.J., SEARLE, F., GLASER, M.G., GREEN, A.J. AND DALE, R.G. (1988). Repeated antitumour antibody therapy in man with suppression of the host response by cyclosporin A. *Br. J. Cancer* **58**, 654–657.

LEE, M.D., DUNNE, T.S., SIEGEL, M.M., CHANG, C.C., MORTON, G.O. AND BORDERS, D.B. (1987a). Calicheamins, a novel family of antitumor antibiotics. 1. Chemistry and partial structure of calicheamicin $\gamma 1^1$. *J. Am. Chem. Soc.* **109**, 3464–3466.

LEE, M.D., DUNNE, T.S., CHANG, C.C., ELLESTAD, G.A., SIEGEL, M.M., MORTON, G.O., McGAHREN, W.J. AND BORDERS, D.B. (1987b). Calicheamicins, a novel family of antitumor antibiotics. 2. Chemistry and solution structure of calicheamicin $\gamma 1^1$. *J. Am. Chem. Soc.* **109**, 3466–3468.

LI, Y.-w., LAWRIE, D.K., THAMMANA, P., MOORE, G.P. AND SHEARMAN, C.W. (1990). Construction, expression and characterization of a murine/human chimeric antibody with specificity for Hepatitis B surface antigen. *Mol. Immunol.* **27**, 303–311.

LIAO, S.-K., HORTON, L., FLAHART, R., O'REAR, L., KIMBRO, B. AND ROBINSON, R.R. (1989). Antibody dependent cellular cytotoxicity (ADCC) of a mouse-human chimeric monoclonal antibody of the human IgG1 subclass with specificity for human adenocarcinomas. *Proc. Am. Soc. Cancer Res.* **30**, 391.

LIND, P., LECHNER, P., HAUSMAN, B., SMOLA, M.G., KOELTRINGER, P., STEINDORFER, P., CESNIK, H., PASSL, R. AND EBER, O. (1991). Development of human antimouse antibodies (HAMA) after single and repeated diagnostic application of intact murine monoclonal antibodies. *Antibod. Immunoconj. Radiopharm.* **4**, 811–818.

LIOU, R.-S., ROSEN, E.M., FUNG, M.S.C., SUN, W.N.C., SUN, C., GORDON, W., CHANG, N.T. AND CHANG, T.W. (1989). A chimeric mouse–human antibody that retains specificity for HIV gp120 and mediates the lysis of HIV-infected cells. *J. Immunol.* **143**, 3967–3975.

LISZEWSKI, M.K., POST, T.W. AND ATKINSON, J.P. (1991). Membrane cofactor

protein (MCP or CD46) newest member of the regulators of complement activation gene cluster. *Ann. Rev. Immunol.* **9**, 431–455.

LIU, A.H., CREADON, G. AND WYSOCKI, L.J. (1992). Sequencing heavy and light chain variable genes of single B-hybridoma cells by total enzymatic application. *Proc. Natl. Acad. Sci. USA* **89**, 7610–7614.

LIU, A.Y., MACK, P.W., CHAMPION, C.I. AND ROBINSON, R.R. (1987a). Expression of mouse:human immunoglobulin heavy-chain cDNA in lymphoid cells. *Gene* **54**, 33–40.

LIU, A.Y., ROBINSON, R.R., HELLSTROM, K.E., MURRAY, E.D., JR., CHANG, C.P. AND HELLSTROM, I. (1987b). Chimeric mouse–human IgG1 antibody that can mediate lysis of cancer cells. *Proc. Natl. Acad. Sci. USA* **84**, 3439–3443.

LIU, A.Y., ROBINSON, R.R., MURRAY, E.D.JR., LEDBETTER, J.A., HELLSTROM, I. AND HELLSTROM, K.E. (1987c). Production of a mouse–human chimeric monoclonal antibody to CD20 with potent Fc-dependent biologic activity. *J. Immunol.* **139**, 3521–3526.

LIU, L.F. (1989). DNA topoisomerase poisons as antitumor drugs. *Ann. Rev. Biochem.* **58**, 351–375.

LOBUGLIO, A.F., SALEH, M.N., LEE, J., KHAZAELI, M.B., CARRANO, R., HOLDEN, H. AND WHEELER, R.H. (1988). Phase I trial of multiple large doses of murine monoclonal antibody CO17-1A. I. Clinical aspects. *J. Natl. Cancer Inst.* **80**, 932–936.

LOBUGLIO, A.F., WHEELER, R.H., TRANG, J., HAYNES, A., ROGERS, K., HARVEY, E.B., SUN, L., GHRAYEB, J. AND KHAZAELI, M.B. (1989). Mouse/human chimeric monoclonal antibody in man: Kinetics and immune response. *Proc. Natl. Acad. Sci. USA* **86** 4220–4224.

LOBUGLIO, A.F., MEREDITH, R., WHEELER, R.H., ROGERS, K.J., LIU, T., ALLEN, L., POLANSKY, A., PLOTT, G. AND KHAZAELI, M.B. (1991). Chimeric monoclonal antibody studies in colo-rectal cancer. In *Monoclonal Antibodies. Applications in Clinical Oncology* (A. Epenetos, Ed.), pp. 291–295. Chapman and Hall, London.

LUND, J., TANAKA, T., TAKAHASHI, N., SARMAY, G., ARATA, Y. AND JEFFERIS, R. (1990). A protein structural change in aglycosylated IgG3 correlates with loss of huFcγRI and huFcγRIII binding and/or activation. *Mol. Immunol.* **27**, 1145–1153.

LUND, J., WINTER, G., JONES, P.T., POUND, J.D., TANAKA, T., WALKER, M.R., ARTYMIUK, P.J., ARATA, Y., BURTON, D.R., JEFFERIS, R. AND WOOF, J.M. (1991). Human FcγRI and FcγRII interact with distinct but overlapping sites on human IgG. *J. Immunol.* **147**, 2657–2662.

LUND, J., POUND, J.D., JONES, P.T., DUNCAN, A.R., BENTLEY, T., GOODALL, M., LEVINE, B.A., JEFFERIS, R. AND WINTER, G. (1992). Multiple binding sites on the C_H2 domain of IgG for mouse FcγRII. *Mol. Immunol.* **29**, 53–59.

LYONS, A., KING, D.J., OWENS, R.J., YARRANTON, G.T., MILLICAN, A., WHITTLE, N.R. AND ADAIR, J.R. (1990). Site-specific attachment to recombinant antibodies via introduced surface cysteine residues. *Prot. Eng.* **3**, 703–708.

MACH, J.-P., PELEGRIN, A. AND BUCHEGGER, F. (1991). Imaging and therapy with monoclonal antibodies in non-hematopoietic tumors. *Curr. Opin. Immunol.* **3**, 685–693.

MACH, J.-P., CHATAL, J.-F., LUMBROSO, J.-D., BUCHEGGER, F., FORNI, M., RITSCHARD, J., BERCHE, C., DOUILLARD, J.-Y., CARREL, S., HERLYN, M., STEPLEWSKI, Z. AND KOPROWSKI, H. (1983). Tumor localization in patients by radiolabelled monoclonal antibodies against colon carcinoma. *Cancer Res.* **43**, 5593–5600.

MAEDA, H., MATSUSHITA, S., EDA, Y., KIMACHI, K., TOKIYOSHI, S. AND BENDIG, M.M. (1991). Construction of reshaped human antibodies with HIV-neutralizing activity. *Human Antibodies Hybridomas* **2**, 124–134.

MARAVEYAS, A. AND EPENETOS, A.A. (1991). An overview of radioimmunotherapy. *Cancer Immunol. Immunother.* **34**, 71–73.

MARCHITTO, K.S., KINDSVOGEL, W.R., BEAUMIER, P.L., FINE, S.K., GILBERT, T., LEVIN, S.D., WOODHOUSE, C.S. AND MORGAN, A.C. JR (1989). Characterization of a human–mouse chimeric antibody reactive with a human melanoma associated antigen. *Immun. Cancer*, 101–105.

MARIANI, G. AND STROBER, W. (1990). Immunoglobulin metabolism. In *Fc Receptors and the Action of Antibodies* (H. Metzger, Ed.), pp. 94–177. American Society for Microbiology, Washington, DC.

MARKEN, J.S., SCHIEVEN, G.L., HELLSTROM, I., HELLSTROM, K.E. AND ARUFFO, A. (1992). Cloning and expression of the tumor-associated antigen L6. *Proc. Natl. Acad. Sci. USA* **89**, 3503–3507.

MARKS, J.D., HOOGENBOOM, H.R., BONNERT, T.P., MCCAFFERTY, J., GRIFFITHS, A.D. AND WINTER, G. (1991). By-passing immunization. Human antibodies from V-gene libraries displayed on phage. *J. Mol. Biol.* **222**, 581–597.

MARKS, J.D., GRIFFITHS, A.D., MALMQUIST, M., CLACKSON, T.P., BYE, J.M. AND WINTER, G. (1992). By-passing immunization: building high affinity human antibodies by chain shuffling. *Bio/Technology* **10**, 779–783.

MASUCCI, G. AND MELLSTEDT, H. (1991). Combinations of IL-2, GM-CSF, IL-4, IFN-α and TNF-α increase the antibody dependent cellular cytotoxicity (ADCC) of blood mononuclear cells in presence of either mouse or chimeric 17-1A. A pre-clinical evaluation. *Cancer J.* **4**, 168–173.

MASUCCI, G., LINDEMALM, C., FRODIN, J.-E., HAGSTROM, B. AND MELLSTEDT, H. (1988). Effect of human blood mononuclear cell populations in antibody dependent cellular cytotoxicity (ADCC) using two murine (CO17-1A and Br55-2) and one chimeric (17-1A) monoclonal antibodies against a human colorectal carcinoma cell line (SW948). *Hybridoma* **7**, 429–440.

MASUCCI, G., RAGNHAMMAR, P., WERSALL, P. AND MELLSTEDT, H. (1990). Granulocyte-macrophage colony-stimulating factor augments the interleukin-2-induced cytotoxic activity of human lymphocytes in the absence and presence of mouse or chimeric monoclonal antibodies (mAb 17-1A). *Cancer Immunol. Immunother.* **31**, 231–235.

MATHIESON, P.W., COBBOLD, S.D., HALE, G., CLARK, M.R., OLIVERA, D.B., LOCKWOOD, C.M. AND WALDMANN, H. (1990). Monoclonal antibody therapy in systemic vasculitis. *New Engl. J. Med.* **323**, 250–254.

MATSUSHITA, S., ROBERT-GUROFF, M., RUSCHE, J., KOITO, A., HATTORI, T., HOSHINO, H., JAVAHERIAN, K., TAKATSUKI, K. AND PUTNEY, S. (1988). Characterization of a human immunodeficiency virus neutralizing monoclonal antibody and mapping of the neutralizing epitope. *J. Virol.* **62**, 2107–2114.

MATTES, M.J. (1987). Biodistribution of antibodies after intraperitoneal or intravenous injection and effect of carbohydrate modifications. *J. Natl. Cancer Inst.* **79**, 855–863.

MEEKER, T.C., LOWDER, J., MALONEY, D.G., MILLER, R.A., THIELEMANS, K., WARNKE, R. AND LEVY, R. (1985). A clinical trial of anti-idiotype therapy for B cell malignancy. *Blood* **65**, 1349–1363.

MEREDITH, R.F., LOBUGLIO, A.F., PLOTT, W.E., ORR, R.A., BREZOVICH, I.A., RUSSELL, C.D., HARVEY, E.B., YESTER, M.V., WAGNER, A.J., SPENCER, S.A., WHEELER, R.H., SALEH, M.N., ROGERS, K.J., POLANSKY, A., SALTER, M.M. AND KHAZAELI, M.B. (1991). Pharmacokinetics, immune response, and biodistribution of Iodine-131-labeled chimeric mouse/human IgG1, κ 171A monoclonal antibody. *J. Nucl. Med.* **32**, 1162–1168.

MEREDITH, R.F., KHAZAELI, M.B., PLOTT, W.E., BREZOVICH, I.A., RUSSELL, C.D., WHEELER, R.H., SPENCER, S.A. AND LOBUGLIO, A.F. (1992a). Comparison of two mouse/human chimeric antibodies in patients with metastatic colon cancer. *Antibod. Immunoconj. Radiopharm.* **5**, 75–80.

MEREDITH, R.F., KHAZAELI, M.B., PLOTT, W.E., SALEH, M.N., LIU, T., ALLEN,

L.F., RUSSELL, C.D., ORR, R.A., COLCHER, D., SCHLOM, J., SCHOCHAT, D., WHEELER, R.W. AND LOBUGLIO, A.F. (1992b). Phase I trial of iodine-131-chimeric B72.3 (human IgG4) in metastatic colorectal cancer. *J. Nucl. Med.* **33**, 23–29.

MEZES, P., GOURLIE, B. AND MARK, R. (1990). *A novel family of high affinity, modified antibodies for cancer treatment.* EP 0365997.

MEZZANZZANICA, D., GARRIDO, D., NEBLOCK, D., ZURAWSKI, V., ANDRESS, V., COLNAGHI, M., WUNDERLICH, J. AND SEGAL, D. (1991). In *Proceedings of the Second International Conference on Bispecific Antibodies and Targeted Cellular Cytotoxicity*, Seillac, France (J. Romet-Lemonne, M. Fanger and D.M. Segal, Eds), pp. 223–225.

MICHAELSEN, T.E., GARRED, P. AND AASE, A. (1991). Human IgG subclass pattern of inducing complement-mediated lysis depends on antigen concentration and to a lesser extent on epitope patchiness, antibody affinity and complement concentration. *Eur. J. Immunol.* **21**, 11–16.

MICHAELSEN, T.E., AASE, A., NORDERHAUG, L. AND SANDLIE, I. (1992). Antibody dependent cell mediated cytotoxicity induced by chimeric mouse-human IgG subclasses and IgG3 antibodies with altered hinge region. *Mol. Immunol.* **29**, 319–326.

MILENIC, D.E., YOKOTA, T., FILIPULA, D.R., FINKELMAN, M.A.J., DODD, S.W., WOOD, J.F., WHITLOW, M., SNOY, P. AND SCHLOM, J. (1991). Construction, binding properties, metabolism, and tumor targeting of a single chain Fv derived from the pancarcinoma monoclonal antibody CC49. *Cancer Res.* **51**, 6363–6371.

MOI, M.K., MEARES, C.F. AND DENARDO, S.J. (1988). The peptide way of macrocyclic bifunctional chelating agents: synthesis of 2-(p-Nitrobenzyl) — 1,4,7,10 — tetraazacyclododecane — N, N', N'', N''' — tetraacetic acid, and study of its yttrium (III) complex. *J. Am. Chem. Soc.* **110**, 6266–6267.

MOLTHOFF, C.F.M., PINEDO, H.M., SCHLUPER, H.M.M., NIJAM, H.W. AND BOVEN, E. (1992). Comparison of the pharmacokinetics, biodistribution and dosimetry of monoclonal antibodies OC125, OV-TL3, and 139H2 as IgG and F(ab')$_2$ fragments in experimental ovarian cancer. *Br. J. Cancer* **65**, 677–683.

MORRISON, S.L. (1992). *In vitro* antibodies: strategies for production and application. *Ann. Rev. Immunol.* **10**, 239–265.

MORRISON, S.L. AND OI, V.T. (1984). Transfer and expression of immunoglobulin genes. *Ann. Rev. Immunol.* **2**, 239–256.

MORRISON, S.L. AND OI, V.T. (1989). Genetically engineered antibody molecules. *Adv. Immunol.* **44**, 65–92.

MORRISON, S.L. AND SCHLOM, J. (1990). Recombinant chimeric monoclonal antibodies. *Important Adv. Oncol.*, 3–18.

MORRISON, S.L., JOHNSON, M.J., HERZENBERG, L.A. AND OI, V.T. (1984). Chimeric human antibody molecules: mouse antigen-binding domains with human constant region domains. *Proc. Natl. Acad. Sci. USA* **81**, 6851–6855.

MUELLER, B.M., REISFELD, R.A. AND GILLIES, S.D. (1990). Serum half-life and tumour localization of a chimeric antibody deleted of the C_H2 domain and directed against the disialoganglioside GD2. *Proc. Natl. Acad. Sci. USA* **87**, 5702–5705.

MUELLER, B.M., ROMERDAHL, C.A., GILLIES, S.D. AND REISFELD, R.A. (1990). Enhancement of antibody dependent cytotoxicity with a chimeric anti-GD2 antibody. *J. Immunol.* **144**, 1382–1386.

MULLIGAN, R.C. AND BERG, P. (1980). Expression of a bacterial gene in mammalian cells. *Science* **209**, 1422–1427.

MULLIGAN, R.C. AND BERG, P. (1981). Selection for animal cells that express the *Escherichia coli* gene coding for xanthine-guanine phosphoribosyltransferase. *Proc. Natl. Acad. Sci. USA* **78**, 2072–2076.

MUJOO, K., CHERESH, D.A., YANG, H.M. AND REISFELD, R.A. (1987). Disialoganglioside GD2 on human neuroblastoma: target antigen for monoclonal antibody

mediated cytolysis and suppression of tumor growth. *Cancer Res.* 47, 1098–1104.

MUJOO, K., KIPPS, T.J., YANG, H.M., CHERESH, D.A., WARGALLA, U., SANDER, D.J. AND REISFELD, R.A. (1989). Functional properties and effect on growth suppression of human neuroblastoma tumors by isotype switch variants of monoclonal anti-ganglioside GD2 antibody 14.18. *Cancer Res.* 49, 2857–2861.

MURARO, R., KUROKI, M., WUNDERLICH, D., POOLE, D.J., COLCHER, D., THOR, A., GREINER, J.W., SIMPSON, J.E., MOLINOLO, A., NOGUCHI, P. AND SCHLOM, J. (1988). Generation and characterization of B72.3 second generation monoclonal antibodies reactive with the tumor-associated glycoprotein 72 antigen. *Cancer Res.* 48, 4588–4596.

NAKAKI, T., DEANS, R.J. AND LEE, A.S. (1989). Enhanced transcription of the 78,000-dalton glucose-regulated protein (GRP78) gene and association of GRP78 with immunoglobulin light chains in a non secreting B-cell myeloma line (NS-1). *Mol. Cell. Biol.* 9, 2233–2238.

NAKASHIMA, M., NISHIMURA, Y. AND WATANABE, T. (1991). Recombinant human--mouse chimeric monoclonal antibody specific for human adenocarcinoma associated antigen. *Hybridoma* 10, 1–9.

NAKATANI, T., NOMURA, N., HORIGOME, K., OHTSUKA, H. AND NOGUCHI, H. (1989). Functional expression of human monoclonal antibody genes directed against Pseudomonal exotoxin A in mouse myeloma cells. *Bio/Technology* 7, 805–810.

NELSON, H., RAMSEY, P.S., KERR, L.A., MCKEAN, D.J. AND DONOHUE, J.H. (1990). Regional and systemic distribution of anti-tumor x anti-CD3 heteroaggregate antibodies and cultured human peripheral blood lymphocytes in a human colon cancer xenograft. *J. Immunol.* 145, 3507–3515.

NETA, R., DOUCHES, S. AND OPPENHEIM, J.J. (1986). Interleukin 1 is a radioprotector. *J. Immunol.* 136, 2483–2485.

NEUBERGER, M.S., WILLIAMS, G.T., MITCHELL, E.T., JOUHAL, S.S., FLANAGAN, J.G. AND RABBITTS, T.H. (1985). A hapten-specific chimaeric IgE antibody with human physiological effector function. *Nature* 314, 268–270.

NEUBERGER, M.S., CASKEY, H.M., PETTERSSON, S., WILLIAMS, G.T. AND SURANI, M.A. (1989). Isotype exclusion and transgene down-regulation in immunoglobulin-λ transgenic mice. *Nature* 338, 350–352.

NEUMAIER, M., SHIVELY, L., CHEN, F.-C., GAIDA, F.-J., ILGEN, C., PAXTON, R.J., SHIVELY, J.E. AND RIGGS, A.D. (1990). Cloning of the genes for T84.66, an antibody that has a high specificity and affinity for carcinoembryonic antigen, and expression of chimeric human/mouse T84.66 genes in myeloma and Chinese hamster ovary cells. *Cancer Res.* 50, 2128–2134.

NEWMAN, R., ALBERTS, J., ANDERSON, D., CARNER, K., HEARD, C., NORTON, F., RAAB, R., REFF, M., SHUEY, S. AND HANNA, N. (1992). 'Primatization' of recombinant antibodies for immunotherapy of human diseases: A macaque/human chimeric antibody against human CD4. *Bio/Technology* 10, 1455–1460.

NICOLAOU, K.C., DAI, W.-M., TSAY, S.-C., ESTEVEZ, V.A. AND WRASIDLO, W. (1992). Designed enediynes: a new class of DNA-cleaving molecules with potent and selective anticancer activity. *Science* 256, 1172–1178.

NISHIMURA, Y., YOKOYAMA, M., ARAKI, K., UEDA, R., KUDO, A. AND WATANABE, T. (1987). Recombinant human--mouse chimeric monoclonal antibody specific for common acute lymphocytic leukemia antigen. *Cancer Res.* 47, 999–1005.

NITTA, T., SATO, K., YAGITA, H., OKUMURA, K. AND ISHII, I. (1990). Preliminary trial of specific targeted therapy against malignant glioma. *Lancet* 335, 368–371.

NUCCI, M., SHORR, R. AND ABUCHOWSKI, A. (1991). The therapeutic value of PEG-modified proteins. *Adv. Drug Delivery Rev.* 6, 133–151.

NUTI, M., TERAMOTO, Y.A., MARIANI-CONSTANTINI, R., HORAN-HAND, P., COLCHER, D. AND SCHLOM, J. (1982). A monoclonal antibody (B72.3) defines

patterns of distribution of a novel tumor-associated antigen in human mammary carcinoma cell populations. *Int. J. Cancer* **29**, 539–545.

OI, V.T. AND MORRISON, S.L. (1986). Chimeric antibodies. *Biotechniques* **4**, 214–221.

ONG, G.L., ETTENSON, D., SHARKEY, R.M., MARKS, A., BAUMAL, R., GOLDENBERG, D.M. AND MATTES, M.J. (1991). Galactose-conjugated antibodies in cancer therapy: properties and principles of action. *Cancer Res.* **51**, 1619–1626.

ORDER, S.E., KLEIN, J.L. AND LEICHNER, P.K. (1987). Hepatoma: model for radiolabeled antibody in cancer treatment. *NCI-Monogr.* **3**, 7–41.

ORDER, S.E., STILLWAGON, G.B., KLEIN, J.L., LEICHNER, P.K. SIEGELMAN, S.S., FISHERMAN. E.K., ETTINGER, D.S., HAULK, T., KOPHER, K., FINNEY, K. *et al.* (1985). ^{131}I-antiferritin, a new treatment modality in hepatoma. *J. Clin. Oncol.* **3**, 1573–1582.

ODER, S.E., VRIESENDORP, H.M., KLEIN, J.L. AND LEICHNER, P.K. (1988). A phase I study of ^{90}Y-labelled anti-ferritin: dose escalation and tumour dose. *Antibod. Immunoconj. Radiopharm.* **1**, 163.

ORLANDI, R., GUSSOW, D.H., JONES, P.T. AND WINTER, G. (1989). Cloning immunoglobulin variable domains for expression by the polymerase chain reaction. *Proc. Natl. Acad. Sci. USA* **86**, 3833–3837.

ORLANDI, R., FIGINI, M., TOMASSETTI, A., CANNAVO, A., CANEVARI, S., WINTER, G. AND COLNAGHI, M.I. (1991). Biochemical and biological characterization of the chimeric MBr1 antibody. In *Monoclonal Antibodies. Applications in Clinical Oncology* (A.A. Epenetos, Ed.), pp. 31–36. Chapman and Hall, London.

ORTHO, MULTI- CENTER TRANSPLANT STUDY GROUP (1985). A Randomized clinical trial of OKT3 monoclonal antibody for acute rejection of cadaveric renal transplants. *New Engl. J. Med.* **313**, 337–342.

OWENS, R.J., KING, D.J., HOWAT, D., MOUNTAIN, A., HARVEY, A., LAWSON, A., RHIND, S.K., PEDLEY, B., BODEN, J., BEGENT, R. AND YARRANTON, G.T. (1991a). Tumour binding properties of B72.3 Fv fragments. *Antibod. Immunoconj. Radiopharm.* **4**, 459–467.

OWENS, R.J. MOUNTAIN, A., KING, D.J. AND YARRANTON, G.T. (1991b). *Multivalent antigen-binding proteins.* WO 91/19739.

PADLAN, E.A. (1991). A possible procedure for reducing the immunogenicity of antibody variable domains while preserving their ligand-binding properties. *Mol. Immunol.* **28**, 489–498.

PADLAN, E.A., SILVERTON, E.W., SHERIFF, S., COHEN, G.H., SMITH-GILL, S.J. AND DAVIES, D.R. (1989). Structure of an antibody–antigen complex: crystal structure of the HyHEL10 Fab–lysozyme complex. *Proc. Natl. Acad. Sci. USA* **86**, 5938–5942.

PAGE, M.J. AND SYDENHAM, M.A. (1991). High level expression of the humanized monoclonal antibody CAMPATH-1H in Chinese hamster ovary cells. *Bio/Technology* **9**, 64–68.

PAREKH, R.B., DWEK, R.A., RUDD, P.M., THOMAS, J.R., RADEMACHER, T.W., WARREN, T., WUN, T.C., HERBERT, B., REITZ, B., PALMER, M., RAMABADDRAN, T. AND TIEMER, D. (1989). N-glycosylation and *in vitro* enzymatic activity of human recombinant tissue plasminogen activator expressed in Chinese hamster ovary cells and a murine cell line. *Biochemistry* **28**, 7670–7679.

PERVEZ, S., EPENETOS, A.A., MOOI, W.J., EVANS, D.J., ROWLINSON, G., DHOKIA, B. AND KRANSZ, T. (1988). Localization of monoclonal antibody AUA1 and its F(ab′)2 fragment in human tumour xenografts: an autoradiographic and immunohistochemical study. *Int. J. Cancer* (Suppl.) **3**, 23–29.

PLON, S.E. AND GROUDINE, M. (1991). Unravelling immunoglobulin expression. *Curr. Biol.* **1**, 13–14.

PLUCKTHUN, A. (1991). Antibody engineering: Advances from the use of *Escherichia coli* expression systems. *Bio/Technology* **9**, 545–551.

POLJAK, R.J. (1991). Structure of antibodies and their complexes with antigens. *Mol. Immunol.* **28**, 1341–1345.

POSNER, M.R., ELBOIM, H.S., TUMBER, M.B., WIEST, P.M. AND TIBBETTS, L.M. (1991). An IgG human monoclonal antibody reactive with a surface membrane antigen expressed on malignant breast cancer cells. *Hum. Antibod. Hybridomas* **2**, 74–83.

PRESS, O.W. (1991). Immunotoxins. *Biotherapy* **3**, 65–76.

PRIMUS, F.J., PENDURTHI, T.K., HUTZELL, P., KASHMIRI, S., SLAVIN, D.C., CALLAHAN, R. AND SCHLOM, J. (1990). Chimeric B72.3 mouse/human (IgG1) antibody directs the lysis of tumour cells by lymphokine-activated killer cells. *Cancer Immunol. Immunother.* **31**, 349–357.

PRINZ, J., BRAUN-FALCO, O., MEURER, M., DADDONA, P., REITER, C., RIEBER, P. AND RIETHMULLER, G. (1991). Chimaeric CD4 monoclonal antibody in treatment of generalised pustular psoriasis. *Lancet* **338**, 320–321.

QUEEN, C. (1983). A vector that uses phage signals for efficient sythesis of proteins in *Escherichia coli. J. Mol. Appl. Gen.* **2**, 1–10.

QUEEN, C. AND BALTIMORE, D. (1983). Immunoglobulin gene transcription is activated by down stream sequence elements. *Cell* **33**, 741–748.

QUEEN, C. AND SELICK, H.E. (1990). *Chimeric immunoglobulins specific for p55 TAC protein of the IL-2 receptor.* WO90/07861.

QUEEN, C., SCHNEIDER, W.P., SELICK, H.E., PAYNE, P.W., LANDOLFI, N.F., DUNCAN, J.F., AVDALOVIC, N.M., LEVITT, M., JUNGHANS, R.P. AND WALDMANN, T.A. (1989). A humanized antibody that binds to the interleukin 2 receptor. *Proc. Natl. Acad. Sci. USA* **86**, 10029–10033.

RABBITTS, T.H., FORSTER, H. AND MATHEWS, J.G. (1983). The breakpoint of the Philadelphia chromosome 22 in chronic myeloid leukaemia is distal to the immunoglobulin lambda light chain constant region genes. *Mol. Biol. Med.* **1**, 11–19.

RHIND, S.K. (1990). *Cross-linked antibodies and processes for their preparation.* EP 0385601.

RHIND, S.K., MILLICAN, T.A. AND MILLAR, K. (1990). *Cross-linked antibodies and processes for their preparation.* EP 0384624.

RHIND, S.K., KING, D.J., BODEN, J., PEDLEY, R.B., SEARLE, F.A., BEGENT, R.H.J., BAGSHAWE, K.D., ABRAMS, D., YARRANTON, G.T., BODMER, M., SECHER, D.S. AND ADAIR, J. (1990). Rapid clearance of aglycosyl recombinant chimeric B72.3 (γ4) antibody in athymic mice bearing human colorectal xenografts. *Antibod. Immunoconj. Radiopharm.* **3**, 55.

RIECHMANN, L., FOOTE, J. AND WINTER, G. (1988). Expression of an antibody Fv fragment in myeloma cells. *J. Mol. Biol.* **203**, 825–828.

RIECHMANN, L., CLARK, M., WALDMANN, H. AND WINTER, G. (1988). Reshaping human antibodies for therapy. *Nature* **332**, 323–327.

ROBINSON, R.R., CHARTIER, J. JR., CHANG, C.P., HORWITZ, A.H. AND BETTER, M. (1991). Chimeric mouse–human anti-carcinaoma antibodies that mediate different anti-tumour cell biological activities. *Hum. Antibod. Hybridomas* **2**, 84–93.

RODECK, U., HERLYN, D., MOLTHOFF, C., ATKINSON, B., VARELLO, M., STEPLEWSKI, Z. AND KOPROWSKI, H. (1987). Tumor growth modulation by a monoclonal antibody to the epidermal growth factor receptor: immunologically mediated and effector cell-independant effects. *Cancer Res.* **47**, 3692–3696.

ROITT, I., BROSTOFF, J. AND MALE, D. (1987). *Immunology.* Gower Medical, London.

ROSE, B., GILLESPIE, A., WUNDERLICH, D., BARBOSA, J.A., CAHILL, K., DZUIBA, J., SHEDD, D. AND ZERLER, B. (1992a). A chimeric mouse/human anti-IL2 receptor antibody with enhanced biological activities. *Mol. Immunol.* **29**, 131–144.

ROSE, B., GILLESPIE, A., WUNDERLICH, D., KELLEY, K., DZUIBA, J., SHEDD, D., CAHILL, K. AND ZERLER, B. (1992b). Differential effects of a murine and chimeric mouse/human anti-interleukin-2 receptor antibody on human T-cell proliferation. *Immunology* **76**, 452–459.

ROSEN, S.T., ZIMMER, A.M., GOLDMAN-LEIKIN, R., GORDON, L.I. KAZIKIEWICZ, J.M. KAPLAN, E.H., VARIAKOJIS, D., MARDER, R.J., DYKEWICZ, M.S., PIER-GIES, A. *et al.* (1987). Radioimmunodetection and radioimmunotherapy of cutaneous T cell lymphomas using an 131I-labeled monoclonal antibody: an Illinois Cancer council Study. *J. Clin. Oncol.* **5**, 562–573.

ROUTLEDGE, E.G., LLOYD, I., GORMAN, S.D., CLARK, M. AND WALDMANN, H. (1991). A humanized monovalent CD3 antibody which can activate homologous complement. *Eur. J. Immunol.* **21**, 2717–2725.

RYBAK., S.M., HOOGENBOOM, H.R., MEADE, H.M., RAUS, J.C.M., SCHWARTZ, D. AND YOULE, R.J. (1992). Humanization of immunotoxins. *Proc. Natl. Acad. Sci. USA* **89**, 3165–3169.

SAGA, T., ENDO, K., KOIZUMI, M., KAWAMURA, Y., WATANABE, Y., KONISHI, J., UEDA, R., NISHIMURA, Y., YOKOYAMA, M. AND WATANABE, T. (1990). *In vitro* and *in vivo* properties of human/mouse chimeric monoclonal antibody specific for common acute lymphocytic leukemia antigen. *J. Nucl. Med.* **31**, 1077–1083.

SAHAGAN, B.G., DORAI, H., SALTZGABER-MULLER, J., TONEGUZZO, F., GUINDON, C.A., LILLY, S.P., MCDONALD, K.W., MORRISSEY, D.V., STONE, B.A., DAVIS, G.L., MCINTOSH, P.K. AND MOORE, G.P. (1987). A genetically engineered murine/human chimeric antibody retains specificity for human tumor-associated antigen. *J. Immunol* **137**, 1066–1074 (erratum published 1990, *J. Immunol.* **145**, 3150).

SALEH, M.N., LOBUGLIO, A.F., WHEELER, R.H., ROGERS, K., HAYNES, A., LEE, J.Y. AND KHAZAELI, M.B. (1990). A Phase II trial of murine monoclonal antibody 17-1A and interferon-γ: clinical and immunological data. *Cancer Immunol. Immunother.* **32**, 185–190.

SALEH, M.N., KHAZAELI, M.B., WHEELER, R.H., ALLEN, L., TILDEN, A.B., GRIZZLE, W., REISFELD, R.A., YU, A.L., GILLIES, S.D. AND LOBUGLIO, A.F. (1992a). Phase I trial of the chimeric anti-GD2 monoclonal antibody ch14.18 in patients with malignant melanoma. *Hum. Antibod. Hybridomas* **3**, 19–24.

SALEH, M.N., KHAZAELI, M.B., WHEELER, R.H., DROPCHO, E., LIU, T., URIST, M., MILLER, D.M., LAWSON, S., DIXON, P., RUSSELL, C.H. AND LOBUGLIO, A.F. (1992b). Phase I trial of the murine monoclonal anti-GD2 antibody 14.G2a in metastatic melanoma. *Cancer Res.* **52**, 4342–4347.

SANDLIE, I. AND MICHAELSEN, T.E. (1991). Engineering monoclonal antibodies to determine the structural requirements for complement activation and complement mediated lysis. *Mol. Immunol.* **28**, 1361–1368.

SANDLIE, I., AASE, A., WESTBY, C. AND MICHAELSEN, T.E. (1989). C1q binding to chimeric monoclonal IgG3 antibodies consisting of mouse variable regions and human constant regions with shortened hinge containing 15 to 47 amino acids. *Eur. J. Immunol.* **19**, 1599–1603.

SANDRI-GOLDIN, R.M., GOLDIN, A.L. AND LEVINE, M. (1981). High frequency transfer of cloned herpes simplex virus type I sequences to mammalian cells by protoplast fusion. *Mol. Cell. Biol.* **1**, 734–752.

SARAGOVI, H.U., FITZPATRICK, D., RAKTABUTR, A., NAKANISHI, H., KAHN, M. AND GREENE, M.I. (1991). Design and synthesis of a mimetic from an antibody complementarity-determining region. *Science* **253**, 792–795.

SARAGOVI, H.U., GREENE, M.I., CHRUSCIEL, R.A. AND KAHN, M. (1992). Loops and secondary structure mimetics: Development and applications in basic science and rational drug design. *Bio/Technology* **10**, 773–778.

SARMAY, G., LUND, J., ROZSNYAY, Z., GERGELY, J. AND JEFFERIS, R. (1992). Mapping and comparison of the interaction sites on the Fc region of IgG responsible for triggering antibody dependent cellular cytotoxicity (ADCC) through different types of human Fcγ receptor. *Mol. Immunol.* **29**, 633–639.

SCHEINBERG, D.A., STRAUSS, D.J., YEH, S.D., DIVGI, C., GARIN-CHESA, P., GRAHAM, M., PENTLOW, K., COIT, D., OETTGEN, H.F. AND OLD, L. (1990). A Phase I toxicity, pharmacology and dosimetry trial of monoclonal antibody OKB7

in patients with non-Hodgkins lymphoma; effects of tumour burden and antigen expression. *J. Clin. Oncol.* **8**, 798–803.

SCHEINBERG, D.A., LOVETT, D., DIVGI, C.R., GRAHAM, M.C., BERMAN, E., PENTLOW, K., FEIRT, N., FINN, R.D., CLARKSON, B.D., GEE, T.S., LARSON, S.M., OETTGEN, H.F. AND OLD, L.J. (1991). A phase I trial of monoclonal antibody M195 in acute myelogeneous leukemia: specific bone marrow targeting and internalization of radionuclide. *J. Clin. Oncol.* **9**, 478–490.

SCHLIMOK, G., FUNKE, I., HOLZMANN, B., GOTTLINGER, G., SCHMIDT, G., HAUSER, H., SWIERKOT, S., WARNECKE, H.H., SCHNEIDER, B., KOPROWSKI, H. AND RIETHMULLER, G. (1987). Micrometastatic cancer cells in bone marrow: *in vitro* detection with anti-cytokeratin and *in vivo* labeling with anti-17-1A monoclonal antibodies. *Proc. Natl. Acad. Sci. USA* **84**, 8672–8676.

SCHLOM, J., COLCHER, D., SILER, K., THOR, A., BRYANT, G., JOHNSTON, W.W., SZPAK, C.A., SUGARBAKER, P., CARRASQUILLO, J.A., REYNOLDS, J.C., KEENAN, A.M. AND LARSON, S.M. (1990). Tumour targeting with monoclonal antibody B72.3: experimental and clinical results. In *Cancer Imaging with Radiolabeled Antibodies* (D.M. Goldenberg, Ed.), pp. 313–336. Kluwer Academic Publishers, Norwell, MA, USA.

SCHLOM, J., EGGENSPERGER, D., COLCHER, D., MOLINOLO, A., HOUCHENS, D., MILLER, L.S., HINKLE, G. AND SILER, K. (1992). Therapeutic advantages of high-affinity anticarcinoma radioimmunoconjugates. *Cancer Res.* **52**, 1067–1072.

SCHROFF, R.W., FOON, K.A., BEATTY, S.M., OLDHAM, R.K. AND MORGAN, A.C. JR (1985). Human anti-murine immunoglobulin responses in patients receiving monoclonal antibody therapy. *Cancer Res.* **45**, 879–885.

SCOTT, J.K. (1992). Discovering peptide ligands using epitope libraries. *Trends Biochem Sci.* **17**, 241–245.

SEARLE, F., BIER, C., BUCKLEY, R.G., NEWMAN, S., PEDLEY, R.B., BAGSHAWE, K.D., MELTON, R.G., ALWAN, S.M. AND SHERWOOD, R.F. (1986). The potential of carboxypeptidase G2-antibody conjugates as anti-tumour agents. I. Preparation of antihuman chorionic gonadotrophin-carboxypeptidase G2 and cytotoxicity of the conjugate against JAR choriocarcinoma cells *in vitro*. *Br. J. Cancer* **53**, 377–384.

SEARS, H.F., HERLYN, D., STEPLEWSKI, Z. AND KOPROWSKI, H. (1984). Effects of monoclonal antibody immunotherapy on patients with gastrointestinal adenocarcinoma. *J. Biol. Response Modif.* **3**, 138–150.

SEARS, H.F., HERLYN, D., STEPLEWSKI, A. AND KOPROWSKI, H. (1986). Initial trial use of murine monoclonal antibodies as immunotherapeutic agents for gastrointestinal adenocarcinoma. *Hybridoma* **5**. (suppl.) S109–S115.

SEARS, H.F., BAGLI, D.J., HERLYN, D., DeFREITAS, E., SUZUKI, H., STEELE, G. AND KOPROWSKI, H. (1987). Human immune response to monoclonal antibody administration is dose dependent. *Arch. Surg.* **122**, 1384–1388.

SENTER, P.D., SAULNIER, M.G., SCHREIBER, G.J., HIRSCHBERG, D.L., BROWN, J.P., HELLSTROM, I. AND HELLSTROM, K. (1988). Antitumor effects of antibody-alkaline phosphatase conjugates in combination with etoposide phosphate. *Proc. Natl. Acad. Sci. USA* **85**, 4842–4846.

SERAFINI, A.N., GOLDENBERG, D.M., HIGGINBOTHAM-FORD, E.A., SILBERSTEIN, E.B., VAN HEERTUM, R.L., KOTLER, J.A., BALSUBRAMINIAN, N., GARTY, I. AND WLODKOWSKI, T. (1989). A multi-centre trial of cancer imaging with fragments of CEA monoclonal antibodies. *J. Nucl. Med.* **30**, 748.

SHALABY, M.R., SHEPARD, H.M., PRESTA, L., RODRIGUES, M.L., BEVERLEY, P.C.L., FELDMANN, M. AND CARTER, P. (1992). Development of humanized bispecific antibodies reactive with cytotoxic lymphocytes and tumor cells overexpressing the *HER2* protooncogene. *J. Exp. Med.* **175**, 217–225.

SHARPE, M.J., MILSTEIN, C., JARVIS, J.M. AND NEUBERGER, M.S. (1991). Somatic hypermutation of immunoglobulin κ may depend on sequences 3′ of Cκ and occurs on passenger transgenes. *EMBO J.* **10**, 2139–2145.

SHAW, D.R., KHAZAELI, M.B. AND LOBUGLIO, A.F. (1988). Mouse-human chimeric antibodies to a tumour associated antigen: biologic activity of the four IgG subclasses. *J. Natl. Cancer Inst.* **80**, 1553–1558.

SHAW, D.R., KHAZAELI, M.B., SUN, L.K., GHRAYEB, J., DADDONA, P.E., MCKINNEY, S. AND LOBUGLIO, A.F. (1987). Characterization of a mouse-human chimeric monoclonal antibody (17-1A) to a colon cancer tumour-associated antigen. *J. Immunol.* **138**, 4534–4538.

SHAW, D.R., HARRISON, G., SUN, L.K., SHEARMAN, C., GHRAYEB, J., MCKINNEY, S., DADDONA, P.E. AND LOBUGLIO, A.F. (1988). Human lymphocyte and monocyte lysis of tumor cells mediated by a mouse/human IgG1 chimeric monoclonal antibody. *J. Biol. Response Mod.* **7**, 204–211.

SHAWLER, D.L., BARTHOLOMEW, R.M., SMITH, L.M. AND DILLMAN, R.O. (1985). Human responses to multiple injections of murine monoclonal IgG. *J. Immunol.* **135**, 1530–1535.

SHEARMAN, C.W., KANZY, E.J., LAWRIE, D.K., LI, Y.-W., THAMMANA, P., MOORE, G.P. AND KURRLE, R. (1991a). Construction, expression and biologic activity of murine/human chimeric antibodies with specificity for the human α/β T cell receptor. *J. Immunol.* **146**, 928–935.

SHEARMAN, C.W., POLLACK, D., WHITE, G., HEHIR, K., MOORE, G.P., KANZY, E.J. AND KURRLE, R. (1991b). Construction, expression and characterization of humanized antibodies directed against the human α/β T cell receptor. *J. Immunl.* **147**, 4366–4373.

SHERIFF, S., SILVERTON, E.W., PADLAN, E.A., COHEN, G.H., SMITH-GILL, S.J., FINZEL, B.C. AND DAVIES, D.R. (1987). Three-dimensional structure of an antibody-antigen complex. *Proc. Natl. Acad. Sci. USA* **84**, 8075–8079.

SHIN, S.-U., WEI, C.-F., AMIN, A.R., THORBECKE, G.J. AND MORRISON, S.L. (1992). Structural and functional properties of mouse-human chimeric IgD. *Hum. Antibod. Hybridomas* **3**, 65–74.

SHOPES, B., WEETALL, M., HOLOWKA, D. AND BAIRD, B. (1990). Recombinant human IgG1-murine IgE chimeric Ig. Construction, expression, and binding to human Fcγ receptors. *J. Immunol.* **145**, 3842–3848.

SHULMAN, M., WILDE, C.D. AND KOHLER, G. (1978). A better cell line for making hybridomas secreting specific antibodies. *Nature* **276**, 269–270.

SINITSYN, V.V., MAMONTOVA, A.G., CHECKNEVA, Y.Y., SHNYRA, A.A. AND DOMOGATSKY, S.P. (1989). Rapid blood clearance of biotinylated Ig after infusion of avidin. *J. Nucl. Med.* **30**, 66–69.

SKERRA, A. AND PLUCKTHUN, A. (1989). Assembly of a functional immunoglobulin Fv fragment in *Escherichia coli*. *Science* **240**, 1038–1041.

SKERRA, A., PFITZINGER, I. AND PLUCKTHUN, A. (1991). The functional expression of antibody Fv fragments in *Escherichia coli*: improved vectors and a generally applicable purification strategy. *Bio/Technology* **9**, 273–278.

SOUTHERN, P. AND BERG, P. (1982). Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early promoter. *J. Mol. Appl. Genet.* **1**, 327–341.

STARLING, J.J. AND MASTRO, J.M. (1992). A critical assessment of the ability of preclinical human tumour xenograft models to predict the clinical antitumour activity of the monoclonal antibody-vinca alkaloid immunoconjugate, KS1/4-DAVLBHYD. *Antibod. Immunoconj. Radiopharm.* **5**, 148.

STEPHENS, P.E. AND COCKETT, M.I. (1989). The construction of a highly efficient and versatile set of mammalian expression vectors. *Nucl. Acids Res.* **17**, 7110.

STEPLEWSKI, Z., SUN, L.K., SHEARMAN, C.W., GHRAYEB, J., DADDONA, P. AND KOPROWSKI, H. (1988). Biological activities of human–mouse IgG1, IgG2, IgG3 and IgG4 chimeric monoclonal antibodies with antitumour activity. *Proc. Natl. Acad. Sci. USA* **85**, 4852–4856.

STRAMIGNONI, D., BOWEN, R., ATKINSON, B. AND SCHLOM, J. (1983). Differential

reactivity of monoclonal antibodies with human colon adenocarcinomas and adenomas. *Int. J. Cancer* **31**, 543–552.

SUN, L.K., CURTIS, P., RAKOWICZ-SZULCZYNSKA, E., GHRAYEB, J., MORRISON, S.L., CHANG, N. AND KOPROWSKI, H. (1986). Chimeric antibodies with 17-1A derived variable and human constant regions. *Hybridoma* **5** (Suppl. 1), S17–S20.

SUN, L.K., CURTIS, P., RAKOWICZ-SZULCZYNSKA, E., GHRAYEB, J., CHANG, N., MORRISON, S.L. AND KOPROWSKI, H. (1987). Chimeric antibody with human constant regions and mouse variable regions directed against carcinoma-associated antigen 17-1A. *Proc. Natl. Acad. Sci. USA* **84**, 214–218.

SUN, L.K., LIOU, R.S., SUN, N.C., GOSSETT, L.A., SUN, C., DAVIS, F.M., MACGLASHAN, D.W. JR AND CHANG, T.W. (1991). Transfectomas expressing both secreted and membrane-bound forms of chimeric IgE with anti-viral specificity. *J. Immunol.* **146**, 199–205.

SUNG, C., SHOCKLEY, T.R., MORRISON, P.F., DVORAK, H.F., YARMUSH, M.L. AND DEDRICK, R.L. (1992). Predicted and observed effects of antibody affinity and antigen density on monoclonal antibody uptake in solid tumours. *Cancer Res.* **52**, 377–384.

TAKAHASHI, H., HERLYN, D., ATKINSON, B., POWE, S., RODECK, U., ALAVI, A., BRUCE, D.A. AND KOPROWSKI, H. ((1987). Radioimmunodetection of human glioma xenografts by monoclonal antibody to epidermal growth factor receptor. *Cancer Res.* **47**, 3847–3850.

TAKEDA, S., NAITO, T., HAMA, K., NOMA, T. AND HONJO, T. (1985). Construction of chimaeric processed immunoglobulin genes containing mouse variable and human constant region sequences. *Nature* **314**, 452–454.

TAKEUCHI, M., INOUE, N., STRICKLAND, T.W., KOBATA, M., WADE, M., SHIMIZU, R., HOSHI, S., KOZUTSUMI, H., TAKASAKI, S. AND KOBATA, A. (1989). Relationship between sugar chain structure and biological activity of recombinant human erythropoietin produced in Chinese hamster ovary cells. *Proc. Natl. Acad. Sci. USA* **86**, 7819–7822.

TAN, L.K., OI, V.T. AND MORRISON, S.L. (1985). A human–mouse chimeric immunoglobulin gene with a human variable region is expressed in mouse myeloma cells. *J. Immunol.* **135**, 3564–3567.

TAN, L.K., SHOPES, R.J., OI, V.T. AND MORRISON, S.L. (1990). Influence of the hinge region on complement activation, C1q binding, and segmental flexibility in chimeric human immunoglobulins. *Proc. Natl. Acad. Sci. USA* **87**, 162–166 (erratum, 1991, *Proc. Natl. Acad. Sci. USA* **88**, 5066).

TAO, M.H. AND MORRISON, S.L. (1989). Studies of aglycosylated chimeric mouse–human IgG. Role of carbohydrate in the structure and effector functions mediated by the human IgG constant region. *J. Immunol.* **143**, 2595–2601.

TAO, M.-H., CANFIELD, S.M. AND MORRISON, S.L. (1991). The differential ability of human IgG1 and IgG4 to activate complement is determined by the COOH-terminal sequence of the C_H2 domain. *J. Exp. Med.* **173**, 1025–1028.

TAUB, R. AND GREENE, M.I. (1992). Functional validation of ligand mimicry by anti-receptor antibodies: Structural and therapeutic implications. *Biochemistry* **31**, 7431–7435.

TAYLOR, G., FURZE, J., TEMPEST, P.R., BREMNER, P., CARR, F.J. AND HARRIS, W.J. (1991). Humanised monoclonal antibody to respiratory syncytial virus. *Lancet* **337**, 1411–1412.

TEMPEST, P.R., BREMNER, P., LAMBERT, M., TAYLOR, G., FURZE, J.M., CARR, F.J. AND HARRIS, W.J. (1991). Reshaping a human monoclonal antibody to inhibit human respiratory syncytial virus infection *in vivo*. *Bio/Technology* **9**, 266–271.

THOR, A., OHUCHI, N., SZPAK, C.A., JOHNSTON, W.W. AND SCHLOM, J. (1986). The distribution of oncofetal antigen TAG-72 defined by monoclonal antibody B72.3. *Cancer Res.* **46**, 3118–3124.

THOR, A., VIGLIONE, M.J., MURARO, R., OHUCHI, N. AND SCHLOM, J. (1987). Monoclonal antibody B72.3 reactivity with human endometrium: a study of

normal and malignant tissues. *Int. J. Gynecol. Pathol.* **6**, 235–247.

THORPE, P.E. (1992). Deglycosylated ricin A-chaim imunotoxins in the treatment of B-cell Lymphoma. *7th International Conference on Monoclonal Antibody Immunoconjugates for Cancer*, San Diego, USA.

TONEGUZZO, F., HAYDAY, A.C. AND KEATING, A. (1986). Electric field mediated DNA transfer: transient and stable gene expression in human and mouse lymphoid cells. *Mol. Cell Biol.* **6**, 703–706.

TRAMONTANO, A., CHOTHIA, C. AND LESK, A.M. (1990). Framework residue 71 is a major determinant of the position and conformation of the second hypervariable region in the V_H domains of immunoglobulins. *J. Mol. Biol.* **215**, 175–182.

TRANG, J.M., LOBUGLIO, A.F., WHEELER, R.H., HARVEY, E.B., SUN, L., GHRAYEB, J. AND KHAZAELI, M.B. (1990). Pharmacokinetics of a mouse/human chimeric monoclonal antibody (C-17-1A) in metastatic adenocarcinoma patients. *Pharmacol. Res.* **7**, 587–592.

TRAVERS, P. AND BODMER, W. (1984). Preparation and characterization of monoclonal antibodies against placental alkaline phosphatase and other human trophoblast-associated determinants. *Int. J. Cancer* **33**, 633–641.

TULIP, W.R., VARGHESE, J.N., WEBSTER, R.G., AIR, G.M., LAVER, W.G. AND COLMAN, P.M. (1989). Crystal structures of neuraminidase–antibody complexes. *Cold Spring Harbor Symp. Quant. Biol.* **54**, 257–263.

VERHOEYEN, M., BRODERICK, L., EIDA, S. AND BADLEY, A. (1991). Reshaped human anti-PLAP antibodies. In *Monoclonal Antibodies Applications in Clinical Oncology* (A.A. Epenetos, Ed.), pp. 37–43. Chapman and Hall, London.

VERHOEYEN, M., MILSTEIN, C. AND WINTER, G. (1988). Reshaping human antibodies: grafting an antilysozyme activity. *Science* **239**, 1634–1536.

WALDMAN, T.A. (1989). Multichain Interleukin-2 receptor: a target for immunotherapy in lymphoma. *J. Natl. Cancer Inst.* **81**, 914–923.

WALDMAN, T.A. (1991a). The Interleukin-2 receptor. *J. Biol. Chem.* **266**, 2681–2684.

WALDMANN, T. (1991b). Monoclonal antibodies in diagnosis and therapy. *Science* **252**, 1657–1662.

WALKER, M.R., LUND, J., THOMPSON, K.M. AND JEFFERIS, R. (1989a). Aglycosylation of human IgG1 and human IgG3 monoclonal antibodies can eliminate recognition by human cells expressing FcγI and FcγII receptors. *Biochem. J.* **259**, 347–353.

WALKER, M.R., WOOF, J.M., BRUGGEMANN, M., JEFFERIS, R. AND BURTON, D.R. (1989b). Interaction of human IgG chimeric antibodies with the human FcRI and FcRII receptors: requirements for antibody-mediated host cell-target cell interaction. *Mol. Immunol.* **26**, 403–411.

WALLEN, M. AND BORREBAECK, C.A.K. (1991). Affinity repertoire of monoclonal antibodies obtained by primary or secondary *in vitro* immunization. *Hybridoma* **10**, 333–345.

WARD, B.G., MATHER, S.J., HAWKINS, L.R., CROWTHER, M.E., SHEPHERD, J.H., GRANOWSKA, M.I., BRITTEN, K.E. AND SLEVIN, M.L. (1987). Localization of radioiodine conjugated to the monoclonal antibody HMFG2 in human ovarian carcinoma: assessment of intravenous and intraperitoneal routes of administration. *Cancer Res.* **47**, 4719–4723.

WARD, E.S. (1992). Expression and purification of antibody fragments using *Escherichia coli* as a host. In *Antibody Engineering: A Practical Guide* (C.A.K. Borrebaeck, Ed.), pp. 121–137. W.H. Freeman and Co., San Francisco.

WARD, E.S., GUSSOW, D., GRIFFITHS, A.D., JONES, P.T. AND WINTER, G. (1989). Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli*. *Nature* **341**, 544–546.

WARGALLA, U.C. AND REISFELD, R.A. (1989). Rate of internalization of an immunotoxin correlates with cytotoxic activity against tumor cells. *Proc. Natl. Acad. Sci. USA* **86**, 5146–5150.

WAWRZYNCZAK, E.J. (1991). Systemic immunotoxin therapy of cancer: advances and prospects. *Br. J. Cancer* **64**, 624–630.

WEETALL, M., SHOPES, B., HOLOWKA, D. AND BAIRD, B. (1990). Mapping the site of interaction between murine IgE and its high affinity receptor with chimeric Ig. *J. Immunol.* **145**, 3849–3854.

WEISSENHORN, W., WEISS, E., SCHWIRZKE, M., KALUZA, B. AND WEIDLE, U.H. (1991). Chimerization of antibodies by isolation of rearranged variable regions by the polymerase chain reaction. *Gene* **106**, 273–277.

WHITTLE, N., ADAIR, J., LLOYD, C., JENKINS, L., DEVINE, J., SCHLOM, J., RAUBITSHEK, A., COLCHER, D. AND BODMER, M. (1987). Expression in COS cells of a mouse/human chimaeric B72.3 antibody. *Prot. Eng.* **1**, 499–505.

WIGLER, M., PELLICER, A., SILVERSTEIN, S., AXEL, R., URLAUB, G. AND CHASIN, L. (1979). DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells. *Proc. Natl. Acad. Sci. USA* **76**, 1373–1376.

WILLIAMS, W.V., MOSS, D.A., KIEBER-EMMONS, T., COHEN, J.A., MYERS, J.N., WEINER, D.B. AND GREENE, M.I. (1989a). Development of biologically active peptides based on antibody structure. *Proc. Natl. Acad. Sci. USA* **86**, 5537–5541.

WILLIAMS, W.V., WEINER, D.B., COHEN, J.A. AND GREENE, M.I. (1989b). Development and use of receptor binding peptides derived from antireceptor antibodies. *Biotechnology* **7**, 471–475.

WINTER, G. AND MILSTEIN, C. (1991). Man-made antibodies. *Nature* **349**, 293–299.

WOLFF, M.E. AND MCPHERSOIN, A. (1990). Antibody-directed drug discovery. *Nature* **345**, 365–366.

WOODLE, E.S., THISTLETHWAITE, J.R., JOLLIFFE, L.K., ZIVIN, R.A., COLLINS, A., ADAIR, J.R., BODMER, M., ATHWAL, D., ALEGRE, M.-L. AND BLUESTONE, J.A. (1992). Humanized OKT3 antibodies: successful transfer of immune modulating properties and idiotype expression. *J. Immunol.* **148**, 2756–2763.

WRIGHT, E.M., HUMPHREYS, G.O. AND YARRANTON, G.T. (1986). Dual-origin plasmids containing an amplifiable ColE1 ori; temperature-controlled expression of cloned genes. *Gene* **49**, 311–321.

WU, T.T. AND KABAT, E.A. (1970). An analysis of the sequences of the variable regions of Bence Jones proteins and myeloma light chains and their implications for antibody complementarity. *J. Exp. Med.* **132**, 211–250.

XIANG, J. AND CHEN, Z. (1992). Genetic engineering of high affinity anti-human colorectal tumour mouse/human chimeric antibody. *Immunology* **75**, 209–216.

XIANG, J., RODER, J. AND HOZUMI, N. (1990). Production of murine V-human Cr1 chimeric anti-TAG72 antibody using V region cDNA amplified by PCR. *Mol. Immunol.* **27**, 809–817.

XU, M., REAL, F.X., WELT, S., SCHUSSLER, M.H., OETTGEN, H.F. AND OLD, L. (1989). Expression of TAG-72 in normal colon, transitional mucosa, and colon cancer. *Int. J. Cancer* **44**, 985–989.

YARRANTON, G.T. AND MOUNTAIN, A. (1992). Expression of proteins in prokaryotic systems–principles and 'case' studies. In *Protein Engineering* (A.R. Rees, Ed.), pp. 303–325. Oxford University Press, Oxford.

YOKOTA, T., MILENIC, D.E., WHITLOW, M. AND SCHLOM, J. (1992). Rapid tumour penetration of a single chain Fv and comparison with other immunoglobulin forms. *Cancer Res.* **52**, 3402–3408.

YOKOYAMA, M., NISHIMURA, Y. AND WATANABE, T. (1987). Suppression of tumour growth by *in vivo* adminstration of a' recombinant human–mouse chimeric monoclonal antibody. *Jpn. J. Cancer Res. (Gann)* **78**, 1251–1257.

ZARLING, J., MORAN, P.A., GROSMARIE, L.S., MCLURE, J., SHRIVER, K. AND LEDBETTER, J.A. (1988). Lysis of cells infected with HIV-1 by human lymphocytes targeted with monoclonal antibody heteroconjugates. *J. Immunol.* **140**, 2609–2613.

ZEBEDEE, S.L., BARBAS III, C.F., HOM, Y.-L., CAOTHIEN, R.H., GRAFF, R., DEGRAW, J., PYATI, J., LAPOLLA, R., BURTON, D.R. AND LERNER, R.A.

(1992). Human combinatorial antibody libraries to hepatitis B surface antigen. *Proc. Natl. Acad. Sci. USA* **89**, 3175–3179.

ZEMEL-DREASEN, O. AND ZAMIR, A. (1984). Secretion and processing of an immunoglobulin light chain in *Escherichia coli. Gene* **27**, 315–322.

ZIVIN, R.A., XU, D., BROSIUS, R.A., COLLINS, A.M., PULITO, E.S., ADAIR, J.R., BODMER, M., WOODLE, E.S., ALEGRE, M.L., BLUESTONE, J.A. KNOWLES, R.W., KHAZAELI, M.B., LOBUGLIO, A.F. AND JOLLIFFE, L.K. (1992). Antibody humanization and effector function modification of Orthoclone OKT^{TM}3. Second International Conference on Human Antibodies and Hybridomas, Cambridge, UK.

ZOLLER, M. AND SMITH, M. (1983). Oligonucleotide-directed mutagenesis of DNA fragments cloned in M13 vectors. *Methods Enzymol.* **100**, 468–472.