of PMN recruitment to the site. Up-regulation of the selectins allows rolling of the PMN along the endothelial cells and up-regulation of intercellular adhesion molecule-1 (ICAM-1) allows the PMN to become firmly attached to the endothelial cell and then to migrate into the interstitium. Imaging sites of inflammation by antibodies to these endothelial cell adhesion molecules may therefore give additional selectivity. ^{111}In-labelled antibodies to both E-selectin and ICAM-1 have been investigated in animal models with promising results (Keelan *et al.*, 1994; Sasso *et al.*, 1996).

The abundance of CD4 on inflammatory cells present in arthritic joints has led to interest in targeting CD4 to image arthritic joints. Antibodies to CD4 have been used to assess targeting in animal models of arthritis. Design of the reagent is crucial for success, as intact IgG demonstrated no benefit over a non-specific antibody, whereas a ^{99m}Tc-labelled Fab′ led to improved imaging (Kinne *et al.*, 1995). Anti-CD4 reagents may also be useful in monitoring the distribution of CD4 positive lymphocytes, which could be useful in assessing patients with HIV infection (Rubin *et al.*, 1996). RAID has also been investigated for detection of atherosclerotic plaques using an antibody towards proliferating smooth muscle cells (Narula *et al.*, 1995) and lesions in Alzheimer's disease using a cationised antibody towards beta A4 protein (Bickel *et al.*, 1994). Cationisation through chemical modification of the antibody with charged groups is believed to aid transport of antibodies through the blood:brain barrier, which is obviously helpful to image brain lesions in Alzheimer's disease.

4

Monoclonal Antibodies in Therapeutic Applications

4.1 Introduction

The idea of targeted therapy using antibodies dates from the beginning of the twentieth century when it was proposed by Paul Ehrlich, and indeed antibodies have been used in humans for many years as polyclonal antisera, particularly for passive immunisation. Since the discovery of the technology for production of MAbs there has been intense interest in their development as therapeutic agents for human disease. However, until recently progression of many MAb-based therapeutics was limited by the human immune response generated by the administration of murine or rat antibodies. Since the introduction of recombinant chimeric and humanised antibodies, and ultimately human antibodies, interest in developing therapeutics has been revived. There are now five antibody-based products licensed for human therapeutic use, and many more under clinical investigation (Table 4.1).

MAbs can be used in several different modes, depending on the required therapeutic effect. In some cases a simple blocking or neutralising effect may be required, for example in the neutralisation of an inflammatory cytokine or the blocking of a specific receptor. In other applications therapy may require an active role for the antibody by the targeting of an effector function. Therapeutics can utilise natural antibody effector functions such as complement activation, phagocytosis or ADCC, or entirely novel functions can be introduced, as in the case of targeting radioisotopes, drugs or toxins to kill tumour cells. In some cases antibodies may also find a role through their ability to mediate signal transduction from binding to cell surface receptors (Vitetta and Uhr, 1994). Available mechanisms for generation of therapeutic effects can be considered as a range of antibody effector functions (Table 4.2). Many of these effector functions can be designed into, or out of, antibody molecules for specific therapeutic purposes as described in Chapter 2. In this chapter the basis of targeted therapy is illustrated using examples of the various modes of antibody-based therapy in different disease states. The emphasis is placed on the design of antibody-based therapeutics rather than a catalogue of antibodies used in therapeutic studies.

Table 4.1 Commercially available monoclonal antibody therapeutics

Therapeutic agent	Antibody	Disease	Company
Orthoclone OKT3	Murine OKT3	Acute transplant rejection (kidney, liver and heart)	Ortho Biotech
ReoPro	Chimeric 7E3 Fab	Complications of post-coronary angioplasty	Centocor
Panorex	Murine 17-1A	Colorectal cancer	Centocor
Rituxan	Chimeric 2B8	Lymphoma	IDEC/Genentech
Zenapax	Humanised anti-tac	Acute kidney transplant rejection	Protein Design Labs/ Roche

Table 4.2 Effector functions for antibody targeted therapy

Blocking/neutralising	Antigen binding
Natural – Fc mediated effects	Complement fixation ADCC Phagocytosis
Cell signalling	Receptor cross-linking
Natural immune responses	Generation of anti-idiotype response Other 'vaccination' approaches
Artificial effectors	Radioisotopes Toxins – bacterial – plant Cytotoxic drugs Cytokines Enzymes – prodrug activation – direct toxicity
Bifunctional	Cross-linking cytotoxic effector cells Two-step targeting strategies for radioisotopes, toxins, etc.

4.2 Cancer

One of the major targets of antibody-based therapeutics has been in the development of anti-cancer agents. Initially MAbs to tumour-associated antigens were raised and investigated without modification, in attempts to target natural antibody effector mechanisms to tumour cells. Results of such studies were initially disappointing in many cases, particularly with the common solid tumour types. However, beneficial effects were seen when treating patients with minimal disease and in treating lymphomas and leukemias, suggesting that further research to investigate the role of antibodies as anti-cancer agents may lead to improved results. In addition, the use of antibodies to target traditional anti-cancer agents such as cytotoxic drugs and radioisotopes has made steady progress, particularly since the introduction of recombinant antibodies and improved methods of coupling

cytotoxic agents (Chapter 2). As described in Chapter 3, many tumour-associated antigens representative of different tumour types are now characterised (see Table 3.5) which allow therapeutic molecules to be designed.

4.2.1 Cancer therapy with unmodifed (naked) antibodies

The use of unmodified, or naked, antibodies for tumour therapy initially resulted from attempts to harness the immune system through the natural antibody effector functions, such as ADCC or complement-mediated lysis. More recently it has become apparent that cell signalling mechanisms may be involved in many of the anti-tumour effects observed, through arrest of the cell cycle or inducing apoptosis. Design of antibodies to elicit natural effector functions has largely been a process of choosing the optimal isotype to harness human ADCC and complement effects, although as described in Section 2.6.1 the selection of a suitable isotype alone is not sufficient to ensure good cell killing, and suitable antigen targets must be individually tested. Mouse IgG2a is the best of the murine isotypes for eliciting human ADCC, with rat IgG2b also potent. Of the human isotypes, IgG1 and IgG3 are the most potent in cell killing studies. Of these IgG1 is most commonly used for construction of humanised antibodies, partly because IgG3 antibodies are more difficult to purify and to handle *in vitro* due to a tendency to aggregate. The preparation of chimeric or humanised antibodies from rodent antibodies thus has the dual benefit of reduced immunogenicity and the ability to use a constant region best suited to recruitment of human effector functions. Similarly human antibodies isolated from phage display, or other means, can be reconstructed and expressed with the desired isotype constant regions. Expression of the antibody in mammalian cells is required as the glycosylation of the C_{H2} region is required for maintainence of the ability to elicit effector functions. However, cell killing effects are dependent not only on the constant region, but also on the disposition of antigenic sites and other poorly understood mechanisms. Many cells also have protective mechanisms against attack from the immune system, and thus the *in vivo* effects of antibodies capable of eliciting cell killing *in vitro* are often difficult or impossible to predict.

Several alternative mechanisms have also been suggested for tumour cell killing by unmodified antibodies. Antibodies against cell surface markers on many types of tumour cell can act as ligands eliciting anti-tumour effects by signal transduction (Vitetta and Uhr, 1994). Signal transduction can result in arrest of the cell cycle, hence preventing tumour cell growth, or in some cases the induction of programmed cell death known as apoptosis. In addition, antibodies to growth factors or their receptors may exert anti-tumour effects through blocking binding of growth factors needed for tumour cell growth. Growth factors such as EGF (epidermal growth factor) and IL-6 have been implicated in the growth of a number of tumour types, and target tumour cells overexpress large numbers of molecules of the growth factor receptors. Receptor blocking antibodies prevent interaction with the ligand, and can lead to down-regulation of the number of receptor molecules present on the tumour cell such that growth is inhibited. However, unravelling the contribution of each of these factors to effective tumour cell killing is not straightforward and often several mechanisms may operate concurrently. Future research may allow the selection of antibodies based on an improved understanding of the importance of eliciting each of these anti-tumour effects. For example, it may be possible to select combinations of MAbs capable of different mechanisms of cell killing.

As described in Chapter 2, dimeric or polymeric antibodies have been produced either by chemical cross-linking or by recombinant means which have increased activity for both complement activation and ADCC. Chemically cross-linked constructs comprising Fab fragments linked to two Fc regions $FabFc_2$ have also been produced in attempts to increase therapeutic efficacy through effector functions. However, studies comparing such constructs with a humanised antibody for immunotherapy of multiple myeloma showed no advantage in complement activation or ADCC (Ellis *et al.*, 1995).

Homodimerisation of IgG has also been reported to be able to confer signalling activity, leading to cell cycle arrest or apoptosis, to antibodies which are unable to do so in monomeric form (Ghetie *et al.*, 1997a). Chemically cross-linked IgG homodimers were produced for several antibodies and shown to be capable of specific anti-tumour effects, presumably due to cross-linking the antigen and/or a lower dissociation rate. In the case of an anti-CD19 antibody the Fc region was not required for anti-tumour activity, leading to the possibility that small multimeric fragments may be capable of potent anti-tumour effects.

Although many studies with unmodified antibodies for cancer therapy have been disappointing, with no evidence of anti-tumour effects, several antibodies have shown promising effects in the clinic. It is only in the past few years that recombinant chimeric and humanised antibodies have reached clinical evaluation. In these studies it has been apparent that the ability of the human constant regions to interact with the human immune system plus the ability to re-treat without the generation of a prohibitive immune response is leading to more effective therapeutic agents.

The first humanised (CDR-grafted) antibody to be used clinically, CAMPATH-1H, recognises the CAMPATH–1 antigen, also known as CDw52, which is present on most human lymphocytes and monocytes but not stem cells. CAMPATH-1H has been investigated as a potential therapeutic agent in non-Hodgkin lymphoma (B-cell lymphoma) as well as in several inflammatory diseases (Section 4.5.3). Although only limited studies have been reported, use of CAMPATH–1H for the treatment of B-cell lymphoma has led to tumour remissions in some patients (Hale *et al.*, 1988). Studies with different isotypes of the rat parent antibody suggest that human effector cells are involved, as the IgM and rat IgG2a versions, which can activate complement but not ADCC, led to only transient falls in blood cell counts. In contrast, the rat IgG2b version which could bind to and activate human effector cells led to much more efficient depletion of tumour cells *in vivo* (Dyer *et al.*, 1989).

Another antibody, which has shown promising effects in B-cell lymphoma, recognises CD20, a phosphoprotein present on the surface of B cells. A chimeric IgG1 version of the antibody 2B8, termed C2B8, has been shown in *in vitro* assays to be an effective mediator of both complement-mediated effects and ADCC using human effector cells (Reff *et al.*, 1994). In addition, this antibody has been shown to induce transmembrane signalling leading to cell cycle arrest and occasionally apoptosis of CD20 positive cells, and thus several mechanisms may be important in the anti-tumour effects observed (Demiden *et al.*, 1995). Phase II and III clinical studies for relapsed B-cell lymphoma have shown tumour shrinkage of 50% or more in approximately half of the patients studied (Maloney *et al.*, 1997). In addition, C2B8 was found to sensitise resistant lymphoma cells to certain cytotoxic drugs, which may lead to synergistic effects in combination therapy. In a phase II clinical study of C2B8 with standard chemotherapeutic treatment, responses were seen in all patients studied (Czuczman *et al.*, 1995). Although as a single agent, tumour responses were not as impressive as those observed with radiolabelled antibodies to CD20 (Section 4.2.5), treatment with this unmodified antibody was much less toxic and is more

straightforward than handling large amounts of radioisotope. Therefore, it is likely that this agent will find clinical application alongside chemotherapy for B-cell lymphoma treatment.

The HER2 gene (also known as *neu* or *c-erb-B2*) is a proto-oncogene which encodes a transmembrane growth factor receptor ($p185^{HER2}$) which is overexpressed in 25–30% of patients with breast cancer, particularly those with a poor prognosis. A humanised antibody to $p185^{HER2}$ has been developed which has shown significant growth inhibition of cells overexpressing this receptor (Carter *et al.*, 1992b). The humanised antibody was also shown to be more efficient at mediating ADCC with human effector cells than the parent murine antibody. In a phase II clinical study in patients with metastatic breast cancer that overexpressed HER2, objective responses were seen in 5 of 43 patients including one complete remission and 4 partial remissions (Baselga *et al.*, 1996). The mechanisms of action of this agent are also not clear at present. The antibody induced a clear down-regulation of the growth factor receptor which may reverse the malignant phenotype. This antibody is also known to be capable of activation of a signal transduction pathway that leads to inhibition of tumour cell proliferation and possibly cell death, and also elicits ADCC.

4.2.2 *Anti-idiotype antibodies*

MAbs may also be used as vaccines to generate an anti-idiotype response. The idotype of an antibody is made up of a cluster of epitopes at the antigen-binding site. As each MAb has a different antigen-binding site, the idiotype of each MAb is unique. Antibodies can be raised through an immune response to the idiotype which have a conformation that resembles the antigen of the original antibody (Figure 4.1). These anti-idiotype antibodies are also known as anti-id or ab2 antibodies. Immunisation with an ab2 antibody can in turn lead to the elicitation of an anti-idiotype to the ab2 antibody, an anti-anti-id, known as ab3, a proportion of which will have the same or overlapping specificity as the original ab1 antibody (Figure 4.1). Structural studies suggest that anti-idiotype antibodies carry an 'internal image' of the original antigen which allows the development of ab3 responses that recognise the original antigen. The crystallographic structure of an anti-idiotype antibody in complex to an antibody raised against lysozyme showed that the same antigen-binding residues used to bind lysozyme were used for binding to the ab2, and that the ab2 mimicked lysozyme (Fields *et al.*, 1995).

Vaccination with ab2 has been investigated in attempts to raise a human ab3 response to target antigens which may be poorly immunogenic or difficult to prepare or use in immunisation. ab2 may also be able to raise antibodies not raised to the original antigen through breaking immunological tolerance and also give rise to T cell responses which may have therapeutic significance. Many studies, both preclinical and clinical, have been conducted in attempts to achieve therapeutic effects with ab2 vaccines for cancer (reviewed by Herlyn *et al.*, 1996). In several cases antigen-specific immune responses have been achieved, including both antibody and cellular responses. For example, the antibody 105AD7 is an anti-idiotype antibody that mimics a colorectal tumour-associated antigen. Phase I clinical studies demonstrated that a T cell response to tumour was induced on treatment with 105AD7 and this resulted in delayed tumour growth and an increase in survival time compared with patients at the same stage of disease that did not receive the antibody (Buckley *et al.*, 1995).

It is, of course, also possible for antibodies to tumour-associated antigens administered for tumour therapy (ab1) to elicit anti-idiotype responses. The murine MAb 17-1A has

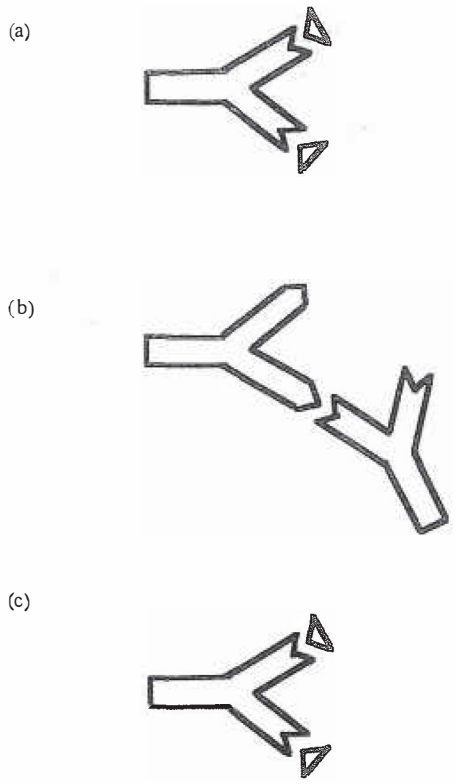


Figure 4.1 Representation of anti-idiotype antibodies: (a) initial antibodies (ab1) recognise the antigen to which they were raised; (b) anti-idiotype antibodies (ab2) recognise the antigen-binding region of ab1 and thus can be considered as containing an 'internal image' of the antigen; (c) anti-anti-idiotype antibodies (ab3) raised against ab2 have similar specificity to the original antibody (ab1) and can thus recognise the same antigen

been extensively studied. It recognises the epithelial membrane antigen (EMA) present on the majority of colorectal tumour cells. Originally developed for its ability as a murine IgG2a to elicit ADCC in humans, early clinical studies revealed occasional responses in patients with advanced disease which were delayed following antibody administration, implying an active immune response. Most patients developed an anti-idiotype response to 17-1A which may have led to the observed therapeutic effects (Fagerberg *et al.*, 1996). In a phase III trial of 17-1A for the treatment of minimal residual disease in colorectal cancer patients following surgical removal of the primary tumour, 17-1A treatment resulted in a statistically significant improval in survival (Riethmuller *et al.*, 1994). After following patients for five years, 17-1A therapy reduced the overall death rate by 30% and decreased the recurrence rate by 27%. These results led to the approval of 17-1A as a therapeutic agent, sold under the name 'Panorex' (Table 4.1). Analysis has identified patients with high levels of ab3 as those that live longer, suggesting that the induction of an anti-idiotype response is important in the mechanism of the therapeutic effect of this antibody (Fagerberg *et al.*, 1996).

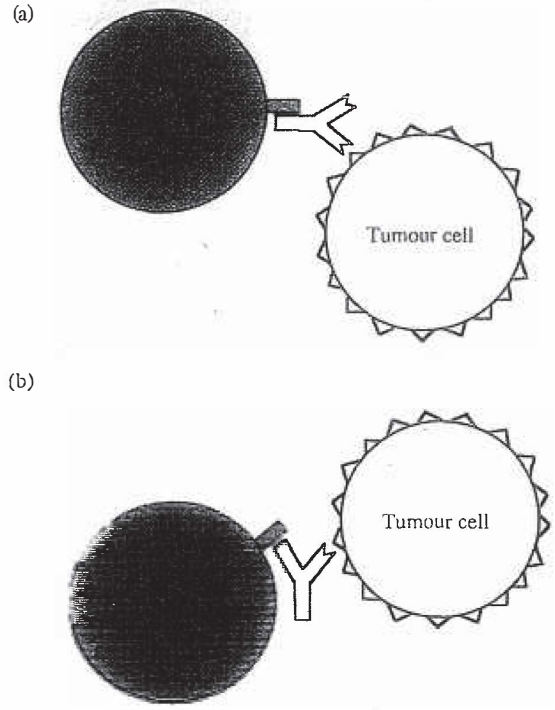


Figure 4.2 Mechanisms of targeting effector cells to tumours: (a) active isotypes which can bind to Fc receptors on the effector cell can be used to target the cell to a tumour-associated antigen; (b) alternatively, bispecific antibodies may be used which bind to both the tumour cell and the effector cell through the antigen-binding arms of the antibody (for further details see text)

4.2.3 *Bispecific antibody-mediated effector cell targeting*

The limited success that has been achieved in using antibodies to elicit ADCC responses has prompted the search for other approaches to recruit effector cells, such as cytotoxic T cells, to the tumour site. One such approach is to use bispecific antibodies with one specificity for a tumour-associated antigen and one for the effector cell such that the effector cell is linked directly to the tumour cell (Figure 4.2). A surface 'trigger molecule' is used as the antigen on the effector cell such that the cell is activated to lead to a cytotoxic response. Several different types of effector cell and trigger molecule have been investigated, including Fcγ receptors and molecules of the CD3/T cell receptor complex (Table 4.3). The advantages and disadvantages of each of these have been reviewed (Renner and Pfreundschuh, 1995). In addition to requiring triggering, cytotoxic effector mechanisms may also require the involvement of accessory molecules and activation via cytokine release.

On monocytes, macrophages, eosinophils and polymorphonuclear neutrophils (PMNs), activation is achieved through linking to Fcγ receptors. Natural killer cells are activated through FcγRIII and CD2, whereas activation of T cells is usually achieved through the

Table 4.3 Cytotoxic effector cells and trigger molecules

Effector cell	Trigger molecules
T cells	CD3 T cell receptor (γ/δ) CD2 CD28
Natural killer cells	Fcγ receptor III (CD16) CD2
Monocytes/macrophages	Fcγ receptor I (CD64) Fcγ receptor II (CD32) Fcγ receptor III (CD16)
Granulocytes (eosinophils, PMNs)	Fcγ receptor II (CD32)
PMNs activated with interferon-γ, GM-CSF or G-CSF	Fcγ receptor I (CD64)

CD3/T-cell receptor complex. The CD3/T-cell receptor complex is normally responsible for antigen-specific T cell responses, but the use of bispecific antibodies allows activation through this complex which can be redirected to the antigen recognised by the other arm of the bispecific antibody. Activation of effector cells is greatly increased by cytokines such as interleukin-2 or interleukin-7, or by co-stimulatory signals. CD2 and CD28 are co-stimulatory molecules which when stimulated increase the activation of T cells. For example, combinations of triggering CD3 and CD28 allow particularly potent anti-tumour effects. Cell killing by a bispecific antibody cross-linking the Hodgkin lymphoma antigen CD30 to CD3 resulted in relatively little tumour cell killing unless co-administered with CF30–CD28 bispecific antibody (Renner *et al.*, 1994). The combination was also far more potent in treatment of xenografted human tumour in immunodeficient mice repopulated with human T cells. Cures of established tumours could only be achieved with the combination of both bispecific molecules (Renner *et al.*, 1994). Similar effects can be achieved by co-administration of anti-CD28 IgG with anti-CD3/anti-tumour bispecific antibodies (Demanet *et al.*, 1996). An alternative approach has been the use of a trispecific antibody, made by cross-linking three Fab′ fragments, with specificity for tumour antigen as well as two different T cell antigens (Tutt *et al.*, 1991b).

Several phase I clinical studies have been carried out with bispecific antibodies with interesting results, although these have been limited by the need to activate T cells *ex vivo* and re-infuse to the patient at the time of bispecific antibody therapy. IL-2 activated cells targeted by an anti-CD3/anti-tumour bispecific antibody administered intracranially to glioma patients resulted in survival of 76% of patients over two years, compared to 33% of those treated with activated cells alone (Nitta *et al.*, 1990). Anti-FcγRIII/anti-tumour antibodies designed to activate natural killer cells and mononuclear phagocytic cells have also been used clinically, with minor responses observed (Weiner *et al.*, 1995).

The production of bispecific antibodies can be achieved using a variety of approaches, including the production of hybrid hybridomas, chemical cross-linking and recombinant approaches (Section 2.5). The affinity of the antibody for the triggering molecule may also be important. Humanised Fab′ fragments to the breast tumour antigen $p185^{HER2}$ and

to CD3 have been expressed and cross-linked to form a bispecific antibody capable of efficient lysis of tumour cells (Zhu *et al.*, 1995). In these studies a high affinity version of the humanised anti-CD3 Fab′ was found to be more effective at tumour cell lysis than a lower affinity version. The same antibody specificities have been used to produce a bispecific diabody which was equally active in tumour cell lysis but was much simpler to produce as it was directly expressed by secretion from *E. coli* at high yield (Zhu *et al.*, 1996). Under some circumstances diabodies may be more effective than bispecific IgG at achieving tumour cell lysis, possibly due to the smaller size of the diabody bringing target and effector cell closer together (Holliger *et al.*, 1996).

4.2.4 *Other approaches to recruit the immune system using MAbs*

Superantigens are bacterial or viral proteins, such as the exotoxin produced by *Staphylococcus aureus*, that stimulate T cells by cross-linking the T cell receptor to MHC class II molecules. Superantigens bind to the T cell receptor outside the antigen recognition site and to MHC class II molecules outside the peptide binding groove normally involved in antigenic recognition, bypassing normal immunological specificity. Superantigens are thus the most potent known activators of T cells. Fab′–superantigen fusion proteins have been produced which stimulate T cells and target to tumour cells. The fusion proteins are intended to replace MHC class II binding with tumour cell binding through the Fab′. Such fusion proteins have been shown to be capable of inhibiting tumour growth in animal models through causing activated T cells to infiltrate and attack the tumour (Dohlstein *et al.*, 1995). One problem with the use of such agents, however, is residual MHC class II binding which leads to relatively high toxicity through systemic T cell activation and accumulation of inflammatory cytokines in serum. Attempts to improve Fab′–superantigen fusion proteins for tumour therapy are thus being made by mutations in the superantigen binding site for MHC class II which prevent binding and reduce systemic toxicity (Hansson *et al.*, 1997).

Other approaches in attempts to improve the recruitment of the immune system by antibodies have included the use of antibody–cytokine fusion proteins. Certain cytokines, such as interleukin-2 (IL-2) are able to activate multiple immune mechanisms, although attempts at tumour therapy with cytokine alone have led to severe systemic toxicity. Fusion proteins have thus been designed to target a high local concentration of cytokine to the tumour site, minimising systemic toxicity. Several different cytokines have been investigated. IL-2 stimulates T cell proliferation and T cell mediated killing and has thus been widely investigated, but tumour necrosis factor α (TNFα), TNFβ, GM-CSF, IL-5, IL-8 and interferon α have also been tested. Fusion proteins have been produced with several antibodies and encouraging pre-clinical results obtained. Chimeric 14.18, an antibody that recognises the GD_2 ganglioside expressed on neuroblastoma, melanoma and certain other tumours, has been used to produce fusions with several cytokines, the most promising of which appears to be IL-2 (Hank *et al.*, 1996). The fusion protein maintained antigen binding and IL-2 activity and was able to exert anti-tumour effects in mice bearing human tumour xenografts of neuroblastoma and melanoma. Activation of human effector cells has also been demonstrated (Hank *et al.*, 1996). Cytokine fusion proteins have also been produced with small antibody fragments such as scFv, which may allow more effective tumour penetration (Dorai *et al.*, 1994). However, whether such fusion proteins will be sufficiently less toxic than free IL-2 when administered to patients remains to be seen.

4.2.5 Radioimmunotherapy

As with diagnostic tumour imaging (Section 3.9.1), the design of antibodies for radioimmunotherapy (RIT) requires careful consideration of the individual components of the immunoconjugate, including the form of the antibody, the radioisotope and the means of attachment of the radioisotope to the antibody. In addition, the choice of a suitable tumour-associated antigen becomes more critical with therapeutic doses of isotope, as the presence of antigen on normal tissues will result in the deposition of activity, which may lead to unacceptable toxicity. Many factors influence the degree of tumour localisation in patients. These include heterogeneous expression of antigen, presence of circulating antigen, tumour vascularisation and penetration of antibody into tumour tissue (Boxer *et al.*, 1992). However, there may not be a need to achieve localisation to each individual tumour cell, as a radioisotope can be chosen with a pathlength of many cell diameters, allowing for 'bystander cell' killing at the tumour site. Nevertheless, a high proportion of tumour cells expressing the antigen is desirable. This is because the major limitation to RIT is the dose of isotope which can be delivered at the tumour site without resulting in unacceptable toxicity. In general, only a small proportion of the dose administered localises to the tumour site in patients. Typically tumour-localisation levels of 10% injected dose per kg of tumour (0.01% injected dose per gram of tumour) are observed in human studies where good targeting is achieved (van Hof *et al.*, 1996). The total administered dose is usually dependent on the toxicity to normal body tissues. Bone marrow is the most sensitive normal tissue to radiation and therefore doses are usually limited by the dose to bone marrow from radiolabelled antibody circulating in the blood (Badger, 1990). Several strategies have been investigated in attempts to reduce bone marrow toxicity, including the use of short-range radioisotopes which require cell internalisation for cytotoxicity (Auger emitters), the use of specific clearing mechanisms to remove circulating activity, two-step targeting strategies and the use of rapidly clearing antibody fragments.

Careful optimisation of an immunoconjugate for RIT, through antibody engineering and development of suitable chemistry for radioisotope attachment, can lead to improved properties both *in vitro* and *in vivo* (King *et al.*, 1994), and it is likely that such optimised immunoconjugates will provide the next generation of molecules of clinical utility for RIT. Some of the parameters which need to be considered in the design of radioimmunoconjugates for therapy are given in Table 4.4.

Table 4.4 Parameters for design of radioimmunoconjugates for therapy

Radioimmunoconjugate component	Properties to consider
Antibody	Specificity, affinity for tumour antigen Lack of cross-reactivity with accessible normal tissues
Form of engineered antibody	Immunogenicity Pharmacokinetics Accessibility/penetration into normal tissues Incorporation of residues for site-specific labelling
Radioisotope	Physical properties (type of emission, energy, half-life) Stability of attachment to antibody Ease of radiolabelling/suitability for clinical protocol Immunoreactivity of final conjugate

Form of antibody

In contrast to tumour imaging *in vivo*, generation of a high tumour : blood ratio is not the major consideration for the optimal form of the antibody for RIT. An important consideration is the absolute amount of antibody localised to the tumour site. Therefore, the ideal molecule would localise to the tumour in large amounts, delivering a high dose of radiation while clearing rapidly from the circulation and the rest of the body, minimising non-specific toxicity. In practice this ideal is not reached and thus there has been much debate over the ideal form of antibody to use. Intact IgG circulates for a long period of time and accumulates high levels of activity at the tumour site, whereas antibody fragments clear more rapidly, sparing the dose to normal tissues but accumulating lower levels of activity at the tumour site.

Comparisons of IgG with $F(ab')_2$ and Fab′ fragments in both animal models and patients have suggested that there may be a significant advantage for $F(ab')_2$ fragments. Studies with radioiodinated $F(ab')_2$ in animal models revealed more effective tumour therapy with less toxicity than IgG (Buchegger *et al.*, 1990; Pedley *et al.*, 1993). More efficient early uptake into tumours has also been found for ^{131}I-$F(ab')_2$ fragments in clinical studies, suggesting a significant advantage in tumour penetration (Lane *et al.*, 1994). Also, theoretical predictions of the optimal molecule for tumour therapy based on pharmacokinetic and tumour accumulation data suggest that $F(ab')_2$ may be the best targeting molecule (Yorke *et al.*, 1991). Smaller fragments such as Fab′- and Fv-based molecules result in higher tumour : blood ratios but significantly less accumulated at the tumour (e.g. Brown *et al.*, 1987; Milenic *et al.*, 1991; King *et al.*, 1994). Although such fragments are useful for imaging applications, the tumour levels reached are unlikely to be useful for achieving therapy. These findings in animal models have been confirmed in clinical studies with scFv in which high tumour : blood ratios were generated although absolute uptake into the tumour was low (Begent *et al.*, 1996).

Further development of antibody fragments for delivery of ^{131}I has suggested that the use of chemically cross-linked $F(ab')_2$ fragments may be particularly useful. This was investigated due to the relative instability of some $F(ab')_2$ fragments where the exposed hinge region may lead to proteolytic and/or reductive breakdown. In addition, different $F(ab')_2$ fragments have different *in vivo* stabilities. In a study comparing human IgG1, 2 and 4 $F(ab')_2$, it was found that IgG4 $F(ab')_2$ was relatively unstable compared to IgG2 $F(ab')_2$, with the stability of IgG1 $F(ab')_2$ intermediate between them (Buchegger *et al.*, 1992). This leads to differences in the efficiency of different $F(ab')_2$ fragments in tumour localisation. The differences may be due to disulphide bond formation in the hinge region. IgG2 has four hinge disulphide bonds whereas IgG1 has two. The least stable $F(ab')_2$, IgG4, also has the potential for two disulphide bonds, although it is known that due to the structure of the hinge region in human IgG4 molecules, a proportion have only one disulphide bond formed, and some none at all (Angal *et al.*, 1993). This conclusion is verified by the finding that the introduction of more disulphide bonds into the hinge region of a $F(ab')_2$ fragment results in increased *in vivo* stability (Rodrigues *et al.*, 1993).

The introduction of a chemical cross-link between two cysteine residues in the hinge region also leads to increased *in vivo* stability (Quadri *et al.*, 1993; King *et al.*, 1994). Chemical cross-linked $F(ab')_2$ can be produced through the use of bis-maleimide linkers (Section 2.4.2, Figure 2.4). The hinge region of recombinant Fab′ fragments can be engineered to contain a single hinge thiol group to allow simple and high-yield production of chemically cross-linked molecules (King *et al.*, 1994). Such chemically cross-linked molecules have been termed DFM, for a di-Fab prepared with a bis-maleimide

linker. DFMs of the antibody chimeric B72.3 which recognises a tumour-associated glycoprotein have been shown to improve targeting to human tumours in a mouse xenograft model, resulting in both higher tumour accumulation and improved tumour : blood ratios compared to $F(ab')_2$ (King *et al.*, 1994). Also, DFM may have higher association rates for antigen than $F(ab')_2$, which may be a consequence of the increased spacing or flexibility of the linker. This was the case for DFM of the anti-carcinoembryonic antigen antibody, A5B7, which led to this being the preferred molecule for RIT applications with ^{131}I as the radioisotope (Casey *et al.*, 1996).

The best form of antibody for tumour targeting also depends on the radioisotope used. Several metallic isotopes, such as ^{90}Y and ^{67}Cu, are potentially attractive for RIT (see below), but the biodistribution of antibody fragments labelled with metallic isotopes is very different. Once taken into cells, metallic isotopes are less readily excreted than halogens such as iodine, which leads to intracellular accumulation (Press *et al.*, 1996). This can be an advantage for metallic isotopes when internalised into tumour cells, as the isotope is retained at the tumour site for longer. However, when taken into normal cells the build-up of isotope can lead to high levels in normal organs. Most antibody fragments are metabolised and excreted through the kidneys. During excretion re-adsorption of protein takes place in the kidney tubules. This can lead to unacceptably high levels of metallic isotopes deposited in the kidney tubule cells. High kidney levels of activity have thus been observed with many antibody fragments labelled with metallic isotopes, including Fab-, $F(ab')_2$- and Fv-based fragments (Brown *et al.*, 1987; Schott *et al.*, 1992). Similar high kidney uptake takes place with DFM (King *et al.*, 1994). However, very interesting data have been obtained with chemically cross-linked tri-Fab conjugates.

Chemically cross-linked tri-Fabs (TFM) have been prepared with several different antibodies using tri-maleimide linkers (Figure 4.3(b)). These molecules localise to tumour cells at high levels, clear from the blood relatively rapidly and yet do not give rise to unacceptably high kidney levels when labelled with ^{90}Y (Figure 4.3(c)). In addition, linkers have been developed for the attachment of ^{90}Y to the cross-linker, which allows site-specific labelling to high specific activity, suitable for RIT, with no loss of antigen-binding activity (King *et al.*, 1994; Antoniw *et al.*, 1996). Pharmacokinetic studies of chimeric B72.3 TFM revealed a longer alpha phase but a similar beta-phase half life to $F(ab')_2$ and DFM (King *et al.*, 1994). The rapid blood clearance of TFM is surprising considering that this molecule has a molecular weight similar to that of intact IgG. However, the absence of an Fc region means that FcRn-mediated recycling does not operate, as it does for IgG, to maintain blood levels (see Section 2.7) and it also suggests that clearance through the kidney is not solely dependent on molecular weight but that shape and possibly charge of the molecule may also be important. The longer pharmacokinetic alpha phase probably contributes to the increased tumour levels obtained relative to other fragments, but TFM molecules also show increased avidity for antigen due to the presence of an extra binding site (King *et al.*, 1994, 1995). Comparative radioimmunotherapy studies in nude mice bearing human tumour xenografts have been carried out with IgG and TFM of the anti-tumour antibody A33 (Antoniw *et al.*, 1996). Results demonstrated that complete cures of established tumours could be achieved with both A33 IgG and TFM when labelled with ^{90}Y. Higher doses were required for TFM to achieve the same therapeutic effect, but these resulted in less toxicity. Humanised versions of A33 IgG and TFM have also been prepared to allow repeat dose human studies (King *et al.*, 1995). Similar results have also been observed for a TFM prepared from the antibody A5B7, suggesting that in this case also ^{90}Y-TFM is an attractive agent for RIT (Casey *et al.*, 1996).

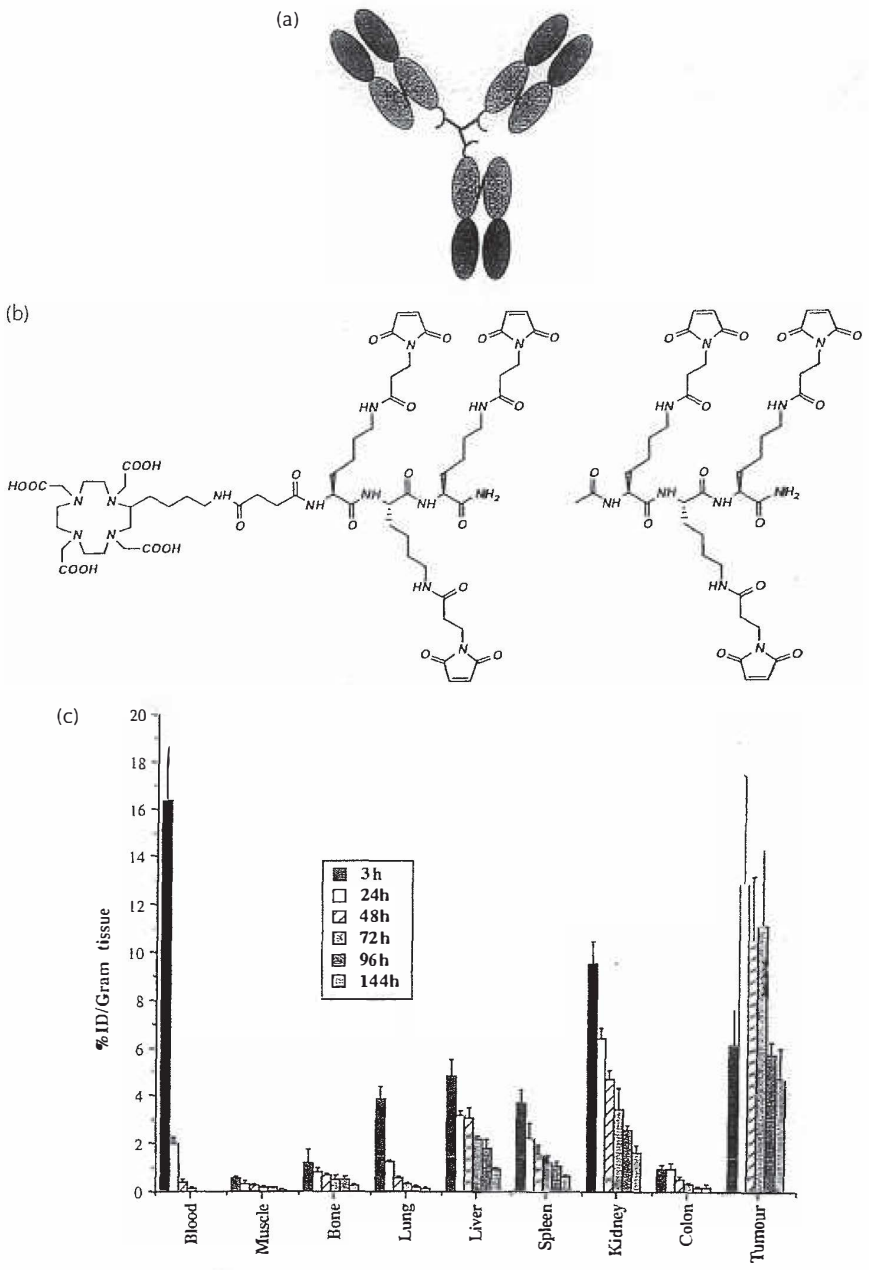


Figure 4.3 Tri-Fab′ production allows improved tumour targeting compared to IgG: (a) tri-Fab′ produced through chemical cross-linking of hinge region cysteines; (b) linkers used to produce tri-Fab′ including one containing a macrocycle for site-specific attachment of therapeutic radioisotopes; (c) biodistribution of yttrium-90-labelled chemically cross-linked tri-Fab′ from the anti-tumour antibody A33 in nude mice bearing SW1222 human tumour xenografts (tissue distribution was determined at 3, 24, 48, 72, 96 and 144 hours post-injection, $n = 4$, error bars indicate standard deviations)

Table 4.5 Some potentially useful radioisotopes for radioimmunotherapy

Radionuclide	Emission	Half-life	E (MeV)	γ (keV)
^{131}I	β	193 h	0.61	364
^{90}Y	β	64 h	2.25	—
^{67}Cu	β	62 h	0.4, 0.48, 0.58	185
^{186}Re	β	89.2 h	1.07, 0.94	137
^{188}Re	β	17 h	2.12, 1.96	155
^{177}Lu	β	162 h	0.50	210
^{111}Ag	β	179 h	1.04	342
^{199}Au	β	75 h	0.29, 0.25	159
^{221}At	α	7.2 h	5.87, 7.45	—
^{212}Bi	α	60.5 min	6.09–8.79	727
^{125}I	Auger	59 days	—	35
^{195m}Pt	Auger	96 h	—	65–130
^{64}Cu	β Auger positron	12.8 h	0.19	511

Radioisotopes

In contrast to imaging applications, radioisotopes for radioimmunotherapy are required to deliver a sterilising dose of radiation to the tumour. Therefore isotopes with a high linear energy transfer (LET, the energy deposited by the isotope over its pathway) are desirable. Three different types of radioisotopes have been investigated: Auger emitters, alpha emitters and beta emitters. Potentially, Auger emitters offer the ability to deliver the highest dose of radiation to the tumour cell but only over a range of less than one cell diameter. Therapy with Auger emitters therefore requires the isotope to be taken into the cell and localised at the nucleus. Alpha emitters also have the potential to deliver a high radiation dose over a short range. In this case the range is about 50 μm in tissue, so little 'bystander killing' of adjacent cells can be expected. Thus both Auger and α emitters require a very homogeneous distribution of the antibody at the tumour to allow a good therapeutic effect. Such homogeneous distribution may be difficult to achieve in many cases. β emitters deposit less energy per cell, but have a longer range and therefore do not require such a homogeneous distribution in tumour tissue. Ranges of β particles in tissue can be over 1 cm for high-energy β emitters such as ^{90}Y. This is favourable for larger tumours although when targeting small micrometastases, much of the energy may be deposited outside the tumour in the surrounding tissue. Candidate radioisotopes for use in RIT are given in Table 4.5.

In addition to suitable energy, candidate radioisotopes also need to fulfil other criteria to allow their use for RIT. A suitable half-life is required which can be matched to the biological half-life of an antibody delivery vehicle, the isotope must not have any hazardous daughter products, suitable methods for stable attachment to antibody must be available and it must be available carrier-free to allow preparation of high specific activity immunoconjugates. Many useful β emitters also have a gamma emission. This is undesirable as the gamma emission contributes little to therapy although it can play a major role in the total body dose (Langmuir, 1992). Also, the presence of large amounts of penetrating gamma radiation may present a significant hazard to medical personnel, and in waste storage and disposal, often making patient handling difficult. However, the gamma emission does allow for imaging studies to be performed using the same labelled

immunoconjugate which allows tumour dose estimates to be determined (DeNardo *et al.*, 1996a).

The majority of radioimmunotherapy studies to date have been carried out using ^{131}I as the therapeutic isotope. Although ^{131}I has a suitable β emission for therapy, it has a relatively long half-life (8 days) and an abundant γ emission, which are less than optimal for an ideal radioimmunotherapeutic. The reasons for the popularity of ^{131}I stem from the long history of its application in the treatment of thyroid malignancies, its ready availability, and the ease of radiolabelling antibodies with ^{131}I without any specialised chemistry. As mentioned above, one problem with radioiodinated antibodies is their relative instability *in vivo*. Several novel methods for radiolabelling antibodies with iodine have been developed in attempts to reduce deiodination or the loss of iodinated metabolites from cells. These include the use of *N*-succinimidyl-3-(tri-*n*-butylstannyl) benzoate (Schuster *et al.*, 1991), dilactitoltyramine, tyramine cellobiose and other non-metabolisable carbohydrate adducts (Ali *et al.*, 1990; Reist *et al.*, 1995) or compounds designed to retain a positive charge to aid intracellular retention (Reist *et al.*, 1996). However, such methods have not yet achieved the high specific activities achievable with conventional iodination methods, and the benefit of such labelling methods in clinical studies remains to be determined. One potential effect of such 'residualising' iodine labelling methods is that the biodistribution of iodinated antibodies will resemble those of radiometal-labelled antibodies, making it difficult to see any advantage for residualising iodine labels over radiometals with more favourable physical properties. Indeed, recent data suggest exactly this, with a similar biodistribution and therapeutic advantage seen to that of radiometals (Stein *et al.*, 1997).

Many alternative isotopes for tumour therapy are now becoming increasingly available together with methods for their attachment to antibodies. Several of these alternative metallic isotopes offer potential advantages, for example in the amount of energy deposited per unit of administered activity, in increased retention in tumour tissue and in the possibility of out-patient treatment with less specialised facilities required.

Apart from ^{131}I the most studied β emitting isotope for RIT has been ^{90}Y. The shorter half-life of ^{90}Y and the higher energy mean that the absorbed dose per unit of activity and the dose rate is higher for ^{90}Y than ^{131}I and the dose is delivered over a shorter time. This may be a particular advantage for RIT as the biological half-life of the antibody delivery agent should also be taken into account. Some useful theoretical examples have been described by Harrison *et al.* (1991). If the labelled antibody remains at the tumour site for infinite time then the percentage of the total dose (182 rad/g/μCi ^{90}Y, 127 rad/g/μCi ^{131}I) delivered over 10 days is 93% for ^{90}Y and 58% for ^{131}I. If a biological half-life of 2.5 days is assumed, the percentage of the dose delivered to tumour in 10 days by ^{90}Y is reduced from 93% to 45% and from 58% to 14.8% for ^{131}I. This may be further reduced for ^{131}I if a residualising label is not used, as described above. In addition, ^{90}Y is a pure β emitter with no gamma component, which is potentially advantageous in terms of patient handling.

In early studies with ^{90}Y attachment to antibody was achieved largely using the chelating group DTPA. However, in common with the imaging isotope, ^{111}In, DTPA is a poor chelator for *in vivo* use and significant leakage of ^{90}Y occurs resulting in deposition, in this case in the bone, leading to unacceptable toxicity at high doses. In clinical studies, administration of EDTA has been investigated to mop up free yttrium which has leaked from the DTPA based immunoconjugate (Stewart *et al.*, 1990). However, subsequently improved chelators have been developed for ^{90}Y, including DTPA derivatives with all eight coordination sites for ^{90}Y retained (Brechbiel *et al.*, 1986). These ligands demonstrated improved stability over DTPA in *in vivo* studies (Washburn *et al.*, 1991). A

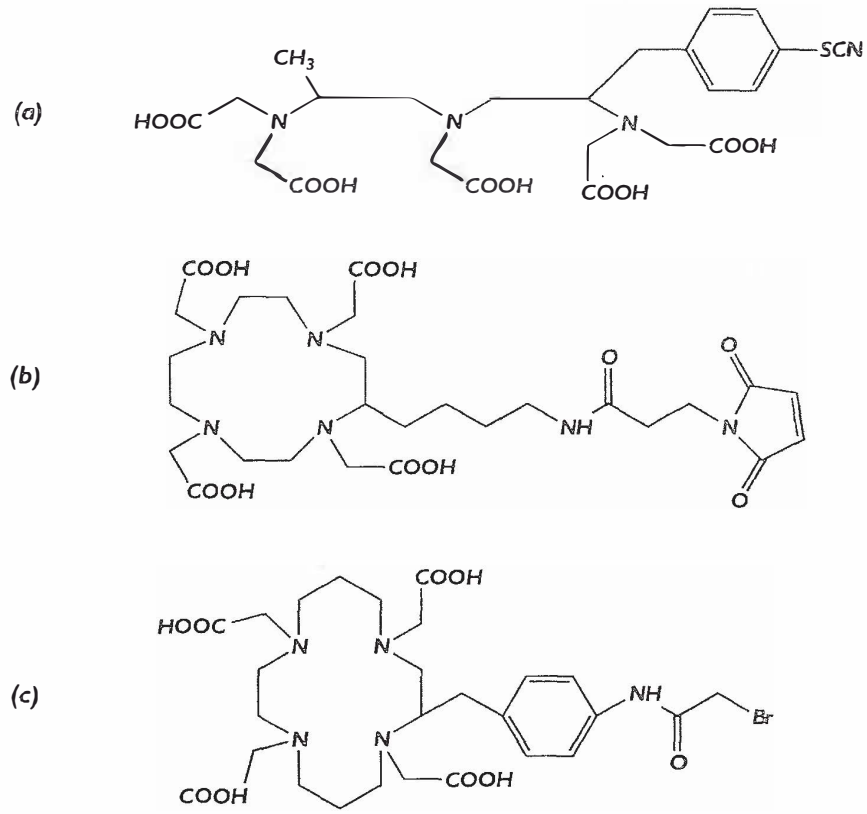


Figure 4.4 Structures of ligands used for attachment of therapeutic radioisotopes: (a) 1B4M-DTPA (Camera *et al.*, 1944); (b) 12N4-maleimide for labelling with yttrium-90 (Harrison *et al.*, 1991); (c) 14N4 derivative for labelling with copper isotopes (Morphy *et al.*, 1989)

further advance came with the development of stable macrocyclic ligands such as tetraazacyclododecane tetraacetate (DOTA or 12N4) (Figure 4.4; Cox *et al.*, 1989; Deshpande *et al.*, 1990). Comparative biodistribution studies have demonstrated improved targeting with ^{90}Y bound through DOTA, and lower levels of activity in normal tissues, especially bone, which is of course important to prevent unnecessary bone marrow toxicity (Harrison *et al.*, 1991). Initial clinical studies using DOTA to chelate yttrium resulted in an immune response against the macrocyclic structure (Kosmas *et al.*, 1992) which led to renewed interest in less stable chelators such as the DTPA derivative CHX-DTPA (Figure 4.4). Studies on the stability and biodistribution of CHX-DTPA and other DTPA derivatives revealed that all were less stable than DOTA (Camera *et al.*, 1994). However, DTPA derivatives have been used to chelate ^{90}Y in clinical studies of RIT using an antibody to polymorphic epithelial mucin in breast carcinoma (Schrier *et al.*, 1995). Partial responses to treatment were observed, although it was necessary to provide autologous stem cell support in some patients to overcome bone marrow toxicity. More recent data suggest that immune responses against DOTA may be less of a problem than first thought: in a study in lymphoma patients immune responses to macrocycles were no more frequent

than those seen with other chelating groups, and were less than those to the antibody (DeNardo *et al.*, 1996b).

The use of DOTA for chelation of yttrium has been combined with a humanised tri-Fab to produce an effective immunoconjugate for tumour therapy (Antoniw *et al.*, 1996). This conjugate was produced using a Fab′ cross-linker which incorporates the DOTA macrocycle, allowing site-specific labelling with ^{90}Y. Versions of cross-linkers have been produced with both DOTA and phosphinate DOTA derivatives, which are equally stable chelators of ^{90}Y (Norman *et al.*, 1995a). Methods for high-specific activity labelling have been developed which operate at >95% labelling efficiency, allowing realistic clinical use of the conjugate (Haines and King, unpublished data).

Stable macrocyclic chelators have also been developed for ^{67}Cu labelling, based on tetraazacyclotetradecane tetraacetate (TETA or 14N4, see Figure 4.4) (Cole *et al.*, 1987; Morphy *et al.*, 1989). ^{67}Cu is potentially an attractive radioisotope for RIT but its widespread application has been limited by poor availability. Nevertheless, antibodies labelled with ^{67}Cu via a macrocyclic ligand have been shown to be effective in therapy of tumours in nude mouse models (Connett *et al.*, 1996), and have shown improved biodistribution characteristics in colorectal cancer patients compared to iodinated antibody (Bischoff-Delaloye *et al.*, 1997). The DOTA macrocycle used for ^{90}Y can also be used for chelation of lutetium-177, and this complex has been investigated attached to the antibody CC49, which recognises a tumour-associated glycoprotein, both in animal models and for the treatment of ovarian cancer in patients (Meredith *et al.*, 1996). In this phase I study in patients, ^{177}Lu-CC49 was well tolerated and showed some evidence of anti-tumour activity, particularly in patients with microscopic disease.

Two isotopes of rhenium, ^{188}Re and ^{186}Re, are of interest for RIT (Table 4.4). Rhenium chemistry is very similar to that of the imaging isotope technetium (see Section 3.9.1), and therefore similar methods for attachment to antibody are used. These include direct labelling through reduced hinge thiol groups, and the use of chelators such as N_2S_2 or N_3S (see Figure 3.15). Encouraging results have been obtained in preclinical studies with ^{186}Re-antibody conjugates. Treatment of small cell lung cancer xenografts with antibody labelled with ^{186}Re via an N_3S chelator resulted in long-term tumour regressions in some mice (Beaumier *et al.*, 1991), and clinical studies with rhenium-labelled antibodies are in progress.

As an alternative to β emitting radioisotopes, α emitters emit their energy over a short range and are therefore attractive for treatment of tumours only if homogeneous distribution of antibody throughout the tumour can be achieved. However, the other implication of short-range isotopes is that relatively little normal tissue damage may result. Application of α emitters has been limited by the need to achieve a homogeneous distribution, and also by their short half-life and the unstable daughter products produced which make working with these isotopes a technical challenge. Nevertheless, α emitters have been examined in a number of animal model studies. α emitters may be well suited to RIT for therapy of easily penetrated tumours such as leukemias, micrometastases and malignancies with sheet-like geometry and free floating tumour cells, such as ovarian cancer or neoplastic meningitis where antibody can be administered directly into the body compartment. Astatine-211 labelled antibody has been shown to be effective in therapy of neoplastic meningitis in a rat model (Zalutsky *et al.*, 1994). Bismuth-212 is also a potentially useful isotope, which has shown efficacy in an adjuvant setting for i.p. tumours (Hartmann *et al.*, 1994). It has been suggested that ^{212}Bi-labelled anti-Tac antibody may be useful for therapy of T cell leukemias. Similarly to ^{90}Y, the biodistribution of bismuth-labelled antibody can be significantly improved by use of DOTA to chelate the

isotope, compared to DTPA or DTPA derivatives such as CHX-DTPA (Junghans *et al.*, 1993). Studies so far have used whole antibody; however, because of the short half-life of ^{212}Bi, it is likely that the use of a rapidly clearing antibody fragment would be significantly better. Studies with Fab′ and F(ab′)$_2$ have been limited by their accumulation in the kidney, and so far experiments with improved antibody forms such as those mentioned above have not been reported.

Auger emitters which are potentially useful as therapeutic radionuclides for RIT include ^{125}I, ^{64}Cu, ^{195m}Pt and some isotopes also used for imaging at low doses such as ^{111}In , ^{99m}Tc and ^{67}Ga. As with alpha emitters, applications are likely to be restricted to conditions in which good tumour distribution is achieved. In this case, internalisation into the cell is required for efficacy and preferably location to the nucleus such that there is a good chance of this intense, short-range energy damaging cellular DNA. Therefore Auger emitters have also been investigated as potential therapeutic isotopes in an adjuvant setting, in leukemias and well-vascularised tumours. Because of the requirement for internalisation for cytotoxic effects, Auger emitters are potentially less toxic in the circulation than beta emitting isotopes. Modelling analysis has suggested that ^{195m}Pt-labelled antibody would be effective at delivering a sterilising dose to blood-borne tumour cells without delivering a toxic dose to bone marrow (Willins and Sgouros, 1995). Clinical studies have been carried out with several ^{125}I-labelled antibodies. Studies with ^{125}I-chimeric 17-1A and ^{125}I–murine A33 in colorectal cancer have demonstrated that high doses of this Auger emitting radionuclide can be given without significant toxicity, although no, or very modest, anti-tumour effects were observed in either case (Meredith *et al.*, 1995; Welt *et al.*, 1996).

^{64}Cu is an attractive isotope as a combined Auger and beta emitter which can be used to radiolabel antibodies using the TETA macrocycle, as for ^{67}Cu. Comparisons of ^{64}Cu and ^{67}Cu-labelled antibody for RIT showed that both had similar anti-tumour effects with good therapeutic effects against small tumours (Connett *et al.*, 1996). ^{64}Cu is also under investigation as an isotope for radioimmunodetection using PET (see Section 3.9). The half-life of ^{64}Cu suggests that improved RIT results might be expected using an antibody fragment. Initial biodistribution experiments have used F(ab′)$_2$, although kidney accumulation of this fragment has precluded RIT studies (Anderson *et al.*, 1995).

An approach to improve therapeutic effects with Auger emitters is to target the isotope to the cell nucleus. One attempt at achieving this has been to prepare a synthetic linker comprising a macrocycle for radioisotope chelation with a known DNA intercalator, such as an acridine (Norman *et al.*, 1995b). When internalised into cells it is likely that many antibodies will be degraded, but macrocyclic ligands are known to be retained in the cell. A DNA intercalator may then bind to DNA in the cell nucleus and hence retain the isotope where the Auger emitter may result in more potent cytotoxic effects.

Clearance mechanisms and two-step targeting

Clearance mechanisms and two-step tumour targeting strategies for RAID have been described in Chapter 3. Many of these strategies have also been suggested as potential approaches to improving the therapeutic ratio achievable in RIT. Clearance mechanisms which clear circulating isotope *in vivo* by the administration of second antibody may reduce bone marrow toxicity but are likely to deliver a large dose to the organ of clearance, usually the liver. Hence immunoadsorption devices have been developed (Norrgren *et al.*, 1991). In these systems, radiolabelled antibody is allowed to bind to tumour antigen and then circulating antibody is removed by passing blood through an extra-

corporeal immunoaffinity chromatography column. For example, an anti-mouse antibody can be immobilised as an immunoaffinity material and packed into a column format (see Section 3.7 for more details of immunoaffinity chromatography). Blood can then be pumped outside the body and through the column which binds radiolabelled murine antibody before the blood is returned to the patient. An alternative system of potential application to human and humanised antibodies is to use biotinylated, radiolabelled antibody for RIT. This can then be removed through an extracorporeal column of immobilised avidin, sparing the radiation dose to bone marrow from circulating activity (Garkavij *et al.*, 1997).

Two-step targeting protocols are also under development for RIT. Antibodies to chelate–isotope complexes, e.g. DOTA–^{90}Y, have been developed which may allow production of bispecific reagents which can be localised to the tumour and allowed to clear from circulation (Goodwin *et al.*, 1994). Radiolabelled chelate can then be injected which is bound to the anti-chelate antibody at the tumour site and readily excreted from the rest of the body. Potential problems with this approach are that a high-affinity interaction is required to bind the radiolabelled ligand from a relatively low concentration in blood as it passes the tumour site. The high-affinity interaction of the avidin:biotin system has therefore been employed in attempts to improve tumour loading levels of isotope. Streptavidin–antibody conjugate is localised to tumour tissue and circulating conjugate cleared using a biotin–protein conjugate. ^{90}Y–DOTA–biotin is then administered which localises to the tumour site and clears from the rest of the body, generating high tumour : blood ratios. Promising results demonstrating cures of human tumour xenografts in nude mice have been presented, and clinical evaluation of this approach is underway (Axworthy *et al.*, 1994).

Clinical results with RIT

The best clinical results with RIT to date have been obtained in leukemias and lymphomas. These hematopoietic tumours have the advantage of being readily accessible to intravenously administered antibody and are often relatively sensitive to radiation. Particularly encouraging results have been obtained in trials for the therapy of non-Hodgkin lymphoma. A high proportion of patients have been found to respond to treatment in phase II studies of ^{131}I–B1, a MAb which recognises CD20 (Press *et al.*, 1995; Kaminski *et al.*, 1996). In these studies patients were screened with a tracer dose of ^{131}I–B1, and those with a favourable biodistribution progressed on to therapy. Therapy with non-myeloablative doses resulted in long lasting, complete responses in 50% of patients (Kaminski *et al.*, 1996). Higher doses could also be used together with autologous bone marrow transplantation to cope with the toxicity of the radiolabelled IgG. In this case responses in a phase II trial were seen in 18 of 21 patients, including 16 complete remissions (Press *et al.*, 1995). Responses have also been observed with ^{90}Y-labelled anti-CD20 antibody therapy of lymphoma. In a dose-escalation study, a dose of up to 40 mCi was found not to be myeloablative and resulted in an overall response rate of 78% even though in this study patients were not pre-screened for a favourable biodistribution (Knox *et al.*, 1996).

Promising results have also been obtained in leukemia studies. An early trial of ^{90}Y-labelled anti-Tac antibody in adult T cell leukemia resulted in responses in 9 of 16 patients treated although a relatively low dose was used (Waldmann *et al.*, 1995). Several studies have been carried out with antibodies to CD33 in acute myeloid leukemia. Both murine and humanised versions of Ml 95 have shown significant anti-tumour effects when labelled with ^{131}I, including complete remissions in several patients. This has led to

the incorporation of these agents into preparative regimens for bone marrow transplantation in which complete remissions were observed in most patients (Jurcic *et al.*, 1995). The drawback of this approach is the myelosuppression seen with high doses of radiolabelled antibody. Complete responses have also been obtained in studies with another anti-CD33 antibody, P67, when labelled with ^{131}I (Applebaum *et al.*, 1992). As this antibody internalises into leukemic cells it is also under investigation for targeting the cytotoxic drug calicheamicin, in an approach to reduce myelosuppression (Section 4.2.7).

Results in the major solid tumour types (colorectal, lung, breast and ovarian cancer) have been less impressive, with few tumour responses. Occasional complete responses to treatment have been reported (e.g. Juweid *et al.*, 1997), but these are the exception rather than the rule. Several trials in colorectal cancer have resulted in only minor responses. In a phase II study of the anti-TAG72 antibody CC49 labelled with ^{131}I, no significant tumour responses were observed (Murray *et al.*, 1994). Another anti-TAG72 antibody, chimeric B72.3, resulted in stable disease in 4 of 12 patients treated with ^{131}I-labelled antibody, with one minor response, approx. 40% reduction in tumour size, seen in a repeat dose study (Meredith *et al.*, 1992b). Antibodies to CEA have also been extensively studied in colorectal cancer. Treatment with ^{131}I–A5B7 resulted in one partial response in a lung metastasis and one complete resolution of liver metastasis (Lane *et al.*, 1994). In a study of ^{131}I-labelled chimeric L6 in 10 women with metastatic breast cancer, partial responses of up to 5 months' duration were seen in four patients, with two other minor responses (DeNardo *et al.*, 1994). Following on from successful results for lymphoma when combining RIT with bone marrow support to overcome the toxicity of the circulating labelled IgG, a study has been carried out in breast cancer with ^{90}Y-labelled BrE3 antibody to polymorphic epithelial mucin (Schrier *et al.*, 1995). In this study of 9 patients, 4 required bone marrow support and partial responses were observed in approximately 50% of patients treated.

There is now increasing evidence that although doses delivered to large tumour deposits are inadequate for therapy, significant tumour doses can be achieved to small tumours. Investigations have been made into the use of RIT as an adjuvant therapy, for the treatment of minimal residual disease following surgical excision of primary tumour, or for treatment after tumour reduction by conventional chemotherapy. A clear correlation between tumour response and size of the tumour has been demonstrated in both animal models and patients, presumably as a result of better tumour penetration into smaller tumours. With ^{131}I-antibody administered intraperitoneally for ovarian cancer, no responses were seen in patients with tumours of diameters greater than 2 cm, two responses were seen from 15 patients with smaller tumours (13% response) and three of six (50% response) patients with malignant cytology but no measurable disease had their malignant cells cleared from the peritoneum (Stewart *et al.*, 1989). Encouraging results have also been observed in a study of ^{131}I-anti-CEA F(ab′)$_2$ in patients with tumours less than 3 cm in diameter (Juweid *et al.*, 1996). Disease stabilisation was observed in approximately half of the patients treated with some tumour shrinkage. However, strong HAMA responses to this murine antibody fragment prevented re-treatment. In another ovarian cancer study with intraperitoneally administered ^{131}I-labelled antibody, treatment of minimal residual disease resulted in complete responses in 5 of 16 patients with a mean disease-free period of over 10 months (Crippa *et al.*, 1995). Also in ovarian cancer, a study with ^{90}Y-antibody to polymorphic epithelial mucin resulted in increased survival in patients treated with the antibody and who had no measurable disease at the time of treatment (Hird *et al.*, 1993). These patients were viewed as having received adjuvant therapy for the minute micrometastases present, and were compared to historical control groups

of patients. Over four and a half years, more than 90% of patients treated survived compared to less than 40% in a large group of historical controls. It is not known how much of this effect is due to cytotoxicity from the radioisotope or to biological effects of the antibody administered, such as the induction of an anti-idiotype response (see Section 4.2.2), and these promising results are thus the subject of further study.

Although initially disappointing, RIT is now beginning to prove clinically useful in some disease situations, particularly the liquid tumour types and as an adjuvant therapy or for treatment of minimal residual disease. The majority of agents tested to date have been murine antibodies, often labelled with ^{131}I, which have not taken advantage of the ability to develop improved targeting agents through the developments in molecular biology and improved chemistry mentioned above. As these developments allow improved agents for RIT to be brought forward for clinical testing, it is likely that clinical benefit will be seen in an increased range of diseases. Combinations of RIT with conventional cancer therapy by chemotherapy or external beam radiation are already beginning to be explored in experimental systems, with synergistic effects observed, allowing better therapy than either approach alone (Tschmelitsch *et al.*, 1997; Vogel *et al.*, 1997).

4.2.6 Immunotoxins

Protein toxins are extremely potent cell killing agents and have been widely investigated as potential cytotoxic agents for antibody targeted cancer therapy. Antibody–toxin conjugates, or immunotoxins, are being evaluated with plant toxins such as ricin and abrin and bacterial toxins such as diptheria toxin (DT) and *Pseudomonas* exotoxin (PE) (Table 4.6). The target of these toxins is the inhibition of protein synthesis. In the case of the plant toxins this is achieved through inactivation of ribosomes by modification of ribosomal RNA, whereas the bacterial toxins inactivate the elongation factor, EF-2, which normally interacts with the ribosomal RNA.

Immunotoxins require internalisation into the cell to exert their cytotoxic effect; however, protein toxins are enzymes, and therefore catalytic, and small numbers of toxin molecules may be sufficient for cytotoxicity. The mode of action of toxins (Figure 4.5) requires not only internalisation but translocation of the toxin across a membrane of the endosome or the trans-golgi network (depending on the toxin used) to reach the site of action in the cytosol. Therefore, although under some conditions a single toxin molecule may be sufficient to kill a cell (Yamaizumi *et al.*, 1978), many more must be delivered to the cell surface as internalisation and translocation to the site of action are inefficient. Many toxins have a subunit or domain of their own which is responsible for cell binding and internalisation and this must be removed or blocked to reduce non-specific toxicity. PE has three domains, one of which is responsible for cell binding. Truncated versions of

Table 4.6 Toxins under evaluation for the preparation of immunotoxins

Plant toxins	Bacterial toxins
Ricin	*Pseudomonas* exotoxin (PE)
Abrin	Diptheria toxin (DT)
Gelonin	
Saporin	
Pokeweed antiviral protein (PAP)	

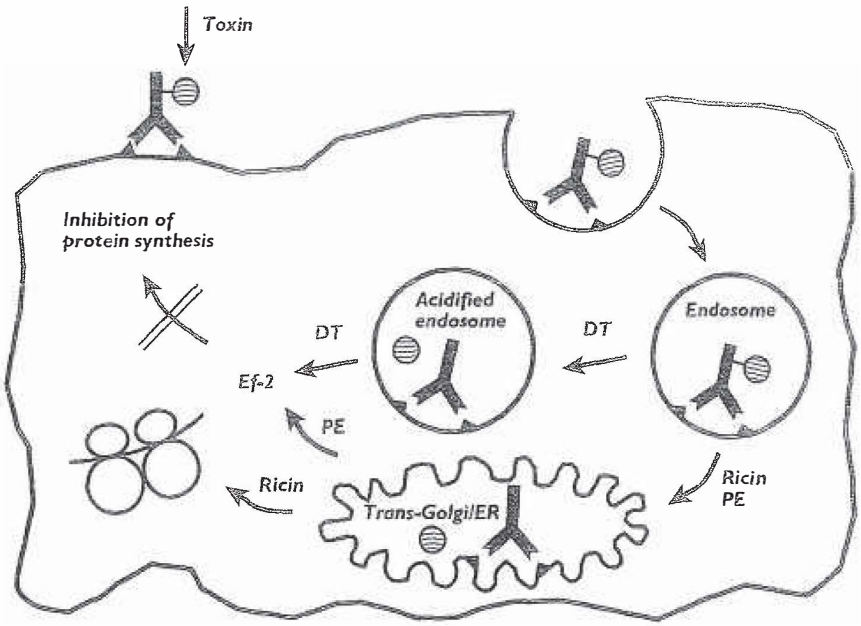


Figure 4.5 Illustration of the mechanism of cytotoxicity of immunotoxins. Immunotoxins bind to cell surface antigen and are internalised by endocytosis. Toxins such as ricin A-chain and *Pseudomonas* exotoxin are routed to the trans-golgi/endoplasmic reticulum network whereas diptheria toxin is routed to an acidified endosome. In these compartments release of toxin is achieved which can be translocated to the cytoplasm and inhibits protein synthesis.

PE have been expressed comprising the catalytic and translocation domains of the toxin, but not the cell binding domain. Ricin comprises an A- and B-chain, the A-chain being responsible for catalytic inactivation of ribosomes whereas the B-chain is responsible for cell binding and internalisation. Immunotoxins have therefore been constructed using ricin A-chain alone, which must be stringently purified away from B-chain and intact toxin. An alternative approach is to block the cell binding site using an affinity ligand. Blocked ricin conjugates have been prepared by covalent linkage of an affinity ligand (Lambert *et al.*, 1991). Blocked ricin conjugates have the advantage that the intact toxin is retained with its full ability to translocate into the cytosol, a property which is reduced in A-chain conjugates.

Immunotoxins can be prepared either by chemical linkage or by expression of recombinant fusions of the toxin gene to the antibody gene. Linkage of toxin to antibody must be stable enough to remain intact in the circulation, but be able to release free toxin inside the cell to allow cytotoxicity. Toxins such as PE contain an internal disulphide bond which is reduced as part of its mechanism of release inside the cell. Stable linkages of PE to antibody therefore result in effective immunotoxins, and these have been prepared through thioether linkages or as recombinant fusions (Pai *et al.*, 1996; Benhar and Pastan, 1995). Ricin A-chain is normally linked to B-chain through a disulphide linkage which is also reduced intracellularly. This cysteine on the A-chain has thus been the site of conjugation to antibody, in the place of B-chain. Initial work with disulphide linked

ricin A conjugates resulted in constructs which were unstable in the circulation, therefore linkers containing 'hindered' disulphide linkages were developed with a methyl or phenyl group adjacent to the disulphide. These are more stable in circulation but still effectively reduced inside the cell (Thorpe *et al.*, 1988). An additional problem with plant toxins such as ricin is their glycosylation which promotes binding to cells of the reticuloendothelial cell system, particularly the liver. Therefore chemically deglycosylated ricin, or A-chain produced by recombinant means in *E. coli* has been used to avoid non-specific toxicity through carbohydrate binding (Thorpe *et al.*, 1988). Alternative linkage of ricin A-chain has been achieved through proteolytically sensitive peptides in a recombinant ricin A-chain fusion protein. When internalised, the proteolytically sensitive site is cleaved to generate free ricin A-chain which is then cytotoxic (O'Hare *et al.*, 1990).

The optimal form of antibody for use in immunotoxins has been less well studied. Intact IgG conjugates can be efficiently internalised and have the highest degree of accumulation at the tumour due to their long circulating half-life. One drawback is their large size which reduces penetration into tumour tissue. Antibody fragments may therefore be advantageous in achieving more effective penetration into tumour tissue. In animal model experiments, antibody fragment toxin conjugates have proved to be superior to intact IgG. Conjugates of BR96, an antibody which recognises a variant of the Le^y antigen on human carcinomas, have been prepared with the intact IgG and PE40 (truncated *Pseudomonas* exotoxin) and as a recombinant scFv fusion protein (Friedman *et al.*, 1993). The IgG conjugate was able to retard the growth of human tumour xenografts in mice, whereas the scFv fusion protein was capable of inducing complete regressions. Similar results have been obtained with immunotoxins prepared with other antibodies. An scFv–PE immunotoxin of the antibody B3, which recognises the same antigen, was shown to be more effective than an IgG conjugate at producing cures of carcinomatous meningitis in a rat model (Pastan *et al.*, 1995). Similarly, a Fab′–PE fusion protein was also shown to be more effective than the equivalent IgG based immunotoxin (Debinski and Pastan, 1995). Both scFv–PE and dsFv–PE immunotoxins have been prepared and shown to be equipotent in their ability to cause long-term regressions of tumour xenografts (Benhar and Pastan, 1995). The scFv–PE conjugate was less stable yet retained more of the binding activity of the IgG, whereas the dsFv was more stable but less immunoreactive, with the overall result that they were approximately equipotent *in vivo*.

Bispecific antibodies have also been suggested as means of delivery of toxins to tumour cells. Antibodies in which one arm recognises a lymphoma-associated antigen and one the toxin saporin have been produced, and complexes of the bispecific antibody with saporin shown to be capable of cell killing (Bonardi *et al.*, 1993). A potential advantage of this approach is that the toxin is held via non-covalent forces in the antibody-binding site and is therefore not damaged through chemical modification. Also, the toxin may be released more easily on internalisation into the cell, and non-specific binding sites on the toxin may be blocked through the antibody interaction. A small clinical study using this aproach for lymphoma therapy has been carried out using an anti-CD22, anti-saporin bispecific di-Fab (French *et al.*, 1995). In this study all of the four patients treated appeared to show rapid and beneficial responses to treatment with only mild toxicity.

Clinical studies have been carried out with a number of different immunotoxins produced by direct linkage of the toxin to antibody (reviewed in Ghetie and Vitetta, 1994). Anti-tumour activity has been reported in lymphomas, and recently in breast and colorectal tumours (Pai *et al.*, 1996). In this phase I study of 38 patients objective anti-tumour activity was observed in 5 patients, 18 had stable disease and 15 progressed. Two major problems have limited efficacy in this and other studies: immunogenicity and toxicity.

The major toxicities seen in immunotoxin trials have been hepatotoxicity, neurotoxicity and often the most limiting, vascular leak syndrome. Vascular leak syndrome is probably caused through effects on endothelial cells, and research is ongoing to attempt to overcome this problem through engineering or inhibition with anti-inflammatory drugs (Siegall *et al.*, 1997).

Protein toxins used to date have been derived from plant or bacterial sources and are highly immunogenic. This immunogenicity has limited the number of doses which can be given in patients and therefore limits anti-tumour effects. Attempts to overcome this through engineering, immunosuppressive agents or PEGylation are being explored. One approach is to use a human enzyme as a toxin which would hopefully be less immunogenic in man. Human ribonucleases which kill cells only after internalisation have been explored as potential immunotoxins both by chemical conjugation to antibody and as recombinant fusion proteins (Gadina *et al.*, 1994). However, the cytotoxicity of such 'humanised immunotoxins' is poor compared to those produced with ricin or PE. Attempts to design improved immunotoxins using scFv–RNAse fusion proteins are a subject of current research (Newton *et al.*, 1996).

4.2.7 Drug conjugates

Chemotherapy using cytostatic or cytotoxic drugs has been the mainstay of cancer therapy over the past 50 years. However, there is little selectivity of these drugs for tumour cells over rapidly dividing normal cells, such as those in the bone marrow, and consequently chemotherapy is limited by normal tissue toxicity, and usually results in severe toxic side-effects. The ability to target chemotherapeutic drugs such that they are inactive until taken into tumour cells is thus an attractive concept with the potential to allow higher doses to tumour cells without exposing normal cells to high concentrations of drug. In common with immunotoxins, drug conjugates require internalisation to be able to kill cells, but they have the potential advantage over toxins of being small molecules which are less likely to raise an immune response. Attempts to address this problem have suggested that choice of suitable chemical linkers, and the use of humanised antibodies, may result in non-immunogenic or only weakly immunogenic conjugates (Johnson *et al.*, 1991a). The other major problem of immunotoxins, non-specific toxicity, may also be overcome with the use of drug conjugates, as many drug derivatives may be screened to identify optimal characteristics.

Initial attempts to produce conjugates of cytotoxic drugs with anti-cancer MAbs focused on drugs used in the clinic. Conjugates have been prepared with many of these, including methotrexate, doxorubicin, mitomycin C, neocarzinostatin, daunorubicin and vinca alkaloids. Early attempts suffered from loss of activity of both the antibody and drug on conjugation, and relatively poorly active immunoconjugates resulted. Several problems were identified, including identifying drugs of sufficient potency given the inefficiency of antibody uptake by solid tumours, and identifying suitable chemistry to generate linkage of the antibody to the drug without a major loss of activity. Since these early experiments, improved linkage methods have been developed (reviewed by Pietersz *et al.*, 1994). The best linkage chemistry varies from drug to drug, but a key feature is to allow stable conjugates in circulation which can also release active drug during antibody degradation inside the cell. In addition, site-specific linkages to antibody have been investigated, for example, to antibody carbohydrate (see Section 2.6.2) which allow retention of antibody activity (Schrappe *et al.*, 1992). However, the low potency of many of

these drugs has required many molecules of drug to be coupled per antibody to achieve sufficient doses for therapeutic effects. Nevertheless, some good anti-tumour effects have been observed with optimised conjugates of several of these drugs in animal models. Long-term growth suppression of glioma xenografts have been observed with conjugates of vinblastine (Schrappe *et al.*, 1992) and cure of xenografted lung, breast and ovarian tumours has been observed in some mice with doxorubicin conjugates of the anti-Le^y antibody BR96 (Trail *et al.*, 1993). In general, however, such responses have required treatment before establishment of the tumour or the use of doses close to the maximum tolerated dose, which may lead to limited benefits over non-conjugated drug in the clinical situation.

Attempts to improve potency have included the use of polymeric carriers to conjugate large numbers of molecules of drug to antibody without loss of antigen-binding properties. In this strategy the polymeric carrier is conjugated to many molecules of drug before site-specific attachment of a single polymer to the antibody. Polymers can be synthetic, proteins such as HSA or carbohydrate such as dextran (Shih *et al.*, 1991). However, results with such conjugates have been disappointing, with little real improvement over directly linked conjugates observed either *in vitro* or *in vivo*.

Attempts have also been made to produce immunoliposomes to target large amounts of the drug to the tumour site. Liposomes are synthetic vesicles consisting of one or more concentric phospholipid bilayers which can be used to encapsulate an aqueous compartment, which can be filled with drug. Antibody can be attached to the surface of the liposome, resulting in an immunoliposome. Interaction of the liposome with the target cell either by direct fusion with the cell membrane or by endocytosis should result in the release of large numbers of drug molecules to the tumour cell. However, immunoliposomes are rapidly taken up by the reticuloendothelial system *in vivo*, and thus PEG-modified phospholipid is often incorporated into the liposome, resulting in 'sterically stabilised' or 'stealth' immunoliposomes which are less prone to reticuloendothelial uptake and can be used to target to tumour cells (Allen *et al.*, 1995). Attempts to deliver doxorubicin using immunoliposomes have shown promising tumour targeting, although little improvement over free doxorubicin has been demonstrated in some studies (Park *et al.*, 1995). Immunoliposome mediated targeting of drugs may find a niche, however, in the treatment of newly established micrometastatic disease or metastatic cells migrating in blood or lymph (Allen *et al.*, 1995).

More impressive results have come with the development of more toxic cytotoxic agents. Several classes of extremely potent new drugs have been identified, at least 100-fold more cytotoxic than drugs used for chemotherapy in the clinic. Such drugs are too toxic for use as the free drug, but can give rise to very potent immunoconjugates. These are principally the enediynes (Nicolaou *et al.*, 1993), maytansinoids (Chari *et al.*, 1992), tricothecenes (Liu, 1989) and analogues of CC-1065 (Chari *et al.*, 1995). Immunoconjugates have been produced with these agents which have impressive anti-tumour effects in mouse xenograft experiments. Calicheamicin, a member of the enediynes, has been conjugated to an anti-PEM antibody, CTM01, and shown cures of established breast and ovarian tumour xenografts in nude mice (Hinman *et al.*, 1993). In this case, several derivatives of calicheamicin were compared to identify the optimal drug for conjugate activity without toxicity. A hindered disulphide was used to allow a stable linkage in circulation which was labile inside the cell, a strategy also used for ricin immunotoxins (see Section 4.2.6). This antibody has been humanised (Baker *et al.*, 1994), and humanised CTM01–calicheamicin conjugates prepared which retain the potent anti-tumour effects of the murine antibody. Clinical trials with this conjugate are under way. A similar

calicheamicin conjugate has been developed for the anti-CD33 antibody, hP67. Early clinical studies with this molecule for the therapy of acute myeloid leukemia have revealed an excellent safety profile and encouraging anti-tumour effects, with several complete and partial responses seen (Bernstein, 1996). Similarly potent anti-tumour effects have been observed with maytansinoid conjugates for therapy of colorectal cancer (Liu *et al.*, 1996). Eradication of established xenograft tumours was achieved even though antigen distribution was heterogeneous, a model in which immunotoxins with PE linked to the same antibody performed relatively poorly. Such potent drug immunoconjugates thus represent considerable promise for cancer therapy.

4.2.8 *Antibody-directed enzyme prodrug therapy (ADEPT)*

ADEPT is a two-step targeting method for cytotoxic drug therapy. Similarly to drug conjugates, ADEPT aims to improve the therapeutic ratio of cytotoxic drugs using antibody targeting, but in this case an enzyme is attached to the antibody and targeted to tumour tissue. The enzyme is then used to convert an inactive prodrug to a cytotoxic drug which generates a high local concentration of drug at the tumour site (Figure 4.6). This an attractive concept, as less antibody conjugate may be required at the tumour site due to the enzyme acting catalytically and being able to turn over many molecules of prodrug to drug. Also, there is less need to target every cell as killing of bystander cells should be possible by the high local drug concentration. The disadvantages of this approach include the fact that free drug is generated which may diffuse away from the tumour site, damaging non-target tissues. Also, an enzyme must be chosen which does not occur in humans, or at least not in extracellular locations, to avoid activation of the prodrug in normal tissues.

Several different enzyme systems have been investigated for ADEPT (Table 4.7) (reviewed by Melton and Sherwood, 1996). The first system to be developed was based on carboxypeptidase G2 (CPG2) cleaving a glutamate derivative prodrug to liberate a nitrogen mustard drug (Searle *et al.*, 1986). Subsequently this and other enzyme systems have shown anti-tumour effects in mouse xenograft experiments. Studies with an anti-CEA $F(ab')_2$ linked to CPG2 revealed only modest anti-tumour effects at first. This was improved by the introduction of a third step to the system, a clearing antibody (Sharma *et al.*, 1990). In this revised three-step system the antibody–enzyme conjugate is administered and allowed to localise to the tumour. After localisation has taken place residual

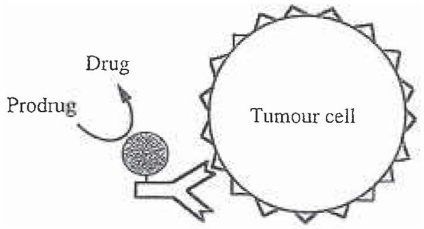


Figure 4.6 Conversion of prodrug to active drug through ADEPT. Antibody–enzyme conjugate is localised to tumour and allowed to clear from the circulation. Inactive drug is then administered which is converted to a cytotoxic drug by the enzyme attached to antibody at the tumour site. This results in a high local concentration of drug with resultant tumour cell cytotoxicity

Table 4.7 Enzymes used for construction of antibody–enzyme conjugates for ADEPT

Enzyme	Origin	Active drug	Substrates
Carboxypeptidase G2	*Pseudomonas*	Benzoic acid mustards	Glutamic acid derivatives
Carboxypeptidase A	Bovine	Methotrexate	Phenylalanine derivatives
Alkaline phosphatase	Bovine	Etoposide, doxorubicin, phenol mustards	Phosphate derivatives
β-lactamase	*E. coli* *Enterobacter*	Nitrogen mustards, vinblastine, taxol	Cephalosporin derivatives
Cytosine deaminase	Yeast	5-fluorouracil	5-fluorocytosine
Penicillin amidase	*Fusarium*	Doxorubicin, melphalan	Hydroxyphenylacetyl derivatives
β-glucuronidase	*E. coli*, human placental	Doxorubicin, epirubicin, aniline mustards	Glucuronide derivatives
β-glucosidase	Sweet almond	Hydrogen cyanide	Amygdalin

circulating conjugate is removed by the administration of a clearing antibody against the CPG2 enzyme. This clearing antibody was galactosylated to allow rapid clearance through galactose receptors in the liver. After clearance of circulating conjugate the prodrug is administered for activation at the tumour site. This approach was found to reduce non-specific activation of prodrug and allowed successful anti-tumour effects in xenografts to be seen.

Early conjugates were prepared by chemical coupling of IgG or $F(ab')_2$ to the enzyme of interest, but subsequently recombinant constructs have been designed and expressed as scFv–enzyme fusions (Goshorn *et al.*, 1993). These have the advantage of smaller size which may assist penetration into tumour tissue and more rapid clearance which may avoid the need for clearing antibody. scFv–β-lactamase conjugates were expressed in *E. coli* in an active form and were therefore relatively simple to produce, and as defined molecules, simpler to characterise compared to chemical conjugates (Goshorn *et al.*, 1993). Disulphide-linked Fv–β-lactamase conjugates have also been produced which are more stable in circulation than the scFv fusion proteins, although a direct comparison has not yet been reported (Rodrigues *et al.*, 1995).

The CPG2 system has been examined in a pilot clinical study which confirmed the suspected major drawback of ADEPT, i.e. immunogenicity of the enzyme in humans (Bagshawe *et al.*, 1995). In this study repeat dosing was limited by immune responses to both the murine $F(ab')_2$ used and the bacterial enzyme, and attempts to suppress the immune reponse with cyclosporin were unsuccessful. Overcoming the immune response to the antibody may be achieved by use of a human or humanised antibody, although overcoming responses to non-human enzymes may be more difficult. A humanised antibody–human enzyme conjugate has been prepared using β-glucuronidase, which has shown anti-tumour effects in mice (Bosslet *et al.*, 1994).

An alternative approach which may be non-immunogenic is the use of catalytic antibodies (Miyashita *et al.*, 1993). Antibodies can be produced to transition state analogues which are capable of catalysing a variety of chemical reactions (see Chapter 6). These can be humanised to reduce immunogenicity, and used to produce a bispecific antibody

with binding specificity for a tumour-associated antigen as well as catalytic activity. Use of such bispecific antibodies may be a feasible approach, and a catalytic antibody capable of activating a carbamate prodrug to a nitrogen mustard has been produced (Wentworth *et al.*, 1996). The catalytic activity of this antibody was too weak to be of use *in vivo*, and therefore it has been used to set up assays to screen phage libraries for improved catalytic activity.

4.2.9 Vascular targeting

The continued growth and viability of a tumour is critically dependent on maintaining an effective blood supply which can supply the oxygen and nutrients required. An alternative strategy for tumour therapy is thus to shut off tumour blood vessels, cutting off nutrient supply which rapidly leads to tumour cell death. Two approaches have been investigated, targeting existing blood vessels or preventing the formation of the new blood vessels required to support growth. The process of blood vessel formation is known as angiogenesis, and thus these two approaches can be termed anti-vascular and anti-angiogenic. As these targets are vascular the problem of accessibility of antibodies to solid tumours is overcome, and thus antibody approaches have played a major part in attempts to develop such therapy.

Several growth factors secreted by tumours are known to be involved in the process of tumour angiogenesis, such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF). Secreted factors cause migration and proliferation of vascular endothelial cells from existing blood vessels to form new tumour vasculature. Blocking this process has been attempted using antibodies which bind to bFGF or VEGF and block receptor interaction. Alternatively, antibodies to the growth factor receptors could be used. Both antibodies to bFGF and VEGF have shown inhibition of tumour growth (Hori *et al.*, 1991; Kim *et al.*, 1993). Particularly exciting data have arisen from inhibition of VEGF-induced angiogenesis which appears to be applicable to several types of tumour. Antibodies to VEGF are capable of inhibiting tumour growth and metastasis in experimental systems (Asano *et al.*, 1995). Complete inhibition of angiogenesis and growth of micro-tumours has been observed following treatment with anti-VEGF, changing the characteristics of a rapidly growing malignancy to a dormant microcolony (Borgstrom *et al.*, 1996).

Cell adhesion interactions, mediated by integrins, are also crucial in the process of angiogenesis. The integrin $\alpha_v\beta_3$ (CD51/CD61) is required on vascular endothelial cells during angiogenesis for their differentiation, migration and division. Blocking $\alpha_v\beta_3$ with a MAb can inhibit the growth of human tumours by causing apoptosis of the proliferating cells while leaving pre-existing blood vessels unaffected (Brooks *et al.*, 1994).

Early anti-vascular therapies were directed at killing tumour endothelial cells. Destruction of tumour blood vessels could lead to the death of many tumour cells, as many cells are supplied by each blood vessel. Animal model studies in which an antibody–toxin conjugate was targeted to tumour vasculature resulted in impressive anti-tumour effects, with eradication of large solid tumours (Burrows and Thorpe, 1993). Another approach is to block tumour blood vessels through inducing blood clots. Targeting of tissue factor, a protein that normally triggers blood clotting at sites of injury, has been shown to be capable of inducing blood clots specifically in the tumour vasculature leading to regression of large tumours (Huang *et al.*, 1997). However, the utility of these approaches may be limited by the availability of markers which are selectively expressed on human tumour

vasculature and not normal vasculature. Although several markers which are at least partially selective for tumour vasculature, such as endosialin and endoglin, have been described (Rettig *et al.*, 1992; Burrows *et al.*, 1995). An anti-endoglin antibody has been raised that cross-reacts with mouse endothelial cells, facilitating studies in mouse tumour models (Seon *et al.*, 1997). The antibody reacted strongly with human endothelial cells and more weakly with mouse vasculature. Nevertheless, anti-tumour effects were seen with an immunotoxin made from this antibody and ricin A chain, with prevention of tumour growth in immunodeficient mice (Seon *et al.*, 1997).

4.3 Infectious disease

4.3.1 Antiviral antibodies

Although most bacterial infections can be well controlled by the use of antibiotics, the control of some viral diseases is far more difficult. Vaccination has allowed the control or even eradication of some viral diseases such as smallpox, poliomyelitis and measles, yet others have been more difficult to control. Antibodies have long been considered as potential protective or therapeutic agents for such conditions and MAbs may have a role to play in some cases. MAbs are under development for therapy of a variety of viral diseases, including respiratory syncytial virus (RSV), rabies, hepatitis B and C (HBV and HCV), herpes simplex viruses (HSV-1 and HSV-2), cytomegalovirus (CMV) and human immunodeficiency virus (HIV).

RSV is the leading cause of pneumonia and bronchitis in infants, and immunity appears to be only partially effective since re-infection can occur within a few weeks. In infants with cardiopulmonary disease or with immunodeficiency RSV can be fatal. Antibodies can protect against serious disease and current therapy uses polyclonal sera enriched with antibodies to RSV. There has been considerable interest in developing MAbs for RSV therapy. MAb preparations allow a higher concentration of specific antibody and are hence more potent therapeutic agents which are easier to use as they can be administered intramuscularly (i.m.) rather than intravenously (i.v.). The major surface antigens of RSV are the F and G proteins, and animal studies suggest that MAbs to certain epitopes on the F protein efficiently neutralise virus. Also, the F protein is conserved between strains and subgroups of RSV, making it an attractive target for a therapeutic MAb. Several anti-RSV antibodies have been humanised and shown to be effective in neutralising virus in animal models (Tempest *et al.*, 1991). The long half-life of humanised IgG is helpful in providing protection against infection for a prolonged period, and humanised IgG is therefore currently under clinical evaluation.

Anti-RSV antibody specificities have also been obtained from phage libraries (Barbas *et al.*, 1992). In some cases isolation of neutralising antibodies from phage libraries may be difficult due to the dominance of a non-neutralising epitope, such that only antibodies to this epitope are isolated. This has been the case for RSV F protein, and has been overcome by use of selection against antigen which is pre-blocked with an antibody to the dominant epitope. An antibody isolated from initial screening has been used to block the F protein dominant epitope such that antibodies to other less dominant epitopes, including neutralising antibodies, can be isolated (Tsui *et al.*, 1996).

Phage libraries have also been used for the isolation of human antibodies to other viral targets including HIV-1 and herpes simplex viruses (Barbas and Burton, 1996). Fab

fragments to the HIV-1 envelope protein gp120 have been isolated. The majority of Fabs were raised to the dominant epitope, the CD4 binding site, although as with RSV it has proved possible to block this epitope to isolate Fabs against other epitopes (Ditzel *et al.*, 1995). Several neutralising Fabs have been isolated, one of which, to the CD4 binding site, has been used to generate a recombinant human IgG and shown to be capable of neutralisation of primary isolates of HIV-1 *in vitro*, and of protecting immunodeficient mice containing human lymphocytes from infection. Attempts to improve binding affinity using mutagenesis have proved successful in some cases. Anti-HIV-1 Fab was improved in affinity by 'CDR walking', in which CDRs are mutated either in turn or in parallel, and favourable mutations combined to develop a high affinity variant (Yang *et al.*, 1995). Antibodies with affinities in the picomolar range have been isolated, and it has been suggested that such high affinity antibodies may have improved *in vivo* potency, possibly extending the time an individual could be protected from disease (Barbas and Burton, 1996). However, the correlation between affinity and potency *in vivo* is not straightforward. Studies with a panel of antibodies to vesicular stomatitis virus (VSV) demonstrated that although there was a clear relationship between avidity and neutralisation *in vitro*, protection *in vivo* was not dependent on avidity provided a minimum threshold value (K_d 2×10^8–5×10^8) was reached (Bachmann *et al.*, 1997). In this study protection depended simply on a minimum serum concentration, suggesting that the isolation of very high affinity antibodies for viral protection may not be beneficial.

Anti-HIV antibodies such as those isolated by phage display, against gp120, may block infection through preventing the gp120/CD4 binding event which is involved in the entry of the virus into T cells. Fc mediated phagocytosis of the virus should also be enhanced. Another approach is to use immunotoxins directed towards the HIV envelope proteins (Pincus and McClure, 1993). Infected cells in which viral replication is occurring express the envelope proteins on the surface of the cell, such that as new virion cores are produced and bud through the cell membrane they become coated with the envelope proteins. Anti-gp120 immunotoxins can therefore be targeted to infected cells in an attempt to kill them before release of further virus. As described in Section 4.2.6, ricin A-chain conjugates can be potent cell killing agents, and anti-gp120–ricin A-chain conjugates can be produced with activity against HIV infected cells (Pincus and McClure, 1993). Antibody conjugates which are capable of virus neutralisation as well as cell killing activity may be additionally beneficial.

4.3.2 *Bacterial sepsis*

Bacterial sepsis is a clinical syndrome associated with severe bacterial infection from a wide variety of conditions ranging from acute diseases such as meningococcal meningitis to chronic conditions such as sepsis associated with cancer or surgical trauma. Once in the circulation, bacterial products such as the cell wall component lipopolysaccharide (LPS or endotoxin) elicit an inflammatory response that results in activation of an array of host defence systems and the release of a complex series of inflammatory mediators, including a variety of cytokines such as TNF and IL-1. In conjuction with the bacterial products over-activation of such systems can lead to shock, organ failure and death. In fact mortality rates in patients with sepsis are high, 25–70%, despite the availability of potent antibiotics for both Gram-positive and Gram-negative infections. There has been much interest in developing MAb based therapy for sepsis, based on antibodies to endotoxin or to mediators such as TNF. Encouraging results have been seen in preclinical and

small-scale clinical studies yet, to date, extensive clinical studies have all resulted in the failure to demonstrate efficacy (reviewed by Quezado *et al.*, 1995).

Endotoxin is a component common to all Gram-negative bacteria, and the lipid A component of endotoxin is conserved across different species. Several antibodies to lipid A have been developed including the murine IgM, E5 and a human IgM HA-1A. Both of these antibodies have been extensively studied and been progressed to large-scale clinical trials. After protective effects were demonstrated in animal models, clinical trials with E5 were believed to show some survival benefit in a subgroup of patients with sepsis who had not gone into refractory shock. However, this finding could not be backed up in a subsequent trial (Bone *et al.*, 1995). HA-1A also demonstrated improved survival in animal models and, in contrast to E5, was believed to have benefit in patients with Gram-negative sepsis accompanied by shock. Again, this finding was not confirmed in subsequent trials (Natanson *et al.*, 1994), and further studies revealed a high degree of non-specific binding of HA-1A to a variety of different antigens and variable results in animal models (Quezado *et al.*, 1995). In addition, mortality was apparently increased in patients without Gram-negative infection.

Antibodies to TNF are under investigation for a range of inflammatory diseases (see also Section 4.5.2) and, as a key mediator in sepsis, inhibition of TNF has also been studied as a potential therapeutic for septic shock. Inhibition of a key mediator may have advantages over antibodies to endotoxin, as both Gram-positive and Gram-negative sepsis should be affected. Selection of TNF as a target to inhibit was based on the evidence that TNF can be measured in the plasma of patients with septic shock, and high levels tend to be prognostic of mortality. The symptoms of septic shock can be reproduced by injection of TNF into animals and anti-TNF antibody can reduce mortality in animal models of septic shock (Beutler *et al.*, 1985). In preclinical studies it has been demonstrated that antibodies with isotypes that are active at recruiting effector functions (human γ1 and murine γ2a) are less effective than inactive isotypes such as human γ4 or murine γ1 (Suitters *et al.*, 1994). This effect may be a consequence of activated complement or effector cells causing the release of additional inflammatory cytokines which in turn potentiate the effects of TNF. However, again studies have failed to show efficacy of anti-TNF antibodies in patients, underlining the complexity and diverse pathology of sepsis and septic shock.

4.4 Cardiovascular disease

4.4.1 *Inhibition of platelet aggregation*

Perhaps the most succesful MAb based therapeutic to date is a mouse/human chimeric Fab fragment from the antibody 7E3. This Fab fragment binds to the gpIIb/IIIa receptor on platelets and blocks the interactions of fibrinogen and von Willebrand factor which are crucial steps in platelet aggregation. Chimeric 7E3 Fab, also known under its tradename ReoPro, is approved for prevention of complications following angioplasty. Angioplasty is an effective treatment for blocked blood vessels which is widely used. In this procedure a balloon catheter is introduced into the vessel and inflated such that the blockage is broken up or displaced. However, some damage to the vascular endothelium usually results, and in a significant proportion of patients, reocclusion of the vessel can occur through activation of platelets leading to their aggregation and adhesion to the damaged endothelium. On activation of platelets there is a conformational change in gpIIb/IIIa

which leads to binding of fibrinogen and hence platelet aggregation. Blocking with c7E3 prevents platelet cross-linking and thus prevents the formation of a platelet-rich thrombus which might otherwise reocclude the vessel.

c7E3 is used as a Fab fragment as the function required is purely a blocking one, bivalent IgG might cross-link cells and the presence of Fc might cause unwanted cell binding through Fc receptors, which would be counter-productive to preventing cellular aggregation. Fab fragments are also known to be less immunogenic than IgG (see Chapter 2) and chimeric Fab was developed to reduce immunogenicity further (Knight *et al.*, 1995). The chimeric Fab was considerably less immunogenic in humans than the murine version, even though the entire variable domains were still of murine origin. This was the case even though most of the response detected to the murine Fab was to the variable region, suggesting that in this case the constant region is responsible for modulation of the response to the variable domains (Knight *et al.*, 1995). CDR-grafted antibodies to gpIIb/IIIa have also been produced which are able to prevent platelet-induced thrombosis (Kaku *et al.*, 1995), although the immunogenicity of c7E3 is sufficiently low to allow its therapeutic use.

The efficacy of c7E3 was demonstrated in a 2099-patient trial for the prevention of acute cardiac complications following high-risk angioplasty. In this trial c7E3 demonstrated a 35% reduction in acute ischaemic events (death, myocardial infarction and urgent coronary intervention) at 30 days and a 23% decrease in ischaemic events (Califf, 1994). Principal adverse events were major bleeding at sites of vascular puncture or coronary artery bypass grafting and associated transfusions. Bolus injection followed by a prolonged infusion produced the best results, indicating the importance of inhibiting platelets for a sustained period. Subsequent trials have been designed which have reduced the problems of associated bleeding through fine tuning of the dose of heparin which is co-administered with c7E3, and have examined the role of c7E3 in patients with unstable angina and in lower-risk angioplasty patients with positive results.

4.4.2 Thrombolysis

The ability to lyse blood clots (thrombi) using thrombolytic therapy with plasminogen activators has led to its widespread use in the treatment of acute myocardial infarction, resulting in a significant reduction in mortality. However, present agents are not ideal and have limitations in their ability to lyse all thrombi, they often lead to high rates of reocclusion and in some cases they result in severe side-effects such as cerebral haemorrhage. The ability to target thrombolytic agents to blood clots may offer advantages, with the potential for increased potency and reduced side-effects. Antibodies to fibrin have been examined for this purpose. Importantly, antibodies that recognise fibrin in clots but not circulating fibrinogen have been isolated and used to produce chemically linked conjugates with plasminogen activators. Both two-chain and single-chain urokinase (uPA) could be made fibrin-selective by conjugation to anti-fibrin IgG or Fab fragments (Bode *et al.*, 1990). Similarly, the efficiency of tissue plasminogen activator (tPA) could also be enhanced by conjugation to anti-fibrin antibody (Runge *et al.*, 1988). Attempts to make active fusion proteins were disappointing with tPA, resulting in material which was less effective at clot lysis; however, fusion proteins of anti-fibrin Fab with uPA were constructed which retained the ability to lyse clots effectively (Runge *et al.*, 1991). Fusion proteins have also been produced in the format of scFv–uPA using a 32 kDa truncated version of uPA, resulting in a single-chain plasminogen activator. This protein demon-

strated increased thrombolytic potency *in vitro* compared to single-chain uPA, but not compared to two-chain uPA (Holvoet *et al.*, 1991).

Bispecific antibodies have also been examined for their ability to target plasminogen activators to clots. Bispecific antibodies have the potential advantage of not causing any damage to the plasminogen activator on conjugation to the antibody or in the construction of fusion proteins. A bispecific antibody with specificity for fibrin and tPA has been produced from a hybrid hybridoma with better thrombolytic activity than tPA alone in a rabbit model of thrombolysis (Kurokawa *et al.*, 1989). The potential to combine thrombolysis with anti-platelet effects in a single agent has also been explored using a bispecific conjugate. The anti-gpIIb/IIIa Fab′, 7E3, described above has been used to prepare a bispecific di-Fab by cross-linking to a Fab′ fragment that recognises tPA (Neblock *et al.*, 1992). The bispecific agent was able to block platelet aggregation as effectively as 7E3 Fab alone, and could recruit tPA to platelets. Such an agent may be useful in preventing reocclusion of vessels as well as hastening reperfusion.

4.5 Disorders of the immune system/inflammatory diseases

4.5.1 The inflammatory response

Inflammation is the body's protective reaction to injury or the presence of foreign material from infection. However, over-activation of the inflammatory response is also associated with a number of human diseases including autoimmune diseases such as rheumatoid arthritis and multiple sclerosis, inflammatory bowel disease, psoriasis, transplant rejection and allergy. The inflammatory response is characterised by increased blood flow to the site, dilation of blood vessels and migration of lymphocytes across the vessel wall into the tissue. The events required to achieve this form a complex series of interactions including both soluble factors such as pro-inflammatory cytokines and the up-regulation of cell adhesion molecules involved in adherence, rolling and migration of leukocytes (reviewed by Springer, 1994; Figure 4.7).

Injury or infection within the tissue causes the release of pro-inflammatory cytokines from macrophages, such as IL-1 and TNF, which activate vascular endothelial cells and induce the up-regulation of the adhesion molecules P-selectin and E-selectin. These selectins are responsible for the initial attachment of leukocytes to the endothelium. Leukocytes have considerable momentum in the circulation and once captured they continue rolling along the vessel wall. The relatively weak attachment allows the leukocytes to respond to chemoattractant signals mediated by a range of substances including leukotriene B_4, PAF, complement fragment C5a, *N*-formylated peptides, MCP-1, MIP-1 and IL-8. Such factors cause up-regulation and activation of adhesion molecules on the leukocyte such as the integrins CD11a/CD18 (LFA-1), CD11b/CD18 (MAC-1) and CD29/CD49d (VLA-4). Such integrins mediate tighter binding to the endothelium through interaction with their receptors CD54 (ICAM-1) and CD106 (VCAM-1). In some cases other adhesion interactions such as L-selectin with MAdCAM-1 may also be involved (Table 4.8). The strongly bound leukocytes are then seen to flatten against the endothelium and migrate through to the injured tissue.

Autoimmune diseases are a result of the immune system reacting to self antigens either systemically or in tissues, leading to chronic inflammation. At the heart of many autoimmune diseases are inappropriately activated T cells which interact with antigen

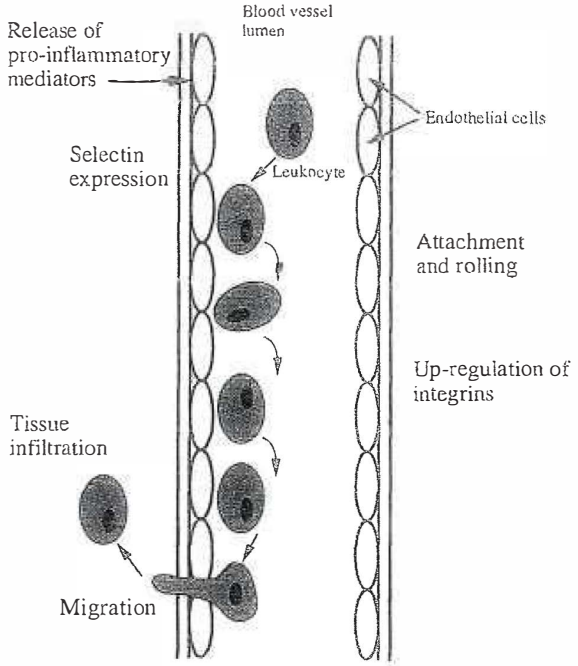


Figure 4.7 Representation of the mechanism of leukocyte-mediated inflammation. The release of pro-inflammatory mediators from tissue macrophages induces the vessel endothelial cells to up-regulate selectins and causes the synthesis of a range of chemokines. Leukocytes are attracted and attach weakly to the endothelium. The flow through the vessel causes rolling of weakly attached leukocytes along the endothelium. As the leukocytes become fully activated, and integrins are up-regulated, the binding becomes stronger and cells flatten and migrate through the endothelial layer to the surrounding tissue. (See text for further details).

Table 4.8 Interactions involved in leukocyte adhesion to the endothelium during inflammation

Endothelium	Leukocyte
P-selectin	PSGL-1, sialyl Lewis^x and others
E-selectin	ESL-1, sialyl Lewis^x and others
CD54 (ICAM-1), CD102 (ICAM-2)	CD11a/CD18 (LFA-1)
CD54 (ICAM-1)	CD11b/CD18 (MAC-1)
CD106 (VCAM-1)	CD49d/CD29 (VLA-4)
GlyCAM-1, CD34	L-selectin
MAdCAM-1	$\alpha 4\beta 7$, L-selectin

presenting cells (APCs) presenting self antigen leading to a number of direct and indirect effects mediated through cytokine release including the infiltration of activated cells described above. Antibodies to T cells and APCs which could block activation have therefore also been considered as potential therapeutics.

The inflammatory process offers many points of potential therapeutic intervention with MAbs, including antibodies to block T cell activation, antibodies to cytokines such as TNF and adhesion molecules such as selectins and integrins on either the endothelium or the leukocyte. These are all blocking interactions and as such are characterised by the need for antibodies which efficiently block ligand binding. These events are largely taking place in the vasculature and hence there is little need for fragments which would penetrate tissues more effectively. The therapeutic reagent is often required to be present for a long period and thus the long circulating half-life of IgG makes this an ideal form for therapy. Neutral isotypes of IgG are also desirable in most cases as triggering effector functions would exacerbate inflammatory processes.

One problem with developing therapy in such situations is that many antibodies to human proteins do not cross-react with the same protein in animal species. Animal model studies are therefore difficult and usually rely on the use of parallel reagents – antibodies to the equivalent animal protein, to generate information on potential efficacy. Parallel reagents may bind to different epitopes with different affinity to the agent intended for clinical use, and thus great care is needed in extrapolating from animal studies to man.

4.5.2 *Blocking inflammatory mediators*

Anti-TNF antibodies in rheumatoid arthritis and inflammatory bowel disease

Blocking of TNF with MAbs has been investigated for the therapy of rheumatoid arthritis and inflammatory bowel disease as well as for septic shock (see Section 4.3.2).

Rheumatoid arthritis (RA) is a common autoimmune disease with a prevalance of about 1% of the population, yet it is still poorly understood in molecular terms. RA is characterised by inflammation of synovial joints which can progress to joint damage and eventually destruction. Affected joints are heavily infiltrated with cells, especially T lymphocytes and macrophages, and elevated TNF levels have been found in joint synovial fluid in more than 50% of patients. TNF has many biological effects, including not only endothelial activation and adhesion molecule expression but also granulocyte activation resulting in increased phagocytosis, degranulation and generation of oxygen radicals and prostaglandin E2, stimulation of fibroblast growth, stimulation of cytokine production, and co-stimulation with IL-2 of T cell proliferation. All of these may be important in the RA disease process.

Antibodies to mouse TNF completely prevent collagen-induced arthritis even after the inflammatory process has begun (Williams *et al.*, 1992), and two recombinant anti-human TNF antibodies, a mouse:human chimeric IgG1 (cA2) and an engineered human IgG4 (CDP571) are being examined in clinical trials for RA. CDP571 has been examined at doses up to 10 mg/kg in a double-blind study in patients with active RA (Rankin *et al.*, 1995). The treatment was well tolerated, and patients treated at the top dose demonstrated significant improvements in disease severity measured through reduction in pain, number of tender and swollen joints and serum markers. Similar encouraging results have been seen with cA2 in both open and double-blind clinical studies with rapid improvement in disease symptoms following treatment (Elliott *et al.*, 1994).

Anti-TNF antibodies are also under investigation for the therapy of inflammatory bowel disease (IBD). IBD consists of two histologically distinct disorders, namely ulcerative colitis and Crohn's disease. Crohn's disease is an inflammatory disease that can

affect any portion of the gastrointestinal tract whereas ulcerative colitis is restricted to the large bowel. TNF is believed to be pivotal to the disease process, particularly in Crohn's disease and both CDP571 and cA2 have been examined in early clinical trials. In a 31-patient double-blind study, a single dose of 5 mg/kg CDP571 was found to reduce disease activity for two weeks, with some patients experiencing sustained remission (Stack *et al.*, 1997). cA2 treatment also resulted in significant improvements in Crohn's disease. In a 108-patient trial, clinical responses were observed in 81% of patients treated with 5 mg/kg compared to 17% in the placebo group (Feldmann *et al.*, 1997). There was apparently little benefit of higher doses, as response rates of 50% and 64% were seen with 10 and 20 mg/kg doses respectively. Beneficial effects of anti-TNF antibody have also been reported in ulcerative colitis, with a consistent reduction in disease activity determined in a phase II trial.

Anti-C5

Activated components of the complement system are potent mediators of inflammation. Antibodies that block the complement cascade at C5, block the generation of the major chemotactic and pro-inflammatory factors C5a and C5b-9. In animal models neutralisation of C5 can both prevent the establishment of arthritis and alleviate established disease (Wang *et al.*, 1995). Similarly, amelioration of disease has been seen in a mouse model for the autoimmune disease systemic lupus erythematosus (Wang *et al.*, 1996). A humanised antibody for such chronic indications has been developed, but for acute conditions where complement mediated damage is indicated, such as myocardial infarction, stroke or cardiopulmonary bypass, it has been suggested that a single-chain Fv may be more suitable due to rapid tissue penetration. An anti-C5 scFv fragment has been shown to inhibit complement activity both *in vitro* and *in vivo*, and is currently under clinical evaluation (Evans *et al.*, 1995).

4.5.3 Blocking adhesive interactions

Blocking adhesion interactions of the vascular endothelium with leukocytes results in anti-inflammatory effects through inhibition of the process of leukocyte adhesion as described above. Antibodies have been described to all of the adhesion molecules known to be involved in the inflammatory process and several of these are under active investigation as anti-inflammatory agents in a number of different diseases.

Binding of leukocytes to E-selectin on the vascular endothelium is an early event in leukocyte recruitment (Springer, 1994). An engineered human antibody to E-selectin has been produced which is capable of blocking leukocyte accumulation into TNF-stimulated baboon skin (Owens and Robinson, 1995). The antibody was designed such that effector functions such as complement binding and Fc receptor binding were not likely to take place, as these might exacerbate the inflammatory process. The relatively neutral isotype, IgG4, was used, and a mutation in the C_{H2} domain, leucine 235 to alanine, was introduced to make the sequence similar to an IgG2. This mutation has been shown to reduce high affinity Fc receptor binding even further (Lund *et al.*, 1991). This antibody is under clinical investigation for treatment of the inflammatory skin disease psoriasis.

Antibodies to the leukocyte integrins, CD11a/CD18 (LFA-1), CD11b/CD18 (MAC-1) and CD49d/CD29 (VLA-4) as well as to receptors on the endothelium such as CD54 (ICAM-1), CD102 (ICAM-2) and CD106 (VCAM-1) (Table 4.8) have been widely

investigated. RA treatment with 5 doses of anti-CD54 was shown to result in clinical improvement in 13 of 23 patients, in 9 of which benefit persisted until day 60 at the end of the study (Kavanaugh *et al.*, 1994). Antibodies to CD18 and CD49d/CD29 have also been shown to improve disease in animal models of arthritis (Issekutz *et al.*, 1996).

The problem of antibodies which do not cross-react with the same protein from animal tissues can be approached in several ways. One approach recently developed for a humanised anti-human CD11a antibody has been the re-engineering of the antibody to allow binding to rhesus monkey CD11a, allowing pre-clinical studies to be carried out (Werther *et al.*, 1996). Alanine scanning mutagenesis was initially carried out and CDR residues important for binding human and rhesus CD11a identified. An engineered variant was then made in which four amino acids in CDR2 of the heavy chain, found to be important in the difference between binding human and rhesus CD11a, were changed. Although it did not prove possible to develop an antibody which bound both human and rhesus CD11a effectively, the resulting antibody was able to bind rhesus CD11a and could be used as a parallel reagent for pre-clinical studies. Anti-CD11a antibodies are currently under development as immunosuppressive therapy of autoimmune diseases and transplant rejection. Several antibodies to CD18 have also been humanised (e.g. Sims *et al.*, 1993) and are currently under evaluation.

Antibodies to CD49d/CD29 (VLA-4) are under development as therapeutic agents for multiple sclerosis (MS). MS is an autoimmune disease characterised by lesions (demyelination) of the central nervous system which lead to the clinical symptoms of weakness of the limbs, paraesthesia and visual disturbances. MS commonly progresses through a series of relapses separated by remissions, to a progressive decline. As disease progresses further symptoms are revealed such as spasticity, fatigue, ataxia, tremor, loss of bladder and bowel control and neuropsychological abnormalities. Lesions in the CNS are caused by activated T cells crossing the blood:brain barrier and initiation of a series of events leading to activation of endothelial cells, recruitment of additional leukocytes, release of pro-inflammatory cytokines and subsequent demyelination of nerve cells. MAb to CD49d has been shown to block binding to CD106 (VCAM-1) and inhibit leukocyte migration. In an animal model of MS, experimental autoimmune encephalomyelitis (EAE), anti-CD49d was able to block onset of the disease (Yednock *et al.*, 1992). In addition, reversal of established EAE has been demonstrated with clearance of leukocytes from the central nervous system (Kent *et al.*, 1995). A humanised form of the antibody has been produced with retention of blocking activity and the ability to reverse active EAE in guinea pigs (Leger *et al.*, 1997). Human IgG4 constant regions were chosen to minimise effector function and maximise half-life. A phase I trial demonstrated that the antibody was well tolerated, and phase II studies are currently in progress.

4.5.4 *Antibodies which directly inhibit T cell activation and proliferation*

The murine IgG2a MAb OKT3 was the first to be licensed for human therapy. It recognises the ε-subunit of the CD3 complex (the T cell receptor) on the surface of T cells and is a potent immunosuppressive agent. As such it has found application for the prevention of acute rejection in kidney, liver, heart and lung transplant patients. OKT3 also has T cell activating properties which are thought to be due to cross-linking of T cells via CD3 to Fc receptor bearing cells leading to massive cytokine release. Cytokine release can cause severe side-effects on treatment with OKT3 and these have limited its use in less

acute conditions such as autoimmune diseases. Also, an immune response is rapidly generated to OKT3 which can block its effectiveness. Several humanised forms of OKT3 have been produced in attempts to overcome immunogenicity which retain full CD3 binding ability and immunosuppressive properties (Adair *et al.*, 1995). Human IgG4 constant regions were used to minimise Fc receptor binding of the humanised antibody, yet Fc receptor mediated cross-linking still occurs. Mutation of a residue in the Fc receptor binding site, leucine 235 to glutamate, resulted in a 100-fold decrease of the affinity of the antibody for Fc receptor bearing cells (Alegre *et al.*, 1992). This resulted in a marked reduction in T cell activation as measured by proliferation and cytokine release, and may offer an attractive profile for human therapy, maintaining strong immunosuppression while minimising toxicity.

CD4 functions as a co-receptor for stabilising the T cell receptor with antigen presented by the MHC class II molecule and is also involved in signal transduction pathway which leads to the activation of a T helper cell. The binding of anti-CD4 antibodies leads to a down-regulation of T cell activity and suppression of immune responses. Antibodies to CD4 have been widely investigated in a number of inflammatory conditions, including RA, MS, psoriasis, diabetes and systemic lupus erythematosus as well as in prevention of transplant rejection. Antibodies that either deplete all CD4 positive cells or merely block the CD4 molecule have been shown to be effective in preventing and reversing the symptoms of autoimmune disease in animal models. Small open trials with two depleting antibodies, one murine and one chimeric, showed promising results, but large randomised placebo-controlled trials in RA did not show any effectiveness of these antibodies (Wendling *et al.*, 1996; van der Lubbe *et al.*, 1995). Non-depleting anti-CD4 antibodies may have an advantage in their ability to temporarily inhibit T cell activation without major inhibition of other immune functions. Several non-depleting antibodies have been humanised (Pulito *et al.*, 1996) and a monkey–human chimeric antibody, termed a primatised antibody, has also been produced (Newman *et al.*, 1992). The primatised antibody may be less immunogenic than mouse:human chimeric antibodies due to the increased homology of monkey and human variable regions. Clinical results with such antibodies seem to be more promising. For example, a double-blind, placebo-controlled study with the primatised antibody in RA resulted in clinical improvement in 77% of patients at the highest dose compared to 17% in the placebo group (Levy *et al.*, 1996). CAMPATH-1H, which recognises the CDw52 antigen, has been described above (Section 4.2.1) as a potential therapeutic antibody in non-Hodgkin lymphoma. As the antigen is also widely expressed on T cells, there has been interest in its use to deplete T cells in vivo for therapy of autoimmune diseases. This humanised antibody binds to an epitope of CDw52 close to the membrane, which may contribute to its effectiveness in mediating cell lysis. Treatment with this antibody leads to severe lymphocyte depletion from the blood followed by a gradual repopulation. Encouraging effects in early RA trials were seen with some significant clinical benefits (Isaacs *et al.*, 1992). However, subsequent larger studies revealed significant toxicity due, at least in part, to cytokine release, suggesting T cell activation takes place (Brett *et al.*, 1996). In addition, treatment resulted in dose-related susceptibility to infection, suggesting lymphocyte depletion with this antibody may be too severe for use in RA.

Interaction between IL-2 and its receptor is required for the activation of cytotoxic T cells and for their proliferation. The IL-2 receptor is made up of three subunits (α, β and γ), and antibodies to both the α and β chains have been investigated as potential immunosuppressive therapies to prevent transplant rejection. IL-2 binds to all three subunits individually but it binds with much higher affinity to the non-covalently associated

combination of all three chains. It was hoped that transplant rejection would be reduced through targeting those T cells involved in immune rejection; antibodies to the IL-2 receptor might be expected to inhibit antigen activated T cell responses without suppressing natural immunity. Initial studies used antibodies to the α chain, also known as the Tac receptor (T activated cell) or CD25. A humanised antibody to the α chain has been produced which retains high antigen-binding affinity and can activate ADCC with human effector cells (Junghans *et al.*, 1990). The humanised antibody was more effective than the murine parent antibody in prolonging survival of monkeys undergoing heart transplants (Brown *et al.*, 1991). However, although graft survival was prolonged without toxic side-effects, antibody treatment was not sufficient alone to prevent eventual rejection. The IL-2 receptor β chain is shared by several cytokine receptors including that for IL-15 which is also involved in T cell proliferation. A humanised antibody to the β chain has also been produced and shown to prolong primate heart transplant survival (Tinubu *et al.*, 1994). Antibody to the β chain showed synergistic effects with antibody to α chain *in vitro*, leading to increased inhibition of T cell proliferation. However, there was no beneficial effect of combined therapy *in vivo* (Tinubu *et al.*, 1994). A humanised bispecific antibody comprising one arm to the α chain and one to the β chain has also been produced (Pilson *et al.*, 1997). This was more active in inhibiting proliferation *in vitro* than the mixture of the two parent antibodies, though *in vivo* studies remain to be carried out. Antibodies to the γ chain may also be attractive as this subunit is also shared by several other cytokine receptors involved in T cell responses, including those for IL-4 and IL-7; however, the effects of blocking the γ chain have not yet been reported.

An attempt to combine the properties of anti-CD3 and anti-IL-2 receptor α chain (CD25) antibodies has been made by the preparation of a bispecific antibody with specificity for both CD3 and CD25 (MacLean *et al.*, 1995). The antibody, produced from a hybrid hybridoma (Section 2.5), was as effective an immunosuppressant as the anti-CD3 alone and more effective than anti-CD25 alone. In addition the toxicity of the anti-CD3 antibody was greatly reduced through the inability to activate T cells via CD3 cross-linking on the surface of the cell. Alternatively, inhibition of IL-2 interaction may be important, although this was less likely as combination of the two original antibodies did not have the same effect. Whether univalent CD3 binding molecules, or $F(ab')_2$ fragments, would be equally effective was not addressed.

Anti-CD25 antibodies have also been investigated as potential therapeutics for graft versus host disease. Graft versus host disease (GvHD) is a life-threatening complication of bone marrow transplantation which is widely used in treatment of leukemia, immunodeficiency and certain other diseases. In GvHD mature T cells carried in the marrow graft recognise the recipient's tissues as foreign and cause immune attack leading to multi-organ damage. GvHD can be reduced by T cell depletion of the marrow before transplantation to the recipient. However, this often leads to increased incidence of graft rejection, relapse of leukemia and delayed reconstitution of the immune system, leading to susceptibility to infection. Antibodies have been investigated as potential treatments to selectively target T cell populations carried in the marrow graft. Treatment with humanised anti-CD25 antibody resulted in improvement in approximately 40% of patients (Anasetti *et al.*, 1994). This relatively poor response may be due to loss of the IL-2 receptor on memory T cells.

CD5 is a marker found on all T cells and a subset of B cells, and antibodies to CD5 have been used in attempts to deplete T cells for therapy of RA and GvHD. Anti-CD5 immunotoxins have been produced in which ricin A-chain was coupled to the antibody as used for anti-cancer immunotoxins (Section 4.2.6). Anti-CD5 immunotoxins were

effectively internalised into T cells and depleted cells effectively *in vitro*. However, little therapeutic benefit was seen in clinical trials for RA, and although short-term benefit has been observed in GvHD, long-term benefit did not result (Martin *et al.*, 1996). Anti-CD3 immunotoxins are also under investigation for GvHD, and in animal models anti-CD3 $F(ab')_2$–ricin A-chain conjugates have proved more effective (Vallera *et al.*, 1995). The use of $F(ab')_2$ conjugates rather than intact IgG removes Fc receptor mediated cross-linking which is at least in part responsible for toxicity of anti-CD3 antibodies as described above. Recombinant immunotoxins consisting of anti-CD3 scFv linked to truncated diphtheria toxin have also been produced, although attempts at therapy of GvHD resulted in only temporary alleviation of symptoms, probably due to the short half-life of this molecule *in vivo* (Vallera *et al.*, 1996).

4.5.5 *Antibody treatment of allergy*

The allergic reaction is an excessive immune response to a common substance which is normally harmless. These substances, termed allergens, include pollen, some foods, dust mites, animal fur and some drugs. In some individuals, termed atopic, who have been sensitised by exposure to an allergen, a second exposure can result in a hypersensitivity reaction. Atopic individuals have high levels of circulating IgE and produce large amounts of allergen-specific IgE in response to the allergen. The clinical symptoms depend on the route of exposure and can include atopic dermatitis or eczema (skin contact), allergic rhinitis or asthma (inhalation) or food allergy (ingestion). The mechanism of allergic reactions is shown in Figure 4.8. On first contact with allergen, allergen-specific IgE is produced which is bound to high-affinity receptors present on mast cells in tissues and on basophils in the circulation. On second exposure, allergen can cross-link two receptor bound IgE molecules leading to signal transduction and degranulation – the release of a range of inflammatory mediators including histamine, cytokines and others, which cause the inflammatory reaction.

MAbs to IgE have been raised which bind to IgE at the Fcε receptor binding site and block binding to basophils and mast cells. Importantly, such antibodies do not bind to IgE bound to the receptor as this would result in cross-linking, signalling and subsequent degranulation (Presta *et al.*, 1994). A humanised version has been produced with high affinity for IgE which is effective at inhibition of allergic reactions *in vitro* (Presta *et al.*, 1993). Allergic reactions may be more difficult to block *in vivo*, but recent evidence suggests that treatment with anti-IgE also results in down-regulation of Fcε receptors on basophils in treated patients, presumably as receptor levels are regulated by levels of IgE (MacGlashan *et al.*, 1997). The combination of reduced IgE levels and reduced receptor levels may allow significant therapeutic effects, and clinical studies in allergic rhinitis and asthma are ongoing.

Other approaches to therapy of allergic diseases include the use of antibodies to specific allergens which might neutralise them in the circulation and prevent IgE receptor cross-linking. Human antibodies have been generated to a number of allergens such as pollen, either from immunised donors or by phage display (De Lalla *et al.*, 1996). Alternatively, recombinant IgA molecules can be constructed which can bind the allergen on mucosal surfaces, such as the nasal linings or the lower airways, and inhibit the entry of the allergen across the mucosal epithelium (Sun *et al.*, 1995). Such approaches suffer from the drawback of being specific to one allergen, and thus of limited general utility.

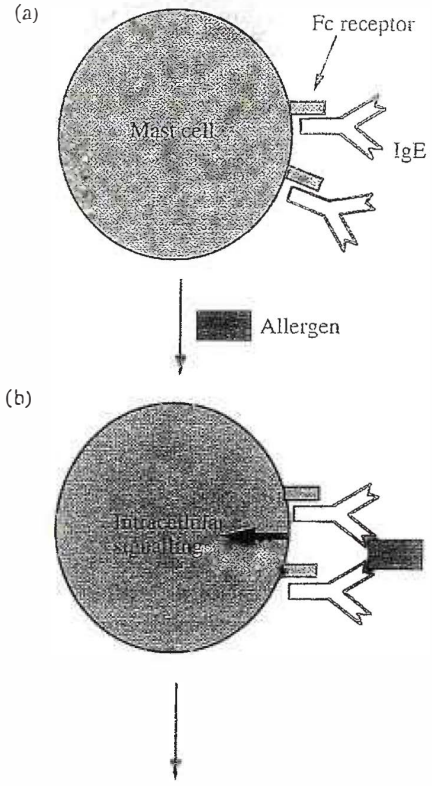


Figure 4.8 Allergen-induced activation of mast cells (see text for details)

A more general approach to asthma therapy may be the neutralisation of key mediators such as IL-5. Eosinophils infiltrate into the lungs during asthma and may cause the damage associated with inflammation of the lung. In animal models antibodies to IL-5 inhibit eosinophil infiltration in the lung and prevent tissue damage and hyperreactivity (Egan *et al.*, 1995). A humanised antibody to IL-5 has been produced which is also effective and is currently under clinical evaluation. Similarly, antibodies to adhesion molecules which are involved in eosinophil migration into the lung may also be effective. Antibodies to CD49d/CD29 (VLA-4) have been shown to inhibit eosinophil accumulation and asthmatic response in a guinea pig model of asthma (Sagara *et al.*, 1997).

5

Production of Monoclonal Antibodies

5.1 Introduction

The ability to apply MAbs in any situation is obviously dependent on the ability to make them! The purpose of this chapter is to review the expression and purification of MAbs, particularly with regard to the ability to manufacture antibodies in an economically viable manner.

The requirements of the production system depend on the form of the antibody required and its intended application. The application will determine the scale of production: for example, MAbs for diagnostic purposes may be required at a scale of tens to hundreds of grams per year, whereas some therapeutic antibodies may be required at tens to hundreds of kilograms per year. In addition, the application will determine the specification for purification of the antibody. The requirement for highly purified antibody is more stringent for antibodies to be used in humans compared to those used for *in vitro* applications. Therefore, it is important at the beginning of a project to develop antibodies with the manufacturing system in mind, including at the design stage where choices can impact on eventual manufacturing processes.

5.2 Expression of antibodies in mammalian cells

Conventional mouse and rat MAbs are expressed from hybridoma cells (see Chapter 1). The selection of the MAb in the first place is often partially dependent on the productivity of the hybridoma for expression and secretion of the antibody. Typical yields of antibody from hybridoma cells are 10–100 $\mu g/10^6$ cells/day leading to accumulation of between 50 and 500 mg per litre depending on the culture medium used and the cell biomass available (Brown *et al.*, 1992). Other important factors for antibody production are the stability of the cell line and the absence of undesirable adventitious agents such as viruses or mycoplasma. Adventitious agents can sometimes be removed by a number of methods followed by repeated cloning and selection, but unstable or low productivity hybridomas are often more problematic. The simplest solution to unstable lines is to clone the antibody genes from the hybridoma and re-express them as a recombinant antibody. Although in

many cases re-expression of antibody genes in myeloma cell lines has led to lower yields than those seen in hybridomas, high level production systems are now available in both myelomas and nonlymphoid cells (see below), and use of such an improved expression system can lead to higher yields of antibody production, from cells which are suitable for large-scale manufacturing.

The expression of IgG is achieved with high efficency in mammalian cells, and mammalian cells are the only system currently used for large-scale production of intact monoclonal antibodies. Mammalian cells have the most appropriate cellular machinery to allow assembly and secretion of IgG as well as the ability to carry out appropriate post-translational modifications, notably glycosylation. As described in Chapter 1, glycosylation of IgG can be important for maintaining the conformation of the C_{H2} region of the antibody, and lack of glycosylation leads to a loss of Fc effector functions (Lund *et al.*, 1990). The type of sugar residues attached is also important to allow maintenance of effector function; for example, the attachment of high-mannose carbohydrate by yeast cells in place of the complex carbohydrate attached by mammalian cells leads to loss of the ability to activate complement (Horwitz *et al.*, 1988). Subtle differences in glycosylation between different types of mammalian cells, or between different types of growth conditions, may also be important in some cases (see below).

Two types of expression system need to be considered, transient expression and stable expression. Cloned DNA can be introduced into the nuclei of mammalian cells by several transfection techniques, where it may persist for several days in a high proportion of cells resulting in transient expression of the genes present on the vector. Alternatively, low-frequency integration events can be selected by use of an appropriate marker gene, during which the vector DNA becomes inserted into random sites within the genome. In this case the DNA is replicated along with the host cell genome, and a permanently transfected stable cell line is produced. A substantial investment in time and effort (usually 2–3 months) is required to produce a stable transfected cell line capable of producing large amounts of antibody. Therefore, transient expression systems are very valuable in allowing the rapid generation of small amounts of recombinant antibody, up to a few milligrams, to allow initial analysis of multiple engineered forms of antibody.

5.2.1 *Transient expression systems*

COS cells are the most commonly used cell type for transient expression of antibody genes. COS cells are derived from a monkey kidney cell line, CV1 transformed with an origin-deficient SV-40 virus (CV1 Origin-deficient SV40). COS cells express the SV40 T antigen which is the only protein required to support SV40 replication in these cells. Therefore a plasmid containing the SV40 origin of replication will be replicated to approx. 10^4–10^5 copies per cell within a few days. This high copy number allows high-level transient expression of genes on the vector. Commonly the genes for light and heavy chain are introduced on separate plasmids, each containing the SV40 origin. This facilitates a range of different combinations of heavy and light chains to be tested with each other, to determine the best combination. For example, several CDR-grafted heavy chains can be tested with the same light chain, and vice versa, to select the best combination of chains for further study. A strong promoter/enhancer is required for efficient expression, such as the adenovirus major late promoter in combination with the SV40 enhancer, or the MIE (major immediate early) promoter/enhancer from human cytomegalovirus (hCMV) (Stephens and Cockett, 1989). Yields of antibody achieved in a few days from transient

expression in COS cells depend on the particular antibody but are usually in the range 0.1–10 μg/ml (Whittle *et al.*, 1987; Emtage *et al.*, unpublished data).

Alternative transient expression systems have also been developed which use the hCMV-MIE promoter in an adenovirus transformed human embryo cell line, 293 cells, together with a transcriptional activator. One of the early adenovirus proteins, E1a, enhances transcription from a number of promoters including hCMV-MIE when coexpressed in the same cell and allows high levels of transient expression: 7–15 μg/ml has been reported (Carter *et al.*, 1992b). Similarly, a Chinese hamster ovary (CHO) cell line has been developed for transient expression by introducing the E1a gene into the genome, resulting in a system capable of similar expression levels (Cockett *et al.*, 1991).

5.2.2 *Stable expression systems*

The most successful cell lines for stable expression of antibodies have been the myeloma lines SP2/0 and NS0, and CHO cells. Most recombinant antibodies produced on a large scale have been produced using myeloma cells. As these are the fusion partners used in hybridoma production, they are known to be capable of secreting large amounts of antibody and they grow well in suspension culture in fermentors. CHO cells have also been developed as a system capable of producing large amounts of antibody; they are readily transfectable and grow well in both attached and suspension culture. In addition, as described below, efficient expression systems based on vector amplification have been established for CHO cells (Bebbington, 1991).

Development of stable cell lines requires the use of a selectable marker to select cells in which the relatively rare event of vector integration into the host cell genome has taken place. Bacterial genes are widely used which are incorporated into the vector with mammalian transcription signals. The *gpt* gene encodes the xanthine–guanine phosphoribosyl transferase enzyme from *E. coli* and can be used to confer resistance to mycophenolic acid. This enzyme allows guanosine monophosphate (GMP) synthesis from xanthine via the salvage pathway when *de novo* GMP synthesis is blocked by mycophenolic acid (Mulligan and Berg, 1981). Alternatively the *neo* gene can be used, as this confers resistance to the antibiotic G418, an analogue of the antibiotics neomycin, kanamycin and gentamycin that block protein synthesis (Southern and Berg, 1982). A third, though less commonly used, system is the *hph* gene from *E. coli* which confers resistance to hygromycin (Gorman, 1990).

The use of these markers in combination allows the selection of cells containing two vectors, and therefore the separate transfection of heavy and light chain genes. This can be useful if several variants of an antibody are to be made such as an IgG isotype series and Fab′ variants. In this case a light chain expressing cell line can be made which can be re-transfected with the heavy chain genes as appropriate (e.g. King *et al.*, 1992a). It should be remembered when using this sequential approach that the light chain must be introduced first. This is because heavy chain alone is retained in the endoplasmic reticulum through association with the heavy chain binding protein, grp78, and accumulation of large amounts of heavy chain in the endoplasmic reticulum is usually toxic to the cell (Hendershot *et al.*, 1988). On the other hand, free light chain is readily secreted by the cell.

Vectors which contain both heavy and light chain on the same plasmid are often preferred, as these offer a more balanced production of heavy and light chain (reviewed by Bebbington, 1991). The two genes should be arranged such that there is not interference

between them as this might lead to an imbalance of chains. In particular transcription from the first promoter may extend beyond the end of the first gene and interfere with transcription initiation at a second promoter downstream. For this reason the light chain is commonly put upstream of the heavy chain, such that excess heavy chain is less likely to be produced. Also a transcriptional terminator can be introduced between the two genes to minimise interference (Stephens *et al.*, 1995).

Immunoglobulin promoters and enhancers have often been used to direct expression in myeloma cells. Early recombinant antibody expression used V regions obtained from hybridomas by genomic cloning which normally cloned the Ig promoter and enhancer at the same time. Use of these to direct expression of recombinant antibody genes usually results in considerably lower expression levels than seen in the parent hybridoma, with yields often in the range 1–5 $\mu g/10^6$ cells/day accumulating antibody from 1–40 milligrams per litre (Sahagan *et al.*, 1986; Crowe *et al.*, 1992). This is because sequences distant from the V region genes are required for efficient expression. Sequences with additional enhancer activity have been identified for both heavy and light chain genes which are several kilobases downstream of the coding regions (Meyer and Neuberger, 1989; Pettersson *et al.*, 1990). Improved expression levels may be achieved by using strong viral or cellular promoters such as hCMV-MIE or the mouse metallothionein promoter (Gillies *et al.*, 1989a).

Expression levels are also highly variable from one transfectant to another, and many cell lines must be screened to identify the best producers. This is probably dependent on the site of integration into the genome and the level of transcription in that region. For example, the identification of a cell line expressing 5 $\mu g/10^6$ cells/day of the humanised antibody CAMPATH–1H required screeening of 700 transfectant clones (Crowe *et al.*, 1992). Attempts have been made to simplify this process using homologous recombination to target gene integration to a highly transcribed region of host DNA. An extensive region of homology between the vector and a sequence on the host cell genome can lead to homologous recombination resulting in the integration of the vector sequences at a defined site. This is a relatively rare event compared to random integration, but nevertheless this approach has resulted in high-level expression in some cases (Yarnold and Fell, 1994).

The most productive cell lines have been produced using selection for gene amplification to obtain increased copy number of vector sequences integrated into the genome. Gene amplification is a relatively common event in mammalian cells, leading to multiple tandem repeats of large regions of a chromosome. These can be selected for by use of a marker such as an essential enzyme which can be inhibited using a selective inhibitor. Two systems have been widely used, based on dihydrofolate reductase (DHFR) and glutamine synthetase (GS), although others such as adenosine deaminase (ADA) are also available. DHFR is a key enzyme in nucleoside biosynthesis which can be inhibited by methotrexate. Following transfection using a vector containing the DHFR gene, selection is achieved by adding methotrexate and resistant colonies, in which the DHFR gene has been amplified (hopefully along with the antibody genes), are isolated. This is a widely used system for expression in CHO cells. For example, chimeric anti-CD20 antibody has been produced at 30 $\mu g/10^6$ cells/day using a single round of amplification in the presence of methotrexate (Reff *et al.*, 1994) and the humanised antibody CAMPATH-1H has been produced at up to 100 $\mu g/10^6$ cells/day, accumulating 200 mg per litre of antibody (Page and Sydenham, 1991). DHFR mediated amplification can also be used in myeloma cells, although because myeloma cells contain endogenous DHFR activity, a gene containing a methotrexate resistant DHFR has been used, and high levels of methotrexate, up to

500 μM, were required to achieve amplification (Dorai and Moore, 1987). Alternatively DHFR expression can be driven from a stronger promoter, allowing selection against the background of endogenous enzyme.

Glutamine synthetase (GS) catalyses the formation of glutamine from glutamate and ammonia and provides the only pathway for the biosynthesis of glutamine. Therefore, in the absence of endogenous glutamine, GS is an essential enzyme. Methionine sulphoximine (MSX) is a selective inhibitor of GS which can be used to select for gene amplification. CHO cells express sufficient GS to grow without added glutamine, but high-expressing cell lines can be isolated using low levels of MSX. Cell lines producing 15 μg/10^6 cells/day, and accumulating 200 mg/litre of chimeric B72.3, have been produced using GS (Bebbington, 1991), and accumulated yields of antibody greater than 500 mg/litre have now been reached using this system in CHO cells (Brown *et al.*, 1992). Myeloma cells do not express sufficient GS of their own and thus have an absolute requirement for glutamine. Therefore when grown in glutamine-free media, GS selection can be used at lower levels of MSX than required for CHO cells. Expression of chimeric B72.3 in NS0 cells reached 10–15 μg/10^6 cells/day, and could accumulate 560 mg/litre (Bebbington *et al.*, 1992). Yields of other recombinant antibodies have also been very high using GS selection in NS0 cells, for example 700 mg/litre of hA33 (King *et al.*, 1995) and over 1 g/litre for hCTM01 (Baker *et al.*, 1994).

Vectors which contain a selectable, amplifiable marker can also be used to select for integration into particularly active transcriptional sites. For example, the GS expression vector has been set up with GS under the control of the relatively weak SV40 promoter. If a low concentration of MSX is added to suppress GS activity during the selection process, then clones in which the GS gene is integrated at a particularly active site are selected. This reduces the number of transfectants isolated, yet increases the proportion which are high-level producers. Characterisation of such a transcriptionally active region of the chromosome in a clone of a high-yielding NS0 cell line selected using GS identified a site in the IgG2a genomic region as the integration site (Hollis and Mark, 1995). Vectors which allowed targeting to this region via homologous recombination were then produced which increased the frequency of integration at this site and thus the frequency of high-level producers.

NS0 cells are particularly well suited to growth in fermentation due to their ability to grow to high cell density in suspension (approx. 10^7 cells/ml). NS0 cells have also been shown to be capable of maintaining stable expression over many generations following transfection and selection using the GS vector system (Brown *et al.*, 1992). The combination of these factors has allowed the development of fed-batch fermentation systems capable of producing titres of antibody routinely over 500 mg/litre and often between 1 and 2 grams per litre (Figure 5.1) (Brown *et al.*, 1992; Bibila and Robinson, 1995). Although more difficult to scale up to fermentation growth, after fermentation development DHFR-amplified CHO lines have also been shown to be capable of producing antibody titres in excess of a gram per litre (Trill *et al.*, 1995).

Comparisons of NS0 and CHO cells for production of antibodies have been carried out by several groups. Expression of cB72.3 IgG4 from both CHO and NS0 cells using the GS system resulted in CHO cells being approximately half as productive as NS0 cells in fed-batch fermentation (Adair *et al.*, 1993). There was no apparent difference in antigen binding or physical properties of the antibodies produced, and pharmacokinetic and biodistribution studies of the antibodies in mice showed identical results. A detailed comparison of the production of an anti-CD4 antibody has been carried out using DHFR amplified CHO cells compared to GS amplified NS0 cells (Peakman *et al.*, 1994). In cell

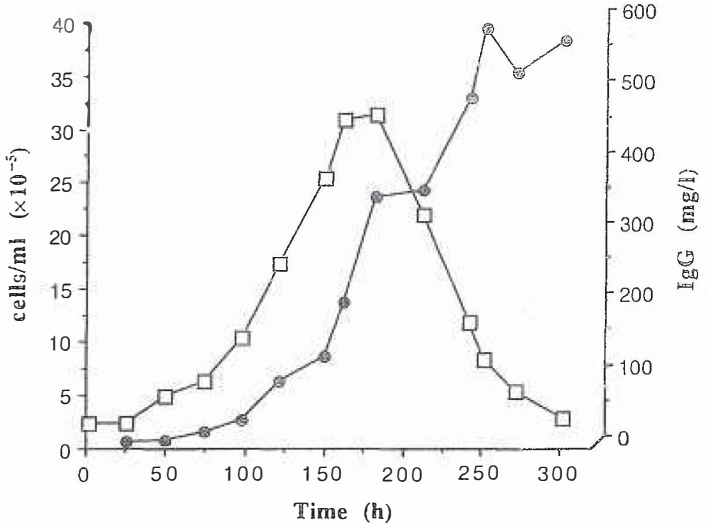


Figure 5.1 Production of chimeric IgG4 in NS0 cells using GS system (see text for details): fed-batch 5 litre fermentations carried out in serum-free air-lift fermenters (adapted from Bebbington *et al.*, 1992)

lines producing equivalent amounts of antibody, the copy number of antibody genes in the CHO cells was significantly higher than the NS0 cells, though mRNA levels produced were similar, suggesting that more active transcriptional sites were targeted using GS. Antibody from both types of cell line was identical in antigen binding and effector function.

One potential difference between cell types is the nature of the glycosylation of the antibody molecule. Subtle differences in the structure of the carbohydrate attached by different cell types, and even between different growth conditions for one cell type, have been observed which may affect the ability of the antibody to elicit effector functions (Lund *et al.*, 1996). In addition, it has been shown in one case for a human IgM that alteration in culture conditions could affect the glycosylation of the antibody leading to alteration of pharmacokinetic properties (Maiorella *et al.*, 1993).

Although there is considerable microheterogeneity in the carbohydrate structure of antibodies produced in mouse myeloma or CHO cells, this is also reflected in the analysis of normal human IgG, and overall the structures are rather similar. One difference is the presence of a bisecting *N*-acetylglucosamine residue between the two 'antennae' of the complex carbohydrate chain which is seen in human IgG and not in mouse or hamster antibodies. As expected, humanised antibodies produced in myeloma cells have sugar groups characteristic of the mouse cell, i.e. without the bisecting *N*-acetylglucosamine (Ip *et al.*, 1994). In addition, myeloma cells have been shown to attach a terminal α(1,3)-galactose residue in some cases, which is not present in human IgG or those produced from CHO cells (Lund *et al.*, 1993). For IgG the carbohydrate is buried within the structure of the paired C_{H2} domains, and alterations may affect biological properties, probably through changes in the conformation and disposition of the domains relative to each other and to the rest of the molecule. At present it is not known whether subtle differences will lead to differences in performance of antibodies in their intended applications,

particularly *in vivo*. Concern has been raised over the presence of terminal α(1,3)-galactose residues in antibodies for human therapy because humans often have pre-existing antibodies which recognise this structure (probably as a result of cross-reacting *E. coli* antigens) (Borrebaeck *et al.*, 1993). However, during studies of the many chimeric and humanised antibodies produced in myeloma cells and administered to humans, an immune response to the constant region has not been detected (see Chapter 2).

5.2.3 *Expression of antibody fragments in mammalian cells*

Co-expression of light chain with Fd or Fd′ results in the secretion of Fab or Fab′ fragments. High yields of Fabs (greater than 100 mg/litre) can generally be achieved by mammalian cell expression in much the same way as IgG expression is achieved. For example, chimeric B72.3 Fab′ has been expressed to 200 mg/litre in CHO cells (King *et al.*, 1994), and humanised A33 Fab′ to 500 mg/litre in NS0 cells (King *et al.*, 1995). In common with bacterial expression (Section 5.3), there is generally little material expressed as $F(ab')_2$ unless hinge modification is undertaken (see Chapter 2). However, oxidation of Fab′ produced in CHO cells to $F(ab')_2$ has been achieved with high yield, demonstrating that the hinge cysteine residues are present (King *et al.*, 1992a).

Smaller antibody fragments such as Fv and scFv have also been expressed in mammalian cells, though in general this has been more difficult, and yields have been lower. Expression of Fv fragments in both myeloma and CHO cells has been achieved (Riechmann *et al.*, 1988b; King *et al.*, 1993). A direct comparison of the same Fv fragment expressed in CHO cells and *E. coli* (see Section 5.3 below) revealed that only 4 mg/litre could be achieved from CHO cells compared to 40 mg/litre in *E. coli* shake-flask culture, rising to 450 mg/litre in fermentation. Similarly, expression of scFv is far less efficient in mammalian cells than in *E. coli*, with yields of up to 10 mg/litre reported in myeloma cells after amplification (Dorai *et al.*, 1994). Mammalian cells may be more useful for scFv-based fusion proteins, particularly if the protein being fused to the scFv is structurally complex or requires glycosylation. Several fusion proteins have been expressed and shown to be active, including scFv-IL2 and scFv-B domain from Staphylococcal protein A (Dorai *et al.*, 1994).

5.3 Expression in *Escherichia coli*

The most widely used and convenient host for gene expression remains the simple prokaryote *Escherichia coli*. *E. coli* has several advantages for gene expression, including the availability of a wide range of expression vectors which can be readily introduced into the cell and tested. *E. coli* is also easy and quick to grow, allowing protein expression to be evaluated rapidly. *E. coli* does, however, suffer two major disadvantages for gene expression; its inability to carry out post-translational modification such as glycosylation, and the tendency of proteins expressed in the cell to accumulate as insoluble aggregates known as inclusion bodies. In many cases, soluble active protein can be recovered from inclusion bodies by *in vitro* protein solubilisation and refolding, although this is a time-consuming and often inefficient process which is not always successful. An alternative approach is to attach a bacterial signal sequence to the gene of interest in an attempt to direct secretion of the expressed protein into the cell periplasm or surrounding

medium. In some cases, including those of antibody fragments, this allows the recovery of soluble active protein directly without the need for complicated refolding protocols.

Intact IgG is not readily produced in soluble form in bacterial expression systems. Although full-length heavy and light chain have been co-expressed intracellularly in *E. coli* and refolded material could be associated to form IgG, yields of assembled IgG were low (Boss *et al.*, 1984; Cabilly *et al.*, 1984). Also, as *E. coli* does not contain the cellular machinery to produce glycosylated proteins, the heavy chain is produced in unglycosylated form. Therefore, research has switched to the investigation of *E. coli* as an expression system for antibody fragments. Rapid progress in this field has taken place since the development of *E. coli* secretion systems which allow soluble, functional antibody fragments to be produced in high yields (Skerra & Pluckthun, 1988; Better *et al.*, 1988), so that now *E. coli* expression has become the system of choice for producing many antibody fragments.

In contrast to expression in mammalian cells, expression of smaller antibody fragments is particularly efficient in *E. coli*, and this has enabled research on novel fragments such as Fv and scFv to progress rapidly. In addition, production of antibody fragments in *E. coli* is relatively inexpensive compared to mammalian cells, leading to more economic manufacture.

5.3.1 *Intracellular expression of antibody fragments in* E. coli

Intracellular expression of antibody fragments can lead to the rapid accumulation of large amounts of insoluble protein and has been used to prepare a range of antibody fragments including Fv, scFv and Fab fragments (Field *et al.*, 1989; Bird *et al.*, 1988; Buchner and Rudolph, 1991). However, refolding of solubilised protein is complex and often has to be developed specifically for individual antibody fragments. Therefore it is often the optimisation of a refolding procedure and the demonstration of a homogeneous, fully functional state for the refolded product which limits the speed and efficiency of the production of antibody fragments by this route. Nevertheless, this method of production can be valuable for antibody fragments for which the secretion route fails, or for the production of fusion proteins which are toxic to the cell in native form, such as immunotoxins.

In common with expression of any foreign gene in *E. coli*, cDNA for the antibody fragment is inserted into the expression cassette of an expression vector. Suitable vectors contain a plasmid origin of replication, an antibiotic selectable marker and an expression cassette comprising a strong promoter and transcriptional terminator separated by a multicloning site with an efficient translational initiation region. Expression of foreign proteins exerts a considerable strain on the cells and leads to strong selection pressure in favour of cells which have lost the expression plasmid (segregational instability) or which have deletions in the expression cassette (structural instability). It is therefore desirable to use a tightly controlled promoter to prevent expression during most of the growth phase of the culture. There is little difference in the transcriptional rate achieved from a number of strong promoters available such as ptac, λpL and T7. The method of transcriptional regulation varies, however, with expression from lac based promoters such as tac induced by IPTG (isopropyl-β-D-thiogalactopyranoside) or by a switch of carbon source to lactose, whereas the λ promoters are controlled by a temperature switch. Translation of mRNA is achieved efficiently through use of an appropriately positioned ribosome binding site sequence which is complementary to ribosomal RNA. A spacing of 6–10 base

pairs between the ribosome binding site and the initiation codon usually allows efficient translation. Many commercially available expression vectors contain these features, and allow the accumulation of large amounts of expressed protein.

Inclusion bodies are readily isolated by cell lysis and centrifugation, and antibody fragments solubilised using high concentrations of denaturants such as guanidine hydrochloride or urea, usually in the presence of reducing agents to ensure that all incorrectly formed disulphide bonds are reduced. Refolding is then achieved by a variety of methods depending on the particular antibody fragment expressed. Many variables need to be examined to identify the optimal procedure for refolding, including solubilisation agent, pH and redox conditions, protein purity and concentration, temperature and rate of change to native conditions. The main problem is preventing the re-aggregation of partially unfolded protein before the native state can be achieved. For some scFvs, renaturation has been achieved by simple dilution or dialysis into a non-denaturing buffer, although the exact procedures required for individual scFvs vary and yields are often low, in the range of 5–15% of the solubilised material (Bird *et al.*, 1988; Huston *et al.*, 1988). The inclusion of a redox coupling system (comprising reduced and oxidised glutathione in a ratio of 10 : 1) to catalyse disulphide interchange may allow improved yields through permitting reshuffling of disulphides in incorrectly folding structures. After optimisation of refolding conditions using such a procedure, native Fab has been recovered with a yield of 40% of the expressed protein (Buchner and Rudolph, 1991). In cases of refolding Fv or Fab′ fragments with two protein chains it has generally been found advantageous to purify the individual chains before attempting final refolding and association to active material (Field *et al.*, 1989; Buchner and Rudolph, 1991).

An alternative method, developed for scFv, has attempted to form disulphide bonds before refolding into the active conformation. The presence of correct disulphides might be expected to improve the recovery of native material, while incorrect disulphide bonds will lead to non-native material. scFv solubilised in sodium lauroylsarcosine was allowed to form disulphide bonds by air oxidation before gradual removal of denaturant, resulting in a yield of 50% of the solubilised protein (Kurucz *et al.*, 1995).

After refolding, the purified material must be characterised to demonstrate that soluble material is in the native state. Soluble, inactive forms of the protein can be produced through alternative protein folding pathways, and may be difficult to separate. In many cases it is difficult to determine the presence of these. For example, when producing an scFv for the first time, a loss of antigen-binding activity is sometimes observed compared to the parent IgG or monomeric antibody fragments produced from the IgG (e.g. Bird *et al.*, 1988). In cases where the scFv has been produced by refolding it is difficult to ascertain whether this is a result of interference in the binding site conformation as a result of the scFv format or a proportion of incorrectly refolded protein.

The uncertainties in refolding efficiencies which can be achieved make it difficult to compare yields achieved using this system. The yield reported may often depend on the procedure used and the amount of development work carried out to achieve high-efficiency refolding. Yields of active protein reported vary from less than a milligram per litre to typically 1–10 mg per litre. It has been reported that higher yields of dsFv can be obtained compared to scFv (Webber *et al.*, 1995). A yield of 1.6 mg/litre has been reported for anti-Tac scFv whereas the same antibody in dsFv format could be refolded with a yield of 7 mg/litre. Similarly, fusion proteins of this antibody with the bacterial toxin PE38 could be produced at 10–40 mg/litre in the scFv format, whereas 30–70 mg/litre was achieved with dsFv-PE38 (Reiter *et al.*, 1994).

5.3.2 Secretion of antibody fragments from E. coli

High-yielding systems have been described for the secretion of Fv, scFv, Fab and other antibody fragments to the *E. coli* periplasm (Skerra and Pluckthun, 1988; Better *et al.*, 1988). Soluble, active protein can often be purified directly from the periplasm and in some cases from the medium. Recovery from the medium is generally a result of accumulation of the antibody fragment in the periplasm causing the cell outer membrane to become leaky, leading to leakage of the antibody fragment into the medium (e.g. King *et al.*, 1993). A signal sequence from an efficiently exported bacterial protein is fused to each antibody gene to direct secretion. There appears to be little difference in the effectiveness of those signal sequences commonly used including pelB, ompA, phoA or stII.

Antibody fragments are efficiently transported across the inner membrane, the signal sequence is efficiently processed to form the mature protein chains and disulphide bonds appear to form efficiently. There is, however, a tendency for some antibody fragments to accumulate as insoluble protein in the periplasm, probably due to the aggregation of folding intermediates (Whitlow and Filpula, 1991). Aggregation of folding intermediates competes with correct folding, and is often the limiting factor in the yield of soluble antibody fragment (Knappik *et al.*, 1993). The extent of this problem varies markedly between fragments of different antibodies and between different fragments of the same antibody. In general, smaller fragments are less prone to insolubility in the periplasm, with Fv-based fragments produced more efficiently than Fab-based fragments of the same antibody. For example, B72.3 Fv could be expressed at 40 mg/litre in soluble form in shake-flask culture whereas a Fab fragment of the same antibody was expressed at 5 mg/litre (King *et al.*, 1992a). The main determinant of insolubility appears to be the primary sequence of the antibody fragment itself. After comparing the sequences of antibody fragments which fold well or poorly, point mutation studies could identify residues which, when changed, improved folding and consequently the yield of soluble protein recovered (Knappik and Pluckthun, 1995). Similar findings have been made with other residues which affect folding pathways (Kipriyanov *et al.*, 1997b).

Several other protein engineering strategies have been used to improve expression of soluble antibody fragments. Switching Fab constant regions has been shown to result in higher yields in some cases (MacKenzie *et al.*, 1994). scFv variants have been engineered by mutation of residues in the hydrophobic patch created in the scFv where the variable/constant domain interface is normally present. Engineered scFv variants were isolated with improved folding properties resulting in higher yields of soluble protein (Nieba *et al.*, 1997). A particularly useful strategy for achieving high-level secretion of active antibody fragments has been exemplified by Carter *et al.* (1992a). A well expressed, soluble humanised Fab′ fragment has been identified which allowed yields of 1–2 grams per litre to be accumulated in fermentation culture. The subsequent CDR-grafting of other antibodies onto the same Fab′ framework region allowed high-level secretion of other binding specificities (Carter *et al.*, 1992a).

Control of cell growth parameters can also aid expression of soluble secreted protein. Growth at lower temperature during gene expression can lead to improved yields, probably due to decreased periplasmic aggregation (Skerra and Pluckthun, 1991). Also, the addition of certain non-metabolisable sugars may be beneficial in reducing aggregation. The yield of soluble scFv was increased over 100-fold by addition of 0.4 M sucrose to the growth medium in shake-flask culture (Kipriyanov *et al.*, 1997a).

The rate of protein synthesis is critical to achieving high yields of soluble secreted protein. This can be modulated either through control of induction of transcription or by

altering translational efficiency. Maximal induction of gene expression may lead to protein synthesis being too rapid to allow the antibody fragment to fold efficiently, leading to increased insolubility through aggregation of rapidly produced folding intermediates. Similarly, translational rate has been demonstrated to be of critical importance for the secretion of heterologous proteins from *E. coli* (Simmons and Yansura, 1996), the optimal rate for secretion of active material being less than the maximum achievable, and variable for different proteins. The optimal yield is therefore often achieved through reducing the rate of protein synthesis by partial induction of expression at a rate which is optimal for secretion and folding. Lower inducer concentrations have been shown to allow higher levels of soluble scFv to be accumulated (Sawyer *et al.*, 1994), and Fab fragments can also be accumulated in higher yields when expression is induced in a controlled fashion over a prolonged period (Yarranton and Mountain, 1992). Accumulation of high levels of foreign protein in the periplasm often leads to the outer membrane becoming leaky, and eventually to cell lysis. Suitable control of induction can also reduce the amount of cell lysis and lead to improved recoveries.

Suitable promoters which allow tight control of expression during cell growth and which can be induced to allow regulation of transcriptional rate are therefore desirable. These include the wild type lac promoter/operator, lacUV5 and the tac promoter, all of which can be induced with the chemical inducer IPTG or by a switch in carbon source to lactose (e.g. Yarranton and Mountain, 1992), the phoA promoter which is induced by phosphate starvation (Carter *et al.*, 1992a), the araB promoter induced by arabinose (Better *et al.*, 1988), and temperature inducible systems such as that described using the trpE promoter controlled by a temperature-induced increase in plasmid copy number titrating out chromosomally expressed trp repressor (Yarranton and Mountain, 1992).

Co-expression of proteins involved in assisting protein folding in *E. coli* has also been investigated in attempts to boost yields of soluble periplasmic protein. The co-expression of the intracellular chaperonins GroES/L had no effect on soluble scFv expression (Ducnas *et al.*, 1994). Similarly, over-expression of the periplasmic proteins proline cis–trans isomerase and dsbA, a protein involved in disulphide bond formation in the periplasm, does not result in improved yields (Knappik *et al.*, 1993). The presence of dsbA is required for assembly of Fab fragments in the periplasm, but does not appear to be limiting. The co-expression of the human enzyme protein disulphide isomerase (PDI) has been shown to improve the yield of a relatively poorly produced Fab′ with γ4 constant regions (Humphreys *et al.*, 1996). The same antibody with γ1 constant regions was produced in higher yield, and in this case there was no effect of PDI co-expression. There are several other periplasmic proteins known to be involved in disulphide formation in *E. coli*, currently dsbA, B, C and D have been described, but as yet studies of their role in folding and assembly of expressed antibody fragments have not been described.

Growth of cells to high cell density in fermentation has led to very high yields of antibody fragments in several cases (Figure 5.2). Fermentation culture also allows greatly improved control of the parameters governing cell physiology such that optimal production levels can be achieved. For example, expression of a chimeric Fab has been improved from 1 mg/litre in shake-flask culture to 500 mg/litre in optimised fermenters (Better *et al.*, 1990), and B72.3 Fv expression from 40 mg/litre to 450 mg/litre (King *et al.*, 1993). Bispecific diabodies have been produced at 935 mg/litre in fermentation culture (Zhu *et al.*, 1996). Yields of over a gram per litre have been observed for humanised Fab′ fragments (Carter *et al.*, 1992a), and a bivalent scFv construct has been expressed at over 3 g/litre in functional form (Horn *et al.*, 1996). In such cases expression of antibody fragments in *E. coli* is a highly competitive option compared to mammalian cell expression.

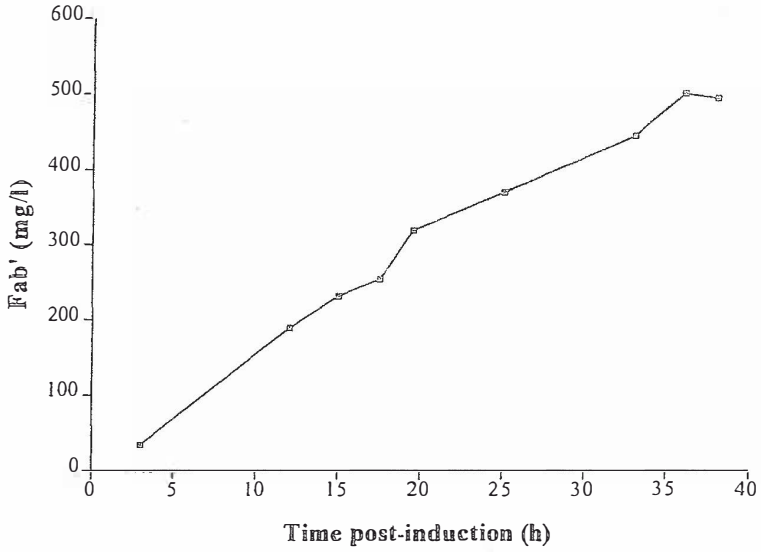


Figure 5.2 Expression of soluble engineered human Fab' in the periplasm of *E. coli*: data from a 1 litre fermentation by courtesy of Neil Weir and Volker Lang (Celltech Therapeutics)

E. coli grows rapidly; transformed cells can be readily produced and cloned leading to shorter development times than those seen with mammalian cells. Fermentation of microbial cells at very large scale is a well-established industrial procedure which is possible at much larger scale than the scale at which mammalian cells can be grown currently. This could lead to significant economic advantages of using *E. coli* for the expression of antibody fragments at the scale required to satisfy large potential markets.

5.4 Expression in other microbial systems

Alternative microbial hosts have been relatively poorly explored for antibody or antibody fragment expression. The Gram-positive bacterium *Bacillus subtilis* has been shown to be capable of expressing scFv fragments of an antibody to digoxin at 5 mg/litre in shake-flask culture (Wu *et al.*, 1993). In contrast to *E. coli*, *B. subtilis* is capable of secreting large amounts of protein directly into the growth medium, although it can also secrete a variety of extracellular proteases which can lead to breakdown of heterologous secreted proteins. Multiple protease deficient strains have been developed to allow accumulation of intact protein which may be isolated simply from the growth medium. Expressed scFv was isolated and purified in a single step by affinity purification from the growth medium (Wu *et al.*, 1993).

Eukaryotic microorganisms have also been investigated. The yeast *Saccharomyces cerevisiae* can synthesise, process and secrete heavy and light chains. Expression of a mouse IgM resulted in the production of functional antibody, though secretion was relatively inefficient with most of the assembled antibody remaining inside the cell (Wood *et al.*, 1985). Heavy and light chains were expressed from different plasmids under the control of the strong phosphoglycerate kinase promoter, and expressed heavy chain was

shown to be glycosylated. Chimeric IgG and Fab have also been expressed in *S. cerevisiae*, resulting in the secretion of antibody and Fab fragment with full antigen binding activity (Horwitz *et al.*, 1988). Secreted levels of antibody were low, with light chain measured at 100 ng/ml and heavy chain at 50–80 ng/ml, 50–70% of which was associated with light chain. The expressed IgG was able to mediate ADCC but could not activate complement, probably as a result of the 'yeast type' carbohydrate added to the constant region.

The methylotrophic yeast *Pichia pastoris* has been developed as a high yielding expression system for several heterologous proteins. *P. pastoris* combines fast growth properties with the general features of eukaryotic protein expression and secretion. The alcohol oxidase gene (AOX1) is used as the basis of expression, and can be used to direct either intracellular expression or secretion. The AOX1 promoter is tightly regulated and drives rapid transcription on addition of methanol, the substrate of alcohol oxidase. Use of the AOX1 promoter therefore allows regulation of expression by supplementation of methanol as the carbon source. Use of this system has allowed secretion of an scFv of a rabbit MAb to more than 100 mg/litre, greater than 100-fold more than could be produced with the same scFv from *E. coli* (Ridder *et al.*, 1995). Two murine scFv fragments have also been expressed, in one case reaching 250 mg/litre, which could be easily recovered from the cell growth medium (Eldin *et al.*, 1997). The expression of other formats of antibody fragments in *P. pastoris* has not yet been reported, but such results with scFv expression merit investigation of this system as a potentially productive alternative.

The filamentous fungus *Trichoderma reesei* has also been investigated for the secretion of antibody fragments (Nyyssonen *et al.*, 1993). This organism has a long history in industrial production of hydrolytic enzymes, which it is capable of secreting at levels of 40 g/litre of culture, and has therefore been investigated as a host for heterologous protein production. The major cellulase, cellobiohydrolase, cbh1 represents 50% of secreted protein and is produced from a single-copy gene, and thus the cbh1 promoter is very strong. This has been used along with the cbh1 signal sequence to direct expression and secretion of a Fab fragment (Nyyssonen *et al.*, 1993). Fab was expressed at only approx. 1 mg/litre, but when a cbh1-Fd fusion protein was co-expressed with light chain, secreted levels reached 150 mg/litre. A *T. reesei* protease was able to cleave some of the cbh1-Fd fusion protein in the culture medium, but resulted in only small amounts of assembled Fab.

5.5 Expression in plants

Transgenic plants have been shown to be capable of producing and assembling both antibody fragments and intact antibodies, including the complex secretory IgA molecule. Plant systems are currently being investigated both as a potential source of large quantities of antibodies for diagnostic or therapeutic application and for potential *in situ* uses within the plant itself (Franken *et al.*, 1997). Such plant-derived antibodies, often termed 'plantibodies', have the potential to be manufactured very cheaply as plant agriculture is the most economical system for production of large quantities of biomass. It has been estimated that expression of antibody in soybean at a level of 1% of total protein (a level already achieved in some cases – see below) could result in production of antibody at a cost of $100 per kilogram (Hiatt, 1990). However, estimation of total cost is difficult as extraction and purification costs following harvest are likely to be significantly higher than other types of production techniques.

The first report of expression of functional antibody from plant cells described the production of intact murine IgG at a level of 1.3% of total protein (Hiatt *et al.*, 1989). The

most common method of transforming plant cells is to introduce the genes of interest into the Ti plasmid of *Agrobacterium tumefaciens*. Recombinant bacteria can then be used to infect plant cells, introducing the gene of interest. Tobacco plant (*Nicotiana*) cells are often used due to their relative ease of transformation using this system. Heavy and light chain cDNAs including the signal sequences were introduced into individual leaf segments and used to regenerate mature plants. The plants expressing heavy and light chain were then crossed and progeny producing assembled antibody isolated (Hiatt *et al.*, 1989). The signal sequences were required for production of functional assembled antibody, and resulted in accumulation of antibody in the intercellular spaces, which is a stable environment in plants and may facilitate antibody isolation. In plant cell suspension cultures the antibody was produced at approx. 20 mg/l in the culture medium.

Both scFv and Fab′ fragments have also been produced in plants at levels similar to those achieved with intact IgGs (Owen *et al.*, 1992; DeNeve *et al.*, 1993). It has also proved possible to produce secretory IgA in tobacco plants which requires the co-expression of four protein chains: heavy chain, light chain, joining chain and secretory component (Ma *et al.*, 1995). Each protein chain was introduced into separate plants and a series of crosses carried out to produce plants capable of producing assembled functional sIgA. The antibody used recognises *Streptococcus mutans*, which is largely responsible for dental caries in man. Oral application of the antibody leads to cross-linking of bacteria and protects against dental caries. Secretory IgA is more stable at mucosal surfaces than IgG and may be a suitable molecule for passive immunotherapy under such conditions. The production of such antibodies in plants offers the intriguing prospect of an oral delivery route in edible plant tissue. If expressed in edible plant tissue, a food formulation of antibody may be possible to exert a therapeutic or prophylactic effect in the mouth or gastrointestinal tract, avoiding the need for isolation and purification of antibody (Ma and Hein, 1995).

An alternative plant source of antibody may be seeds. scFv fragments have been shown to be capable of production in seeds at 0.67% of total seed protein (Fiedler and Conrad, 1995). This also appears to be a stable method of storage of scFv, as seeds containing scFv were stable for a year at room temperature without any loss of scFv protein or antigen-binding ability.

The production of antibodies for therapeutic application in plants has not yet been attempted and thus the regulatory implications are at present unknown. Carbohydrate of plant-produced antibodies is different to that attached to antibodies by mammalian cells, as is also the case with several other expression systems as described above. Some sugar residues may be unique to plants and lead to immunogenicity of administered antibody. However, antigen-binding fragments such as Fab and scFv are not normally glycosylated and their use may overcome such concerns. Plant-specific contaminants such as secondary metabolites may be a cause for concern and would need to be demonstrated to be removed from an antibody product. On the other hand, plant-derived antibodies are not likely to be contaminated with animal viruses or other animal-derived agents. It will be interesting to monitor the progress of transgenic plants as an antibody production system over the next few years.

5.6 Production in transgenic animals

The production of MAbs in the milk of transgenic animals is also a potentially low-cost route to the production of antibodies which is currently under development. Genes are

introduced into the fertilised egg by microinjection. A number of heterologous proteins have been expressed in the milk of lactating transgenic animals and current research is engaged in the development of such systems to generate proteins for therapeutic use (Colman, 1996). Transgenic sheep, goats and cows are being investigated for their ability to produce large volumes of milk containing the expressed protein. High-level expression of functional antibody in transgenic goats has been reported, with levels of 10 g/litre produced in some animals (Parkinson, 1995); however, full details have not yet been published. One drawback of this approach is the long lag time required to generate a reasonable number of mature animals capable of high-level expression of foreign protein.

5.7 Expression in insect cells

Insect cell expression systems have been developed using baculovirus expression vectors which allow rapid production of recombinant proteins. High-efficiency expression and secretion can be achieved in a cost-effective manner using the Sf9 insect cell line. Expression vectors have been developed using the promoter from the baculovirus polyhedrin gene and insect cells transfected using this system are capable of signal peptide cleavage, glycosylation and efficient secretion from the cell. The baculovirus expression system produces a lytic infection which kills the cells after a short time and therefore production levels are relatively low, typically in the range 5–10 mg/l (Hu *et al.*, 1995). Advantages of insect cell expression compared to mammalian cells include the absence of mammalian viruses and any possible mammalian DNA encoding oncogenes.

Antibodies have been expressed in insect cells by several groups. Heavy and light chains are efficiently processed and assembled into intact antibody when expressed by coinfection with two recombinat viruses or when heavy and light chain are introduced on a dual transfer vector containing the two transcription units in opposite orientations (Hasemann and Capra, 1990; Zu Pulitz *et al.*, 1990). Insect cell-specific carbohydrate is added to the antibody in place of that seen from hybridoma cells, and effector functions of antibodies produced in this system have not always been maintained (Poul *et al.*, 1995). Nevertheless, expression in insect cells can be useful for rapid generation of milligram quantities of antibody, particularly in laboratories that have the system set up for other purposes.

Murine, chimeric and humanised IgG have been expressed in insect cells, as has functional human IgA (Carayannopoulos *et al.*, 1994). scFv fragments have also been expressed at 32 mg/litre in insect cell fermentation culture (Kretzschmar *et al.*, 1996). scFv from insect cells could be easily purified by a two-step method without the need for affinity purification.

5.8 Production of monoclonal antibodies – cell culture

MAbs from hybridoma cells or recombinant antibodies from alternative mammalian cell types can be grown using a number of different methods. The scale and characterisation of the production process is determined by the requirements of the intended application. For example, the production of MAbs for use in humans requires stringent control of the entire production process from preparation of cell banks to characterisation of the purified product, whereas small-scale preparation of antibody for laboratory use presents a different range of problems, such as requiring rapid production often of many different antibodies for evaluation purposes.

Cell culture at small scale (up to a few litres) can produce reasonable amounts of material from cells grown in either attached or suspension culture. More concentrated antibody can be derived by growing hybridoma cells as ascites tumours in mice or rats (Brodeur and Tsang, 1986). Typically antibody is produced rapidly within 1–2 weeks following hybridoma inoculation into mice at concentrations of 1–10 mg/ml. However, the use of animals for antibody production in this way is difficult to justify on ethical grounds when alternative methods are available, and it is prohibited or restricted in several countries. There is also a risk of introducing adventitious agents from the animal into the preparation of antibody. Many *in vitro* cell culture techniques are available including the use of attached cells in roller bottles, shake-flask or spinner cultures of cells in suspension, and a range of bioreactors at either small or large scale, such as hollow fibre perfusion systems and air-lift fermentors (Jackson *et al.*, 1996; Birch *et al.*, 1987). Bioreactors can also be scaled up for industrial manufacture, and both airlift fermentors and perfusion systems are currently used for commercial scale manufacture of MAbs with batch sizes of several thousand litres.

Process development to maximise the productivity of a cell line in fermentation becomes crucial to allow economic manufacture. Such development requires optimisation of culture media, nutrient supplementation throughout the fermentation and key enviromental parameters such as pH, dissolved oxygen concentration and temperature (Brown *et al.*, 1992; Bibila and Robinson, 1995). The degree of process development undertaken in the manufacture of a particular MAb will depend on the balance between the requirement to develop the best process at the lowest cost and the time available.

5.9 Purification of monoclonal antibodies

5.9.1 Purification of IgG

For most applications purified MAbs are required, and for therapeutic puposes they must meet a very high purity specification as described below. Purification of MAbs uses the molecular properties of the immunoglobulin molecule in the same way as any protein purification process relies on the molecular properties of the protein of interest. Affinity chromatography is commonly used because of the high degree of purification which can be achieved in one step. This can be based on the specific binding properties of the immunoglobulin either through Fc-binding ligands such as the bacterial immunoglobulin-binding proteins, Staphylococcal protein A and Streptococcal protein G, or using the antigen-binding site itself through binding to immobilised antigen. Alternatively a number of lower resolution methods can be used either alone or in combination, such as ion-exchange purification based on overall molecular charge, hydrophobic interaction chromatography based on hydrophobicity and gel filtration based on the size of the molecule.

The design of the purification process can be considered in two stages: sample preparation, also known as primary recovery, and purification. After production in cell culture the antibody sample must be prepared by removal of the cells and particulate matter, typically by centrifugation and filtration. At a small scale such steps are relatively trivial and easily accomplished using standard laboratory equipment. At large scale such steps may represent more of a challenge, requiring the use of continuous centrifuges which are not capable of delivering the high g forces obtained in laboratory equipment. Precipitation of antibodies, for example, with ammonium sulphate may also be used in primary recovery. This allows concentration of the antibody in the sample as well as a partial

Table 5.1 Binding of immunoglobulin G subclasses to Staphylococcal protein A and Streptococcal protein G

Species	IgG subclass	Protein A	Protein G
Mouse	IgG1	+	+
	IgG2a	++	++
	IgG2b	++	++
	IgG3	++	++
Rat	IgG1	+	+
	IgG2a	—	++
	IgG2b	—	+
	IgG2c	++	++
Human	IgG1	++	++
	IgG2	++	++
	IgG3	—	++
	IgG4	++	++

purification and is often used on a small scale. This method is difficult to use on a large scale because of the requirement for handling and centrifuging the volumes involved. Before purification, the sample may also need to be in solution under certain conditions of pH or ionic strength, depending on the particular method to be used, and therefore the solution may need to be adjusted (conditioned) before purification takes place. Purification is usually achieved through column chromatography using one or more steps. For most laboratory purposes a single step of affinity chromatography is usually sufficient, typically producing MAb at >90% purity. For therapeutic purposes it is necessary to improve the purity to >95% and this is commonly achieved by a combination of affinity chromatography with other techniques such as ion-exchange chromatography or gel filtration.

The best place to start in designing a small-scale purification scheme for IgG is with the bacterial immunoglobulin binding proteins. Both protein A from *Staphylococcus aureus* and protein G from group C and G Streptococci bind to the Fc region of antibodies and are widely available immobilised to suitable solid phases for chromatography applications. Protein G is most useful in recombinant form in which the albumin binding domains, also present on natural protein G, have been removed. Different subclasses of antibody bind to protein A and protein G with different affinities, and thus the subclass is of critical importance in selection of the purification method (Table 5.1). The binding sites for both protein A and protein G for IgG Fc have been defined, and mapped to overlapping sites at the C_{H2}/C_{H3} domain interface (Deisenhofer, 1981; Sauer-Ericksson *et al.*, 1995).

Clarified culture supernatant is applied directly to a column of protein A or protein G under conditions in which the immunoglobulin will bind. Antibodies which bind strongly to protein A or G often require little pre-conditioning of clarified culture supernatant, whereas those which bind weakly may require special buffer conditions to promote binding. For example, mouse IgG1 requires the inclusion of 4 M sodium chloride in the sample to allow binding to protein A. After washing, antibody is eluted from the column, commonly using a decrease in pH (Figure 5.3). Some antibodies may be unstable at low pH and thus require the use of alternative eluents such as chaotropic ions or agents such

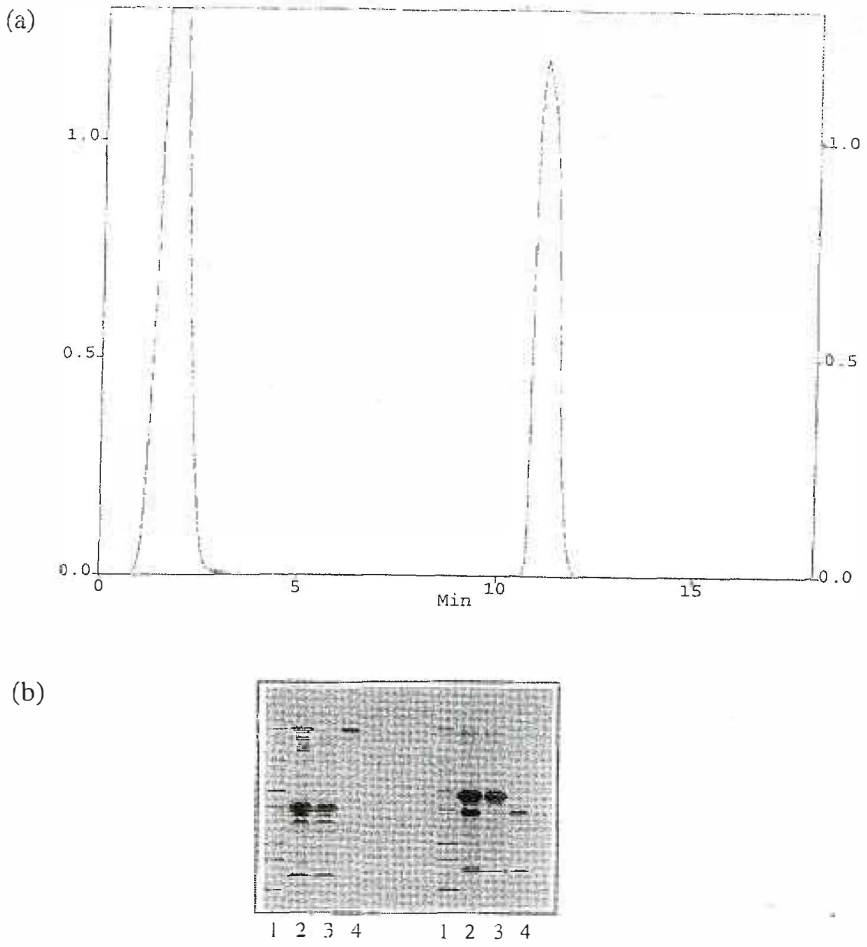


Figure 5.3 Purification of engineered human IgG1 from NS0 cell culture supernatant. Cell culture supernatant was adjusted to pH8 and applied to a column of Poros 50A, immobilised protein A (Perseptive Biosystems) at 600 cm/h which had been pre-equilibrated with 50 mM glycine/glycinate buffer pH8.8. After washing with equilibration buffer, IgG was eluted with 0.1 M glycine–HC1 pH3.5. (a) Absorbance at 280 nm, showing peaks due to non-bound protein (flow through) and eluted IgG. (b) SDS-PAGE under non-reducing and reducing conditions. Lane 1, molecular weight markers; lane 2, culture supernatant sample applied to column; lane 3, flow through peak; lane 4, eluted IgG.

as ethylene glycol (Bywater *et al.*, 1983). Most MAbs will be prepared from clonal cell lines and thus there is little contamination with other rodent or human antibodies when prepared in cell culture. Therefore preparation of total immunoglobulin will allow a high degree of purity to be obtained. Some bovine IgG may be present, however, from the bovine serum often used in cell culture techniques. This can also be removed using bacterial immunoglobulin binding proteins, depending on the subclass of the antibody.

Removal is straightforward from subclasses of antibody which bind strongly to protein A, but more difficult from weakly binding antibodies such as mouse IgG1. In this case it may be necessary to include a separate step specifically to remove bovine IgG, such as ion-exchange chromatography, or the use of anti-bovine IgG affinity chromatography. Purification of antibodies from ascitic fluid may be more difficult for two principal reasons. Firstly, ascitic fluid contains a high proportion of cells and particulate material and also lipid which must be removed by high speed centrifugation, or other means, before purification. Secondly, there is considerable contamination with host mouse IgG which may be difficult to remove from the MAb.

Alternatively, affinity chromatography using antigen can be considered. If sufficient antigen is available, antigen can be immobilised onto a solid phase and used to purify the antibody using immunoaffinity chromatography in the reverse of the immunopurification operation described in Section 3.7. Immobilised antigen can result in a high degree of purification and is one of the few techniques capable of resolving mixtures where several antibodies may be present, such as during the preparation of bispecific antibodies. However, antigen is rarely available in sufficient quantity for more than small-scale operations, and elution conditions required are often harsh for high-affinity antibodies which can lead to losses in affinity of the isolated antibody.

Although the use of protein A or protein G is the first choice purification method in most cases, in others alternative methods may be preferable. The main reasons for choosing an alternative method are the instability of the antibody to elution conditions, and cost. In practice it is usually possible to develop elution conditions which are suitable for retention of activity of the antibody, and antibodies for which this cannot be achieved are rare. Affinity materials using protein A or G are relatively expensive, however, with other materials such as ion-exchange resins being an order of magnitude less expensive in many cases. There is often, therefore, commercial pressure to remove affinity steps from a purification process to minimise costs. However, it is important to consider the cost of the affinity matrix compared to the time and cost of several steps (with consequent yield losses) which might be needed to replace it. It may also be possible to reduce costs through use of repeated cycles on a relatively small column.

Alternatives to affinity chromatography rely on conventional purification techniques. Ion-exchange purification of antibody is a useful technique either as an additional step to affinity chromatography or as an alternative. The isoelectric point of MAbs varies widely from antibody to antibody, in my experience from 4.5 to 8.5, and thus a general method for purification cannot be universally applied. Anion exchange is particularly useful as a second step in purification protocols after affinity chromatography and can lead to the generation of highly purified product. In addition DNA binds very strongly to anion exchange materials and the inclusion of anion exchange steps in purification schemes is useful for therapeutic antibodies, as separation of any residual DNA in the preparation will be achieved.

As a first line antibody purification step anion exchange is often problematical, due to binding of serum albumin which is present in most cell culture fluid at high concentration, and which reduces the capacity of the anion exchange material to bind antibody. In addition, phenol red which is added to small-scale cell cultures as a pH indicator also binds very strongly. Cation exchange has been found to be more suitable for antibody purification, although this is highly dependent on the pI of the particular antibody. In some cases extensive conditioning of the material is needed prior to purification to reduce pH and/or conductivity, often resulting in the need for considerable dilution or the use of diafiltration. The purity of the antibody is not as high as that which is obtained from

affinity chromatography, but the use of high-resolution anion exchange materials which are now available from several suppliers may still allow acceptable purity to be reached in one or two steps (Carlsson *et al.*, 1985). Mixed-mode ion-exchange is also a particularly useful technique. Mixed-mode ion-exchangers, such as Bakerbond Abx (J.T. Baker), contain both weakly anionic and cationic groups. However, over the pH range used for antibody purification (pH5–7 in most cases) the operation of such materials resembles cation exchange. Antibody purity of 70–95% is readily achievable using an Abx mixed-mode ion-exchanger, and mixtures of antibodies may also be resolvable (Ross *et al.*, 1987). Hydroxylapatite, a form of calcium phosphate, is thought to operate along similar principles and can also result in high-purity antibodies after a single step (Stanker *et al.*, 1985). Hydroxylapatite is more difficult to use, however, as lower flow rates must be used and the resin has lower capacity than many other materials. Ion-exchange methods operate without the need for harsh conditions and are therefore particularly useful for antibodies which are sensitive to low pH or other reagents required for elution from affinity chromatography columns.

Hydrophobic interaction chromatography is a commonly used protein purification procedure which separates proteins on the basis of hydrophobicity. The resolution achievable with this technique is relatively low, and therefore it is useful only in combination with purification steps based on other modalities such as ion-exchange. The combination of ion-exchange with hydrophobic interaction chromatography is useful, however, and can result in the generation of highly purified material, equivalent to affinity purified antibody, without the high cost of expensive affinity resins. One problem with some MAbs is precipitation during sample preparation. Binding to hydrophobic interaction columns requires the use of high concentrations of salts such as ammonium sulphate which can cause antibody precipitation and in some cases loss of antigen-binding activity.

Gel filtration chromatography is also widely used as a final clean-up step following purification by affinity chromatography or other methods. It is particularly useful in removing antibody aggregates which are a common problem in antibody preparations. However, gel filtration is time-consuming and difficult to scale-up, and thus processes which can avoid the use of such steps are preferred at large scale.

Thiophilic adsorption of MAbs has also been developed as a method capable of single-step purification (Belew *et al.*, 1987). In this method high salt concentrations promote binding of a wide range of antibody types to a thiophilic resin produced by coupling mercaptoethanol to divinylsulphone activated agarose. Although similar to hydrophobic interaction chromatography in being salt-promoted, binding to this material is believed to be by a different, though unknown, mechanism. Elution from the adsorbent is achieved by reduction of the salt concentration. The preferential affinity of this material for immunoglobulins has allowed mouse MAbs to be obtained directly from culture medium or ascites fluid at purity levels similar to those achieved with ion-exchange chromatography methods.

One approach to simplify purification processes is to remove the need for primary recovery through the use of fluidised, or expanded, bed adsorption. The use of such techniques allows the application of whole cell fermentation harvest directly onto the expanded bed column without removal of cells and particulates, thus avoiding any yield loss due to centrifugation and filtration of the sample. Expanded bed chromatography operates through the use of a dense solid phase which can be suspended in solution, or expanded, such that particulate matter can flow through the column while the antibody binds to the dense particles and remains within the column. The column material is then packed into a conventional packed bed by reversing the flow, allowing elution of bound

material in a sharp peak. Both ion-exchange and protein A materials are available which allow efficient purification of MAbs without clarification (Thommes *et al.*, 1995; Chaplin, 1996). Purification of a murine IgG2a from hybridoma culture was achieved by direct application of the culture to an expanded bed column using immobilised protein A. Material was recovered at a high yield at >95% purity with less than 0.3% aggregates in this single step (Chaplin, 1996). This technology can result in a significant improvement in the efficiency of large-scale operations and is likely to become increasingly important over the next few years.

5.9.2 Purification of IgM

The purification of MAbs of the IgM class is more difficult than IgG, substantially due to their lower stability. However, a number of methods have been devised to obtain pure material, including to the exacting standards required for clinical trials. Ion-exchange chromatography using mixed-mode ion exchangers such as Abx is an efficient method although care must be taken to avoid antibody precipitation at low ionic strength (Chen and Epstein, 1988). The high molecular weight of IgM at 900 kDa also means that gel filtration is an efficient purification method. Few contaminants have similar sizes and thus pure material can be obtained. Selective precipitation can also be used to prepare IgM in its pentameric form, taking advantage of the precipitation of IgM at low ionic strength (Vollmers *et al.*, 1996). In this method culture medium is simply concentrated and dialysed against distilled water, resulting in the selective precipitation of IgM. However, the efficiency of recovery using this precipitation process may be low and a more reliable method may be to use ammonium sulphate precipitation followed by gel filtration.

An affinity chromatography method for purification of IgM is also available based on mannan binding protein. Mannan binding protein (MBP) is a mannose and *N*-acetylglucosamine a specific lectin found in mammalian sera which is structurally similar to the complement component C1q, and binds to IgM and not IgG. Immobilised MBP has been developed in kit form for the purification of IgM (Nevens *et al.*, 1992). Binding to MBP is calcium-dependent and thus elution can be achieved under gentle conditions by use of EDTA. Also, some IgM molecules are capable of purification through the Fab region binding sites on protein A and protein L (see below).

The human monoclonal IgM COU-1 has been purified for clinical use using a four-step chromatographic procedure (Tomoe *et al.*, 1997). In this case antibody was purified from hybridoma cell supernatant using sequential chromatography with hydroxylapatite, hydrophobic interaction, cation exchange and anion exchange. The antibody was substantially pure with regard to protein after step 3 but the fourth step was required to remove contaminating DNA to acceptable levels for human administration. An overall yield of 57% was reported.

5.9.3 Purification of monoclonal antibody fragments

The purification of MAb fragments uses a range of techniques, many of which are similar to those used for IgG purification. The purification required depends on the source of the antibody fragment, with those prepared by digestion of IgG having different requirements to recombinant antibody fragments expressed by mammalian cells or *E. coli*.

After digestion of purified IgG the objective of the purification scheme is to separate the required antibody Fab or $F(ab')_2$ fragment away from any undigested IgG, other

antibody fragments and the enzyme used for digestion. Removal of the enzyme is simplest when it is used in immobilised form linked to agarose beads which can be simply removed by centrifugation or filtration at the end of the digest period. Pepsin and papain are readily available in immobilised form, though the use of alternative enzymes such as bromelain may require use of the enzyme in solution. In some cases a simple negative purification can be successfully carried out using protein A or protein G to bind undigested IgG and Fc fragments and collecting Fab or $F(ab')_2$ in the flow-through. This often results in Fab or $F(ab')_2$ which is pure enough for many purposes. However, some Fab and $F(ab')_2$ fragments bind to protein A and/or protein G (see below) such that this strategy can be unsuccessful. Also a mixture of Fab, $F(ab')_2$ and smaller fragments may be present in the flow through from the column, requiring a further purification step to be carried out. Ion-exchange chromatography is usually the most reliable purification method, though some optimisation of the method may be required for each individual antibody. Both anion exchange and cation exchange may be useful depending on the particular antibody and fragment (e.g. Mhatre *et al.*, 1995). Ion-exchange also has the advantage of separating soluble enzymes used in the preparation. Gel filtration is also a useful method at small scale, with good separation of Fab and $F(ab')_2$ from IgG and small fragments achieved with either HPLC or conventional gel filtration columns. Separation of Fc fragments from Fab will not be achieved, however, and if Fc is present, an affinity chromatography or ion-exchange step will be required to remove it.

Purification of recombinant antibody fragments is more of a challenge, with the antibody fragment usually present in a complex mixture of proteins either from mammalian cell supernatant or from an *E. coli* extract. Some Fab and $F(ab')_2$ fragments, including several human and humanised fragments, are capable of binding to low-affinity binding sites on protein A and/or protein G. The successful purification of recombinant humanised Fab's expressed in both mammalian cells and *E. coli* has been demonstrated using immobilised protein A (Carter et *al.*, 1992b; King *et al.*, 1995). Similarly, protein G can be successfully used for Fab purification from either of these expression systems (Proudfoot *et al.*, 1992; King *et al.*, unpublished data). Protein A binds to Fab's, particularly of the human gene family V_{H3}, and the sequences involved in binding have been localised to the second CDR region and framework regions 1 and 3 in the heavy chain V domain (Potter *et al.*, 1996). Some Fab fragments from other species including mouse, rabbit and guinea pig have also been shown to bind to protein A (Young *et al.*, 1984). The region of Fab interaction with protein G has been defined by structural studies and shown to lie within the C_{H1} domain of the heavy chain (Derrick and Wigley, 1992). Approximately 50% of human Fab fragments are believed to bind to protein G (Erntell *et al.*, 1983). At present it is difficult to determine which Fab fragments will bind to these proteins, which would greatly simplify purification, except by trial and error. In the author's laboratory we routinely test all newly expressed Fab fragments for their ability to bind to protein A and protein G before beginning purification studies.

For Fab fragments which do not bind protein A or G, an alternative bacterial Ig binding protein may be used, protein L. Protein L from *Peptostreptococcus magnus* binds specifically to the variable region of light chains and can be used for the purification of many human and mouse IgG, IgM and IgA antibodies and Fab fragments (Nilson *et al.*, 1993). In addition, humanised antibodies could be produced using light chain variable region frameworks designed to bind to protein L. Protein L has also been shown to be useful for the purification of scFv fragments of several, though not all, antibody types. Human light chain subtypes of the $\kappa 1$, $\lambda 2$ and $\lambda 3$ families were found to bind to protein L, whereas $\kappa 4$ and $\lambda 1$ scFvs were unable to bind (Akerstrom *et al.*, 1994). As expected,

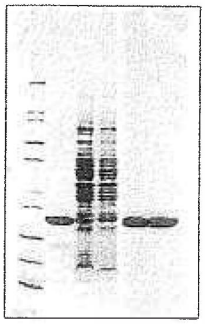


Figure 5.4 Reducing SDS-PAGE analysis of the purification of engineered human Fab′ from *E. coli* cell extract using Streamline A (Pharmacia) in expanded bed mode: lane 1, molecular weight markers; lane 2, Fab′ standard; lanes 3 and 4, cell extract; lanes 5 and 6, eluted Fab′

many scFv fragments containing VH3 heavy chain domains were also able to be purified using protein A. Novel Ig binding proteins have also been produced by gene fusion techniques in attempts to widen the number of Ig molecules which can be purified using a single material. A fusion of protein G and protein L, protein LG, has been prepared which can be used to purify a wide range of MAbs and fragments (Vola *et al.*, 1995). It is likely that the use of such proteins for small-scale purification of antibodies and their fragments will become more widespread as they become increasingly commercially available.

The use of immobilised protein A in expanded bed mode is particularly valuable for the purification of Fab′ fragments from *E. coli*. It is possible to apply crude *E. coli* extract to an expanded bed column and recover pure Fab′ in a single step (Figure 5.4) (Chapman *et al.*, 1996). Extracts from *E. coli* often contain a large amount of particulate material, and although this can be removed by a combination of centrifugation and filtration, this is often at risk of a large yield loss. For Fab′ and other antibody fragments which do not bind to protein A, expanded bed absorption using an ion-exchange material is a valuable first step in the purification process.

Ion-exchange chromatography can also be used to purify recombinant antibody fragments, though this requires a purification protocol involving several steps to be developed. A combination of cation exchange and anion exchange methods may be used to give pure Fab′ fragment expressed from mammalian cells (King *et al.*, 1992a). Alternatively, combinations of ion-exchange and hydrophobic interaction which are suitable for scale-up may be developed which allow high-purity material to be purified from *E. coli*, suitable for clinical use (King *et al.*, unpublished data). Although more steps are required, such protocols are likely to be considerably cheaper than the use of protein A or other Ig binding proteins. This is particularly important when considering the economic advantages of expression in bacterial systems.

Affinity chromatography using the antigen-binding specificity of the antibody fragment to bind to immobilised antigen is an effective purification method and is particularly useful for small antigen-binding fragments such as Fv or scFv which may be difficult to purify by other means (King *et al.*, 1993). As mentioned above, the disadvantages of such purification methods are that antigen may be expensive or simply not available in sufficient quantity and that elution conditions required to dissociate the antibody fragment are

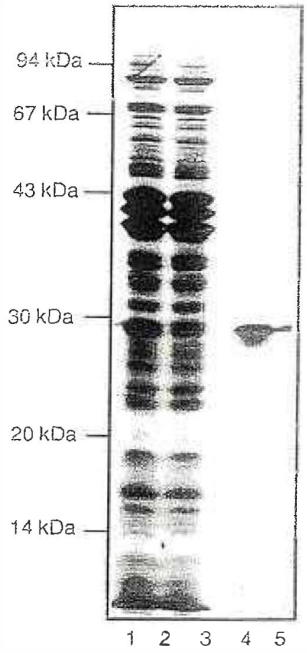


Figure 5.5 SDS-PAGE analysis of the purification of scFv from *E. coli* using immobilised antigen: lane 1, cell extract; lane 2, column flow-through; lane 3, wash fraction; lanes 4 and 5, eluted scFv (positions of molecular weight markers are indicated)

partially denaturing using extremes of pH or chaotropic agents. Monomeric antigen-binding fragments have an advantage in this respect as they can often be eluted under gentler conditions than those required for divalent fragments. When suitable methods are developed, the degree of purification which can be achieved is very high, as demonstrated for the purification of an Fv fragment expressed in *E. coli* (Figure 5.5). Alternatively, immunopurification using an antibody directed against the antibody fragment to be purified can be used. The use of an immobilised anti-human light chain antibody to purify a chimeric Fab′ fragment has been described (King *et al.*, 1992a).

The purification of scFv and other small antigen-binding fragments where antigen is not available can be difficult by conventional means. The most popular approach to achieving their purification has become the use of purification 'tags' which are engineered into the protein specifically for the purpose. A short peptide sequence can be added to the antibody fragment designed to allow simple purification under gentle conditions. Suitable peptides include hexa-histidine which allows purification in a single step of immobilised metal ion affinity chromatography (Skerra *et al.*, 1991); a biotin mimetic peptide, known as strep-tag, which can be used to bind to immobilised streptavidin (Schmidt and Skerra, 1994); and peptides recognised by specific antibodies for which gentle elution systems have been developed, such as the FLAG system described in Section 3.7. Of these the hexa-histidine tag appears to be the most universally applicable, and it has allowed the simple preparation of scFv for clinical application (Casey *et al.*, 1995). Nickel, copper or zinc ions can be used which are bound to the resin through the use of immobilised chelators such as iminodiacetic acid. Clusters of histidine residues

allow specific binding to such metal ions through coordination to unused metal coordination sites. Bound protein can then be eluted under gentle conditions with excess imidazole to compete for metal-binding sites.

5.9.4 Purification for therapeutic use

When antibodies and antibody fragments are purified for use in humans, particular care must be taken to ensure safety by achieving high purity and the absence of potentially harmful contaminants. The stringent purity requirements normally require several purification steps to be integrated into a process designed to achieve high purity in a reproducible manner. Guidelines for the preparation of antibodies for use in humans are available from the regulatory authorities. Particular care must be taken not just to remove protein contaminants but also DNA and endotoxin. DNA and endotoxin are both highly charged materials and thus ion-exchange chromatography is useful for their removal. For mammalian cell derived products, specific viral inactivation and removal steps may also be required, even though cell banks need to be characterised with respect to any adventitious agents present. Many viruses are inactivated by low pH, such as used in elution from protein A columns. Specific filtration steps may also be used to improve clearance (Maerz *et al.*, 1996). During process development small-scale replicas of the process can be set up and spiked with large amounts of DNA, viruses or endotoxin to ensure they can be efficiently removed. Processes also need to be carried out under clean conditions such that contamination does not occur, and sterile product can be generated. Good manufacturing practice (GMP) is required as this ensures the preparation of high-quality material reproducibly from batch to batch. Additionally, purified material needs to be well characterised with specific assays required for antibody identity, purity and activity as well as checking for specific contaminants. Specific contaminants may include materials used during the purification. For example, in the case of protein A purified antibodies, specific measurement of protein A levels in the product may be required.

6

Prospects for Engineered Antibodies in Biotechnology

6.1 Gene therapy

6.1.1 *Intracellular antibodies*

New avenues are already under investigation to extend the range of applications for which the exquisite specificity of MAbs can be exploited. The use of antibodies to interfere with cellular processes inside the cell, i.e. intracellular targeting, is one new area in which the potential of antibodies is being explored for both research and therapeutic purposes. Antibody genes can be introduced into a cell and expressed to produce intracellular antibody which can be used, for example, to modify cellular properties through blocking interactions between macromolecules, to modulate enzyme action through fixing the enzyme in an active or inactive state, or to bind proteins and prevent them from reaching their normal cellular compartments. Attempts to express intact IgG within the cell have been less successful than the expression of a single gene encoded molecule such as a single-chain Fv. scFv can be directed to different cellular compartments using intracellular trafficking signals. For example, the sequence Lys-Asp-Glu-Leu (KDEL) at the C-terminus can retain the scFv in the ER (Beerli *et al.*, 1994), while removal of the signal sequence will direct the protein to the cell cytosol (Biocca *et al.*, 1994). In some cases cytoplasmic Fabs may be more suitable than scFvs due to increased stability in the cytoplasm (Levin *et al.*, 1997). Intracellular antibodies can also be directed to the nuclear compartment through the use of nuclear localisation signals (Mhashilkar *et al.*, 1995).

The specific inhibition of expression of cell surface molecules has important potential applications both in the analysis of the role of cell surface molecules and for therapeutic effects through down-regulation of cell surface receptors. The erb-b2 receptor tyrosine kinase, important in the growth of many breast and ovarian tumour cells, can be inhibited by expression of an ER-located scFv which prevents the transit of erb-b2 through the ER to its cell surface location, resulting in reversal of the transformed phenotype (Beerli *et al.*, 1994). Similarly, cell surface expression of the IL-2 receptor α chain can be inhibited using an ER-located scFv, resulting in cells which are no longer responsive to IL-2 (Richardson *et al.*, 1995), therefore opening up the possibility of controlling the growth of IL-2 receptor-dependent tumour cells. Inhibition of cytosolic oncoproteins such as

$p21^{ras}$ has also been demonstrated to be a feasible approach, through directing a suitable antibody to interfere with cytoplasmic protein function (Biocca *et al.*, 1994). However, the development of gene therapy techniques which can be used to achieve *in vivo* transfection of large numbers of tumour cells is beyond current technology, preventing therapeutic use of such approaches at present. Nevertheless, such techniques are powerful tools in the analysis of the function of cytosolic or cell surface proteins, complementing other techniques such as antisense RNA and gene disruption. For example, an scFv against the integrin VLA-4 has been used to prevent cell surface expression and analyse adhesive interactions (Yuan *et al.*, 1996). The KDEL ER retention signal is not always necessary, and secreted scFv molecules which are well expressed dwell sufficiently in the ER to prevent cell surface expression in some cases (Greenman *et al.*, 1996).

Intracellular antibodies are also under investigation as potential therapeutics against infectious agents such as HIV. Expression of an intracellular scFv against the HIV envelope protein gp120 resulted in disruption of virus assembly and a large decrease in the infectivity of virus particles released (Marasco *et al.*, 1993). Other HIV targets have also been studied including the regulatory proteins *rev* and *tat*, reverse transcriptase and the matrix protein p17 (Duan *et al.*, 1994; Mhashilkar *et al.*, 1995; Maciejewski *et al.*, 1995; Levin *et al.*, 1997). Not all antibodies are effective, however, and the choice of antibody or epitope may be critical to blocking function. For example, one scFv against tat was effective in blocking HIV replication and a second one to a different epitope was ineffective (Mhashilkar *et al.*, 1995). Again, this approach is currently limited by the availability of effective gene transfer methods.

6.1.2 Other applications of MAbs in gene therapy

The development of gene therapy in general, as well as the case of intracellular antibodies above, has been limited by the ability to produce vectors which can deliver DNA to cells *in vivo* in a form which can be subsequently expressed to produce a therapeutic effect. MAbs themselves may also play a role in improving selective gene delivery. At present two types of vector are under investigation for the delivery of genes: engineered viruses and non-viral systems such as DNA bound to polycations or liposomes. A major problem with both of these systems is the ability to target the vector to the required tissue *in vivo*. The targeting specificity of antibodies is one approach which is being investigated to overcome this. Antibodies coupled to the vector of choice, which specifically bind to, and are internalised into, target cells can be used to deliver the therapeutic gene. Antibodies have been linked to engineered viruses (Roux *et al.*, 1989), to liposomes encapsulating DNA (Leonetti *et al.*, 1990), to polycationic substances which bind DNA tightly, such as polylysine (Merwin *et al.*, 1995), or directly to DNA (Poncet *et al.*, 1996). All of these approaches have advantages and disadvantages, and at present it is too early to say which may be most successful in achieving targeted gene delivery *in vivo*. Although preliminary results have shown that all of these approaches may be successful at low-efficiency gene transfer, high-efficiency delivery has not been achieved. Engineered antibodies can also be designed with desirable properties, for example a Fab′–protamine fusion protein can bind plasmid DNA encoding a toxin gene which can be used to achieve selective cell killing (Chen *et al.*, 1995).

Gene therapy may open up alternative formats for using the specificity of antibody variable regions. For example, the delivery of genes for antibody variable regions in the form of novel fusion proteins has been investigated. Fusions of scFv to intracellular

signalling domains can lead to expression of novel cell surface receptors which can use the specificity of the antibody-binding region to activate intracellular pathways. One example of this is the construction of chimeric T cell receptors for the activation of cytotoxic T lymphocytes (Eshhar *et al.*, 1993). This is a similar therapeutic approach to the use of bispecific antibodies to re-target effector cells to tumours as discussed in Section 4.2.3. In the case of chimeric receptors the T cell is the object of gene transfer, and is transfected with a gene encoding an scFv to a tumour-associated antigen fused directly to transmembrane and signalling domains of the T cell receptor. In this way a T cell is produced which is directly activated by binding of the scFv to its antigen. Such transfected T cells can lyse tumour cells in an antigen-dependent manner. For example, chimeric receptors produced from an scFv against $p185^{HER2}$ and the ζ-signal-transducing subunit of the T cell receptor or the γ-signal-transducing subunit of the Ig Fc receptor CD16 were transfected into T cells and shown to be able to lyse $p185^{HER2}$ expressing cells (Stancovski *et al.*, 1993). Similarly, a chimeric receptor produced from an scFv against a renal carcinoma antigen fused to the γ chain of the FcεRI receptor was transfected into human T cells using a retrovirus, and shown to be capable of tumour cell lysis (Weijtens *et al.*, 1996). T cells transfected with such chimeric receptors have been re-introduced into animal models and have shown some anti-tumour activity, but the extension of this technology to transfection of T cells *in vivo*, resulting in anti-tumour activity, has yet to be achieved.

6.2 Applications of antibodies in plants

In situ applications of antibodies in plants are being increasingly investigated. Resistance to viral attack may be conferred by antibody expression in the plant. A cytoplasmically expressed scFv against artichoke mottled crinkle virus could reduce infection and delay onset of symptoms even though expression levels were low (Tavladoraki *et al.*, 1993). An intact IgG has also been shown to protect against tobacco mosaic virus attack when secreted from plant cells (Voss *et al.*, 1995). However, secreted antibody has not been shown to be effective at protecting plants in all cases. For example, antibody against the harmful salivary secretions of a nematode could not protect the plant from attack (Baum *et al.*, 1996). This may be because the nematode saliva is injected directly into the cell cytoplasm, and a cytoplasmically expressed antibody may be more effective. Research is ongoing on the use of antibodies to protect against attack from a range of plant pathogens, and intracellular antibodies may also be used to affect the action of plant hormones. Expression of an scFv to abscisic acid, targeted to the endoplasmic reticulum of the plant cell, led to a similar wilty phenotype to that seen in abscisic acid deficient mutants (Artsaenko *et al.*, 1995).

6.3 Catalytic antibodies

Antibodies are thought of principally as binding molecules which can target a range of effector functions to their antigen. However, antibodies are also capable of catalysing a number of chemical reactions, in a similar manner to enzymes. Such catalytic antibodies, also known as abzymes, were first prepared by raising MAbs to transition state analogues of hydrolytic reactions (Tramontano *et al.*, 1986; Pollack *et al.*, 1986). For example, during hydrolysis of an ester bond, the reaction mechanism requires attack by a hydroxide anion at the carbonyl carbon atom, resulting in a tetrahedral transition state before

cleavage to the carboxylic acid and alcohol products. The production of the transition state is the rate-determining step in the reaction and requires the input of sufficient activation energy. The stabilisation of a transition state lowers the activation energy required and thus catalyses the rate of reaction. Antibodies which bind to, and stabilise, the transition state would therefore be expected to be catalytic. It is usually not possible to raise antibodies directly to transition states of reactions, as by their very nature they are unstable, but antibodies raised against structural analogues of the transition state, which cross-react with the reactants at the transition stage, can be used as catalysts.

Since the early reports the range of chemical reactions which can be catalysed has been greatly extended and the efficiency of many reactions has been improved (Schultz and Lerner, 1995). However, catalytic antibodies remain relatively poor catalysts compared to enzymes, with most rate enhancements in the order of 10^3–10^5 over the uncatalysed reaction, compared to 10^{11} or more for comparable enzymes. Several strategies are being pursued in attempts to improve efficiency, including both random and site-directed mutagenesis, as well as screening large combinatorial libraries of antibodies directly for catalytic activity (Janda *et al.*, 1997). Several chemical transformations which are difficult to achieve by conventional chemistry have already been achieved as well as reactions which are not catalysed by any known enzymes (Schultz and Lerner, 1995), suggesting that catalytic antibodies may find a role in organic chemistry. A particular advantage may be the ability to carry out reactions for selective enantiomers. The ability to produce large amounts of antibody fragments cheaply in bacteria and other systems may be particularly relevant here (see Chapter 5). Applications for catalytic antibodies have also been suggested for converting prodrugs to drugs in cancer therapy (Wentworth *et al.*, 1996; see Section 4.2.8), for other *in vivo* applications such as inactivation of cocaine (Landry *et al.*, 1993), and for *in vitro* applications such as biosensors (Blackburn *et al.*, 1990). However, most of these require a further leap forward in current technology. Whether such applications will ever be realised or whether catalytic antibodies will remain a research curiosity, useful only for studying the mechanisms of catalysis, remains to be seen.

6.4 Towards drug design

As well as being drugs themselves, antibodies can also be used in strategies to design low molecular weight, organic drugs. Antibodies are protein molecules, which restricts their delivery to parenteral routes of administration. For many therapies, low molecular weight drugs which may be orally active are desirable. Antibodies may also assist the development of such drugs in some cases. The desire to produce smaller antigen-binding compounds as a starting point for drug design has prompted several studies of CDR-based peptides. Peptides can be designed based on CDR sequences and structures, or constrained analogues can be produced based on modelling or structural information. Peptides have been designed from an anti-lysozyme antibody based on CDR2 of the heavy chain and CDR3 of the light chain (Welling *et al.*, 1991). Despite relatively low binding affinity, both peptides were immobilised and could be successfully used to 'immunopurify' lysozyme. In a study of an anti-HIV antibody, all six CDR peptides were prepared and compared for their ability to inhibit binding of the parent antibody to the HIV envelope protein (Levi *et al.*, 1993). CDR3 of the heavy chain was most effective, though combinations of the other CDR peptides were synergistic. A cyclised version of the peptide was also produced with improved binding affinity which was able to inhibit HIV replication. CDR-based peptides have also been designed for *in vivo* imaging applications. Peptides based on the heavy chain CDR3 of an anti-platelet antibody were designed and shown to

be able to detect vascular thrombi in animal models (Knight *et al.*, 1994), and a peptide also based on CDRH3, in this case of an antibody to a tumour-associated antigen, has been shown to be able to image breast cancer sites in patients (Sivolapenko *et al.*, 1995). These studies demonstrate that some structural properties of the antigen-binding loops are retained in short peptides, which may have practical applications.

One approach to design is to use the antibody to gather structural information about a target molecule, which may be inaccessible to direct structural analysis, and use the information in attempts to design a peptide or peptidomimetic drug. Antibodies can be raised to the key drug action sites of receptors, enzymes or viruses which contain a complementary image of the structure in question. Raising anti-idiotype antibodies to these may result in antibodies with a positive image of the target site. Information can then be gathered by sequencing the antibody and carrying out structural studies either by modelling or, for example, by solving the three-dimensional structure of the Fab fragment by X-ray crystallography. In some cases sequences of the CDR regions of anti-idiotype antibodies have homology to the initial antigen which can locate key regions. For example, a 17 amino acid peptide from the CDRL2 of an anti-reovirus receptor MAb was found to inhibit binding of the parent antibody to receptor, and sequences within the CDR were shown to be similar to those of the viral haemaglutinin (Williams *et al.*, 1991). Organic mimetic compounds could then be designed using a macrocyclic structure as a framework and introducing synthetic modifications. In this way inhibitors with affinities in the low nM range have been reported (Dougall *et al.*, 1994). Similarly, antibodies to GM-CSF have been used to generate a library of anti-idiotype light chains which could be screened for inhibition of binding of the original antibody to GM-CSF (Monfardini *et al.*, 1995). Weak structural homology was found between the CDR1, CDR2 and framework 3 sequences of an antibody light chain from the screen with helices on GM-CSF. A synthetic peptide based on the CDR1 sequence could then act as an antagonist of GM-CSF binding to cells and blocking bioactivity.

In all cases the binding of the CDR peptides is weak compared to the parent antibody as a consequence of the loss of the large contact region between antibody and antigen. One approach to help maintain binding affinity is to begin with a high-affinity single domain antibody which uses three CDRs rather than six to contact antigen. Both heavy and light chain domains can have high antigen-binding affinity alone in some cases, although isolated domains are poorly soluble due to exposed hydrophobic residues which normally pair with the other V domain (Ward *et al.*, 1989; Masat *et al.*, 1994). Camels have been found to produce antibodies which do not have light chains and thus have evolved single V domains which are more soluble. A crystal structure of a camel antibody fragment in complex with its antigen, lysozyme, has revealed that contacts between antibody and antigen in this case were dominated by one CDR, CDR3, which may be a more suitable starting point for small drug design (Desmyter *et al.*, 1996). Phage display libraries set up to display 'camelised' V_H domains have been used to identify binding domains (Davies and Riechmann, 1995). For example, a camelised V_H against hepatitis C virus protease was isolated to serve as a potential pharmacophore model to design antiviral compounds (Martin *et al.*, 1997).

6.5 Improving affinity

The ability to modulate affinity of antibodies, particularly to increase affinity by protein engineering, has been a desirable objective for some time. Attempts to rationally design

antibodies with increased affinity have been made using modelling techniques and structural information, including crystallographic structures of antibody–antigen complexes. The first report of engineering an antibody which resulted in increased affinity for antigen resulted from a mutation which was predicted from modelling studies to decrease affinity (Roberts *et al.*, 1987). There have subsequently been some modest successes in redesigning the binding site of anti-hapten antibodies to increase affinity based on structural data. The affinity of an anti-phenyloxazolone antibody was increased three-fold by a single amino acid substitution predicted from modelling and NMR studies (Riechmann *et al.*, 1992). However, attempts to rationally design antibodies with increased affinity have not generally been successful. This is probably as a result of our limited understanding of antibody–antigen interactions at the structural level, which may differ between different antibody–antigen pairs. Combinations of hydrophobic interactions and electrostatic interactions may well be involved in antigen binding to different extents in each case, and in some antigen-binding events conformational changes to the antibody variable region are known to take place (see Chapter 1). The ability to redesign variable regions for increased affinity therefore remains a goal for improved modelling based on further understanding of antibody–antigen interaction. Eventually the design of an antibody variable region to an antigen without a pre-existing antibody may even be possible (Rees *et al.*, 1994).

At present the best methodology for improving antibody affinity is the use of phage display to select improved variants. This is achieved by screening large numbers of changes introduced by random mutagenesis of all or part of the variable regions, or by chain shuffling techniques (see Chapter 1). The affinity of antibodies isolated by phage display have been improved in this way, including to very high affinity (Schier *et al.*, 1996; Yang *et al.*, 1995), although, as with any mutagenesis strategy, it is important to ensure that the specificity of the antibody is not altered during this process, as changes to the fine specificity of the antibody can result (Ohlin *et al.*, 1996).

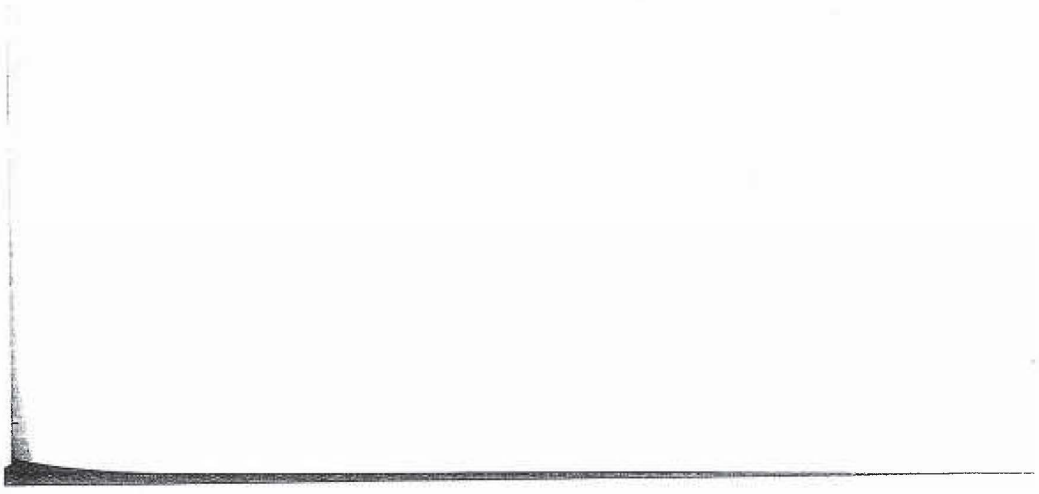
6.6 Summary and prospects

As I have attempted to illustrate, the ability to design and produce antibody-based molecules for specific applications is leading to an increase in both the range and the number of applications in which MAbs can be successfully used. In addition, the ability to generate MAbs in new ways through technologies such as phage display opens up the possibility of generating antibody specificities which were not previously obtainable. New production methods through expression in recombinant cells, or transgenic organisms, may allow the generation of low-cost MAb-based reagents, and the combination of genetic engineering and chemical modification approaches allows a wide range of molecules to be made and tested. As more is understood of the nature of antibody structure and function and the basis of specific interactions, design of molecules with optimal properties becomes achievable, allowing the development of a generation of new reagents for diagnostic and therapeutic applications. In this way many of the problems which have hampered the development of MAb-based therapeutics may be overcome, and entirely new applications such as those described in this chapter will be developed.

The range of applications of MAbs now extends from the research laboratory and the diagnostic laboratory to diagnosis in the home, therapy in the clinic, purification in the biotechnology production plant and potentially in drug design, delivery of therapeutic genes and plant protection.

As therapeutic agents, engineered antibodies will no doubt be of value for a limited time. As injectables, antibodies are not ideal therapeutics, and small molecules which can be taken orally will likely be developed to take their place in many cases. However, the unique targeting ability of antibodies may not easily be replaced with current technology and thus a role for antibody-based therapeutics seems likely for the foreseeable future. Indeed, at present the number of antibody-based therapeutic agents under development is increasing as the benefits of antibody engineering in reducing immunogenicity, targeting novel effector functions and designing appropriate pharmacokinetic properties are realised. In addition, the ability of antibodies to bind cell surface receptors and directly activate intracellular signalling mechanisms is of great potential for developing further therapeutic agents.

Another hurdle in the development of antibody-based agents as therapeutics as well as in other applications has been the cost of antibody production. New methods of production at large scale in microbial fermentation or in transgenic organisms are likely to make antibody-based reagents available at lower cost and in larger quantities than possible previously, and this is likely to be a major factor in developing the role of antibodies in new areas. With such developments and the continued increase in our understanding of the nature of the antibody:antigen interaction and in antibody effector mechanisms, the prospects for further application of engineered antibodies in biotechnology appear bright.



References

ABUCHOWSKI, A., VAN ES, T., PALCZUK, N.C. and DAVIS, F.F., 1977, Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol, *J. Biol. Chem.*, **252**, 3578–3581.

ADAIR, J.R., 1997, Engineered molecules – isolation of antibody variable regions, *The Immunology Methods Manual* (Lefkovits, I., ed.), Academic Press, San Diego.

ADAIR, J.R., ATHWAL, D.S., BODMER, M.W., BRIGHT, S.M., COLLINS, A.M., PULITO, V.L., RAO, P.E., REEDMAN, R., ROTHERMEL, A.L., XU, D., ZIVIN, R.A. and JOLLIFFE, L.K., 1995, Humanization of the murine anti-human CD3 monoclonal antibody OKT3, *Hum. Antibod. Hybridomas*, **5**, 41–47.

ADAIR, J.R., ATHWAL, D.S. and EMTAGE, J.S., 1991, *Humanised antibodies*, International patent application 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, *Protein Engineering of Antibody Molecules for Prophylactic and Therapeutic Applications* (Clark, M., ed.) pp.145–158, Academic Titles, Nottingham.

ADAIR, J.R. and BRIGHT, S.M., 1995, Progress with humanised antibodies – an update, *Exp. Opin. Invest. Drugs*, **4**, 863–870.

ADAMS, G.P., MCCARTNEY, J.E., TAI, M.S., OPPERMANN, H., HUSTON, J.S., STAFFORD, W.F., BOOKMAN, M.A., FAND, I., HOUSTON, L.L. and WEINER, L.M., 1993, Highly specific in vivo tumor targeting by monovalent and divalent forms of 741F8 anti-c-erbB-2 single-chain Fv, *Cancer Res.*, **53**, 4026–4034.

AFEYAN, N.B., GORDON, N.F. and REGNIER, F.E., 1992, Automated real-time immunoassay of biomolecules, *Nature*, **358**, 603–604.

AIZAWA, M., MORIOKA, A., SUZUKI, S. and NAGAMURA, Y., 1979, Amperometric determination of human choriogonadotrophin by membrane bound antibody, *Anal. Biochem.*, **94**, 22–28.

AKERSTROM, B., NILSON, B.H.K., HOOGENBOOM, H.R. and BJORCK, L., 1994, On the interaction between single chain Fv antibodies and bacterial immunoglobulin-binding proteins, *J. Immunol. Methods*, **177**, 151–163.

AKHAVAN-TAFTI, H., SUGIOKA, K., ARGHAVANI, Z., DESILVA, R., HANDLEY, R.S., SUGIOKA, Y., EICKHOLT, R.A., PERKINS, M.P. and SCHAPP, A.P., 1995, Chemiluminescent detection of horseradish peroxidase by enzymatic generation of acridinium esters, *Clin. Chem.*, **41**, 1368–1369.

ALAUDDIN, M.M., KHAWLI, L.A. and EPSTEIN, A.L., 1992, An improved method of direct labeling monoclonal antibodies with ^{99m}Tc, *Nucl. Med. Biol.*, **19**, 445–454.

ALBRECHT, S., BRANDL, H., STEINKE, M. and FREIDT, T., 1994, Chemiluminescent enzyme immunoassay of prostate-specific antigen based on indoxyl phosphate substrate, *Clin. Chem.*, **40**, 1970–1971.

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 immunosuppressive properties of a humanized OKT3 monoclonal antibody, *J. Immunol.*, **148**, 3461–3468.

ALI, S.A., WARREN, S.D., RICHTER, K.Y., BADGER, C.C., EARY, J.F., PRESS, O.W., KROHN, K.A., BERNSTEIN, I.D. and NELP, W.B., 1990, Improving the tumour retention of radioiodinated antibody:aryl carbohydrate adducts, *Cancer Res.*, **50**, 783s–788s.

ALLARD, W.J., MORAN, C.A., NAGEL, E., COLLINS, G. and LARGEN, M.T., 1992, Antigen binding properties of highly purified bispecific antibodies, *Mol. Immunol.*, **29**, 1219–1227.

ALLEN, T.M., AHMAD, I., LOPES DE MENEZES, D.E. and MOASE, E.H., 1995, Immunoliposome-mediated targeting of anti-cancer drugs in vivo, *Biochem. Soc. Trans.*, **23**, 1073–1079.

AMES, R.S., TORNETTA, M.A., MCMILLAN, L.J., KAISER, K.F., HOLMES, S.D., APPELBAUM, E., CUSIMANO, D.M., THEISEN, T.W., GROSS, M.S., JONES, C.S., SILVERMAN, C., PORTER, T.G., COOK, R.M., BENNETT, D. and CHAIKEN, I.M., 1995, Neutralizing murine monoclonal antibodies to human IL-5 isolated from hybridomas and a filamentous phage Fab display library, *J. Immunol.*, **154**, 6355–6364.

ANASETTI, C., HANSEN, J.A., WALDMANN, T.A., APPLEBAUM, F.R., DAVIS, J., DEEG, H.J., DONEY, K., MARTIN, P.J., NASH, R., STORB, R., SULLIVAN, K.M., WITHERSPOON, R.P., BINGER, M.-H., CHIZZONITE, R., HAKIMI, J., MOULD, D., SATOH, H. and LIGHT, S.E., 1994, Treatment of acute graft versus host disease with humanized anti-Tac: an antibody that binds to the interleukin-2 receptor, *Blood*, **84**, 1320–1327.

ANDERSON, C.J., CONNETT, J.M., SCHWARZ, S.W., ROCQUE, P.A., GUO, L.W., PHILPOTT, G.W., ZINN, K.R., MEARES, C.F. and WELCH, M.J., 1992, Copper-64 labeled antibodies for PET imaging, *J. Nucl. Med.*, **33**, 1685–1691.

ANDERSON, C.J., SCHWARZ, S.W., CONNETT, J.M., CUTLER, P.D., GUO, L.W., GERMAIN, C.J., PHILPOTT, G.W., ZINN, K.R., GREINER, D.P., MEARES, C.F. and WELCH, M.J., 1995, Preparation, biodistribution and dosimetry of copper-64-labelled anti-colorectal carcinoma monoclonal antibody fragments 1A3-F(ab')$_2$, *J. Nucl. Med.*, **36**, 850–858.

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 chimeric mouse/human IgG4 antibody, *Mol. Immunol.*, **30**, 105–108.

ANSELL, S.M., TARDI, P.G. and BUCHOWSKY, S.S., 1996, 3-(2-pyridyldithio)propionic acid hydrazide as a cross-linker in the formation of liposome–antibody conjugates, *Bioconj. Chem.*, **7**, 490–496.

ANTON, R. and BEAN, P., 1994, Two methods for measuring carbohydrate-deficient transferrin in inpatient alcoholics and healthy controls compared, *Clin. Chem.*, **40**, 364–368.

ANTONIW, P., FARNSWORTH, A.P.H., TURNER, A., HAINES, A.M.R., MOUNTAIN, A., MACKINTOSH, J., SHOCHAT, D., HUMM, J., WELT, S., OLD, L.J., YARRANTON, G.T. and KING, D.J., 1996, Radioimmunotherapy of colorectal carcinoma xenografts in nude mice with yttrium-90 A33 IgG and tri-Fab (TFM), *Br. J. Cancer*, **74**, 513–524.

APPLEBAUM, F.R., MATTHEWS, D.C., EARY, J.F., BADGER, C.C., KELLOG, M., PRESS, O.W., MARTIN, P.J., FISHER, D.R., NELP, W.B., THOMAS, E.D. and BERNSTEIN, I.D., 1992, The use of radiolabelled anti-CD33 antibody to augment marrow irradiation prior to marrow transplantation for acute myelogenous leukemia, *Transplantation*, **54**, 829–833.

ARMBRUSTER, D.A., HUBSTER, E.C., KAUFMAN, M.S. and RAMON, M.K., 1995, Cloned enzyme donor immunoassay (CEDIA) for drugs of abuse screening, *Clin. Chem.*, **41**, 92–98.

ARMBRUSTER, D.A., SCHWARZHOFF, R.H., HUBSTER, E.C. and LISERIO, M.K., 1993, Enzyme immunoassay, kinetic microparticle immunoassay, radioimmunoassay and fluorescence polarization immunoassay compared for drugs of abuse screening, *Clin. Chem.*, **39**, 2137–2146.

ARNOLD, M.W., SCHNEEBAUM, S., BERENS, A., PETTY, L., MOIZISIK, C., HINCKLE, G. and MARTIN, E.W., 1992, Intraoperative detection of colorectal cancer with radioimmunoguided surgery and CC49, a second generation monoclonal antibody, *Ann. Surgery*, **216**, 11–16.

ARTSAENKO, O., PEISKER, M., ZUR NIEDEN, U., FIEDLER, U., WEILER, E.W., MUNTZ, K. and CONRAD, U., 1995, Expression of a single-chain Fv antibody against abscisic acid creates a wilty phenotype in transgenic tobacco, *Plant J.*, **8**, 745–750.

ASANO, M., YUKITA, A., MATSUMOTO, T., KONDO, S. and SUZUKI, H., 1995, Inhibition of tumour growth and metastasis by an immunoneutralizing monoclonal antibody to human vascular endothelial growth factor/vascular permeability factor 121, *Cancer Res.*, **55**, 5296–5301.

AXWORTHY, D.B., FRITZBERG, A.R., HYLARIDES, M.D., MALLETT, R.W., THEODORE, L.J., GUSTAVSON, L.M., SU, F.M., BEAUMIER, P.L. and RENO, J.M., 1994, Preclinical evaluation of an anti-tumour monoclonal antibody/streptavidin conjugate for pretargeted ^{90}Y radioimmunotherapy in a nude mouse xenograft model, *J. Immunother.*, **16**, 158.

BACHMANN, M.F., KALINKE, U., ALTHAGE, A., FREER, G., BURKHART, C., ROOST, H.P., AGUET, M., HENGARTNER, H. and ZINKERNAGEL, R.M., 1997, The role of antibody concentration and avidity in antiviral protection, *Science*, **276**, 2024–2027.

BADGER, C.C., 1990, Bone marrow toxicity for ^{131}I-labeled antibodies, *Antibody Immunoconj. Radiopharm.*, **3**, 281–287.

BAGSHAWE, K.D., SHARMA, S.K., SPRINGER, C.J. and ANTONIW, P., 1995, Antibody directed enzyme prodrug therapy: a pilot-scale clinical trial, *Tumor Targeting*, **1**, 17–29.

BAILON, P. and ROY, S.K., 1990, Recovery of recombinant proteins by immunoaffinity chromatography. *Protein Purification* (Ladisch, M.R., Willson, R.C., Painton, C.C. and Builder, S.E., eds), pp. 150–167, ACS, Washington, DC.

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 radioimmunotherapy with chimeric B72.3, *Antibody Immunoconj. Radiopharm.*, 4, 799–809.

BAKER, T.S., BOSE, C.C., CASKEY-FINNEY, H.M., KING, D.J., LAWSON, A.D.G., LYONS, A., MOUNTAIN, A., OWENS, R.J., ROLFE, M.R., SEHDEV, M., YARRANTON, G.T. and ADAIR, J.R., 1994, Humanization of an anti-mucin antibody for breast and ovarian cancer therapy, *Antigen and Antibody Molecular Engineering in Breast Cancer Diagnosis and Treatment*, (Ceriani, R.L., ed.), pp. 61–82, Plenum Press, New York.

BARBAS, C.F., III, 1995, Synthetic human antibodies, *Nature Med.*, **1**, 837–839.

BARBAS, C.F. III and BURTON, D.R., 1996, Selection and evolution of high affinity human anti-viral antibodies, *Trends Biotechnol.*, **14**, 230–234.

BARBAS, C.F., III, CROWE, J.E., CABABA, D., JONES, T.M., ZEBEDEE, S.L., MURPHY, B.R., CHANOCK, R.M. and BURTON, D.R., 1992, Human monoclonal Fab fragments derived from a combinatorial library bind to respiratory syncytial virus F glycoprotein and neutralise infectivity, *Proc. Natl Acad. Sci. (USA)*, **89**, 10164–10168.

BARBAS, C.F., III, KANG, A.S., LERNER, R.A. and BENKOVIC, S.J., 1991, Assembly of combinatorial antibody libraries on phage surfaces, *Proc. Natl Acad. Sci. (USA)*, **88**, 7978–7982.

BARNARD, G., KOHEN, F., MIKOLA, H. and LOVGREN, T., 1989, Measurement of estrone-3-glucuronide in urine by rapid, homogeneous time-resolved fluoroimmunoassay, *Clin. Chem.*, **35**, 555–559.

BASELGA, J., TRIPATHY, D., MENDELSOHN, J., BAUGHMAN, S., BENZ, C.C., DANTIS, L., SKLARIN, N.T., SEIDMAN, A.D., HUDIS, C.A., MOORE, J., ROSEN, P.P., TWADDELL, T., HENDERSON, I.C. and NORTON, L., 1996, Phase II study of weakly intravenous recombinant humanized anti-p185^{HER2} monoclonal antibody in patients with HER2/*neu*-overexpressing metastatic breast cancer, *J. Clin. Oncol.*, **14**, 737–744.

BATRA, J.K., KASTURI, S., GALLO, M.G., VOORMAN, R.L., MAIO, S.M., CHAUDHARY, V.K. and PASTAN, I., 1993, Insertion of constant region domains of IgG1 into CD4-PE40 increases its plasma half-life, *Mol. Immunol.*, **30**, 379–386.

BAUM, T.J., HIATT, A., PARROTT, W.A., PRATT, L.H. and HUSSEY, R.S., 1996, Expression in tobacco of a functional monoclonal antibody specific to stylet secretions of the root-knot nematode, *Mol. Plant Microbe Interact.*, **9**, 382–387.

BEAUMIER, P.L., VENKATESAN, P., VANDERHEYDEN, J.L., BURGUA, W.D., KUNZ, L.L., FRITZBERG, A.R., ABRAMS, P.G. and MORGAN, A.C., 1991, ^{186}Re radioimmunotherapy of small cell lung carcinoma xenografts in nude mice, *Cancer Res.*, **51**, 676–681.

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

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 marker, *Bio/Technol.*, **10**, 169–175.

BECKER, W., BAIR, J., BEHR, T., REPP, R., STRECKENBACH, H., BECK, H., GRAMATZKI, M., WINSHIP, M.J., GOLDENBERG, D.M. and WOLF, F., 1994, Detection of soft tissue infections and osteomyelitis using a technetium-99m-labelled anti-granulocyte monoclonal antibody fragment, *J. Nucl. Med.*, **35**, 1436–1443.

BEERLI, R.R., WELS, W. and HYNES, N.E., 1994, Intracellular expression of single-chain antibodies reverts erb-b2 transformation, *J. Biol. Chem.*, **269**, 23931–23936.

BEESLEY, J.E., ed., 1993, *Immunocytochemistry: a Practical Approach*, IRL Press, Oxford.

BEGENT, R.H.J., KEEP, P.A., GREEN, A.J., SEARLE, F., BAGSHAWE, K.D., JEWKES, R.F., JONES, B.E., BARRATT, G.M. and RYMAN, B.E., 1982, Liposomally entrapped second antibody improves tumour imaging with radiolabeled (first) antitumour antibody, *Lancet*, **ii**, 739–742.

BEGENT, R.H.J., VERHAAR, M.J., CHESTER, K.A., CASEY, J.L., GREEN, A.J., NAPIER, M.P., HOPE-STONE, L.D., CUSHEN, N., KEEP, P.A., JOHNSON, C.J., HAWKINS, R.E., HILSON, A.J.W. and ROBSON, L., 1996, Clinical evidence of efficient tumor targeting based on a single chain Fv antibody from a combinatorial library, *Nature Med.*, **2**, 979–984.

BEHR, T.M., BECKER, W.S., BAIR, H.J., KLEIN, M.W., STUHLER, C.M., CIDLINSKY, K.P., WITTEKIND, C.W., SCHEELE, J.R. and WOLF, F.G., 1995, Comparison of complete versus fragmented technetium-99m-labeled anti-CEA monoclonal antibodies for immunoscintigraphy in colorectal cancer, *J. Nucl. Med.*, **36**, 430–441.

BELEW, M., JUNTTI, N., LARSSON, A. and PORATH, J., 1987, A one-step purification method for monoclonal antibodies based on a salt-promoted adsorption chromatography on a thiophilic adsorbent, *J. Immunol. Methods*, **102**, 173–182.

BENHAR, I., PADLAN, E.A., JUNG, S.H., LEE, B. and PASTAN, I., 1994, Rapid humanisation of the Fv of monoclonal antibody B3 by using framework exchange of the recombinant immunotoxin B3(Fv) PE38, *Proc. Natl Acad. Sci. (USA)*, **91**, 12051–12055.

BENHAR, I. and PASTAN, I., 1995, Characterization of B1(Fv)PE38 and B1(dsFv)PE38: single-chain and disulphide-stabilized Fv immunotoxins with increased activity that cause complete remissions of established human carcinoma xenografts in nude mice, *Clin. Cancer Res.*, **1**, 1023–1029.

BERNSTEIN, I., 1996, Presented at American Society of Haematology Meeting, Orlando, FL, December.

BERRY, M.J. and DAVIES, J., 1992, Use of antibody fragments in immunoaffinity chromatography. Comparison of Fv fragments, VH fragments and paralog peptides, *J. Chromatogr.*, **597**, 239–245.

BERRY, M.J., DAVIES, J., SMITH, C.G. and SMITH, I., 1991, Immobilization of Fv antibody fragments on porous silica and their utility in affinity chromatography, *J. Chromatogr.*, **587**, 161–169.

BERRY, M.J. and PIERCE, J.J., 1993, Stability of immunoadsorbents comprising antibody fragments: comparison of Fv fragments and single-chain Fv fragments, *J. Chromatogr.*, **629**, 161–168.

BETTER, M., BERNHARD, S.L., LEI, S.P., FISHWILD, D.M., LANE, J.A., CARROLL, S.F. and HORWITZ, A.H., 1993, Potent anti-CD5 ricin A chain immunoconjugates from bacterially produced Fab′ and $F(ab')_2$, *Proc. Natl Acad. Sci. (USA)*, **90**, 457–461.

BETTER, M., BERNHARD, S.L., WILLIAMS, R.E., LEIGH, S.D., BAUER, R.J., KUNG, A.H.C., CARROLL, S.F. and FISHWILD, D.M., 1995, T-cell targeted immunofusion proteins from *Escherichia coli*, *J. Biol. Chem.*, **270**, 14951–14957.

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.

BETTER, M., WEICKMANN, J. and LIN, Y.L., 1990, Production and scale-up of a chimeric fab from bacteria, *Proceedings of the 1990 Miami Bio/Technology Winter Symposium*, 105.

BEUTLER, B., MISARK, I.W. and CERAMI, A., 1985, Passive immunization against cachectin/tumour necrosis factor protects mice from lethal effects of endotoxin, *Science*, **229**, 869–871.

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.

BIBILA, T.A. and ROBINSON, D.K., 1995, In pursuit of the optimal fed-batch process for monoclonal antibody production, *Biotechnol. Progress*, **11**, 1–13.

BICKEL, U., LEE, V.M., TROJANOWSKI, J.Q. and PARDRIDGE, W.M., 1994, Development and *in vitro* characterization of a cationized monoclonal antibody against beta A4 protein: a potential probe for Alzheimer's disease, *Bioconj. Chem.*, **5**, 119–125.

BIOCCA, S., PIERANDREI-AMALDI, P., CAMPIONI, N. and CATTANEO, A., 1994, Intracellular immunization with cytosolic recombinant antibodies, *Bio/Technol.*, **12**, 396–399.

BIRCH, J.R., LAMBERT, K., THOMPSON, P.W., KENNEY, A.C. and WOOD, L.A., 1987, Antibody production with airlift fermentors, *Large Scale Cell Culture Technology* (Lyderson, B.K., ed.), pp. 1–20, Hanser, New York.

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., VOGEL, C.A., GILLET, M., MACH, J.P., SMITH, A. and SCHUBIGER, P.A., 1997, Comparison of copper-67 and iodine-125-labeled anti-CEA monoclonal antibody biodistribution in patients with colorectal tumours, *J. Nucl. Med.*, **38**, 847–853.

BITOH, S., LANG, G.M. and SEHON, A., 1993, Suppression of human anti-mouse idiotypic antibody responses in hu-PBL-SCID mice, *Hum. Antibod. Hybridomas*, **4**, 144–151.

BLACKBURN, G.F., TALLEY, D.B., BOOTH, P.M., DURFOR, C.N., MARTIN, M.T., NAPPER, A.D. and REES, A.R., 1990, Potentiometric biosensor employing catalytic antibodies as the molecular recognition element, *Anal. Chem.*, **62**, 2211–2216.

BLOOM, J.W., MADANAT, M.S., MARRIOTT, D., WONG, T. and CHAN, S.Y., 1997, Intrachain disulfide bond in the core region of human IgG4, *Protein Sci.*, **6**, 407–415.

BLUMENTHAL, R.D., SHARKEY, R.M., SNYDER, D. and GOLDENBERG, D.M., 1989, Reduction by anti-antibody administration of the radiotoxicity associated with ^{131}I-labeled antibody to carcinoembryonic antigen in cancer radioimmunotherapy, *J. Natl Cancer Inst.*, **81**, 194–199.

BODE, C., RUNGE, M.S., SCHONERMARK, S., EBERLE, T., NEWELL, J.B., KUBLER, W. and HABER, E., 1990, Conjugation to antifibrin Fab′ enhances fibrinolytic potency of single-chain urokinase plasminogen activator, *Circulation*, **81**, 1974–1980.

BOLTON, A.E. and HUNTER, W.M., 1973, The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent. Application to the radioimmunoassay, *Biochem. J.*, **133**, 529–539.

BONARDI, M.A., FRENCH, R.R., AMLOT, P., GROMO, G., MODENA, D. and GLENNIE, M.J., 1993, Delivery of saporin to human B-cell lymphoma using bispecific antibody: targeting via CD22 but not CD19, CD37 or immunoglobulin results in efficient killing, *Cancer Res.*, **53**, 3015–3021.

BONE, R.C., BALK, R.A., FEIN, A.M., PERL, T.M., WENZEL, R.P., REINES, H.D., QUENZER, R.W., IBERTI, T.J., MACINTYRE, N., SCHEIN, R.M.H. and the E5 SEPSIS STUDY GROUP, 1995, A second large controlled clinical study of E5, a monoclonal antibody to endotoxin, *Crit. Care Med.*, **23**, 994–1006.

BORGSTROM, P., HILLAN, K.J., SRIRAMARAO, P. and FERRARA, N., 1996, Complete inhibition of angiogenesis and growth of microtumours by anti-vascular endothelial growth factor neutralizing antibody: novel concepts of angiostatic therapy from intravital videomicroscopy, *Cancer Res.*, **56**, 4032–4039.

BORREBAECK, C.A.K, 1989, Strategy for production of human monoclonal antibodies using in vitro activated B cells, *J. Immunol. Methods*, **123**, 157–165.

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.

BORREBAECK, C.A.K., MALMBORG, A.C. and OHLIN, M., 1993, Does endogenous glycosylation prevent the use of mouse monoclonal antibodies as cancer therapeutics?, *Immunol. Today*, **14**, 477–479.

BOSCHETTI, N., BRODBECK, U., JENSEN, S.P., KOCH, C. and NORGAARD-PEDERSEN, B., 1996, Monoclonal antibodies against a C-terminal peptide of human brain acetylcolinesterase distinguish between erythrocyte and brain acetylcolinesterases, *Clin. Chem.*, **42**, 19–23.

BOSS, M.A., KENTEN, J.H., WOOD, C.R. and EMTAGE, J.S., 1984, Assembly of functional antibodies from immunoglobulin heavy and light chains synthesised in *E. coli*, *Nucl. Acids Res.*, **12**, 3791–3806.

BOSSLET, K., CZECH, J. and HOFFMANN, D., 1994, Tumor-selective prodrug activation by fusion protein-mediated catalysis, *Cancer Res.*, **54**, 2151–2159.

BOSSLET, K., CZECH, J., LORENZ, P., SEDLACEK, H.H., SCHUERMANN, M. and SEEMAN, G., 1992, Molecular and functional characterization of a fusion protein suited for tumour specific prodrug activation, *Br. J. Cancer*, **65**, 234–238.

BOTHWELL, A.L.M., PASKIND, M., RETH, M., IMANISHI-KARI, T., RAJEWSKY, K. and BALTIMORE, D., 1981, Heavy chain variable region contribution to the NP^b family of antibodies: somatic mutation evident in a γ2a variable region, *Cell*, **24**, 625–637.

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 cancer: implications for radioimmunotherapy, *Br. J. Cancer*, **65**, 825–831.

BRAMBELL, F.W.R., HEMMINGS, W.A. and MORRIS, I.G., 1964, A theoretical model of γ-globulin catabolism, *Nature*, **203**, 1352–1355.

BRECHBIEL, M.W., GANSOW, O.A., ATCHER, R.A., SCHLOM, J., ESTEBAN, J., SIMPSON, D. and COLCHER, D., 1986, Synthesis of 1-(p-isothiocyanatobenzyl) derivatives of DTPA and EDTA. Antibody labeling and tumor imaging studies, *Inorg. Chem.*, **25**, 2772–2781.

BREITZ, H.B., WEIDEN, P.L., VANDERHEYEN, J.L., APPLEBAUM, J.W., BJORN, M.J., FER, M.F., WOLF, S.B., RATLIFF, B.A., SEILER, C.A., FOISIE, D.C., FISHER, D.R., SCHROFF, R.W., FRITZBERG, A.R. and ABRAMS, P.G., 1992, Clinical experience with rhenium-186-labeled monoclonal antibodies for radioimmunotherapy: results of phase I trials, *J. Nucl. Med.*, **33**, 1099–1112.

BRENNAN, M., DAVISON, P.F. and PAULUS, H., 1985, Preparation of bispecific antibodies by chemical recombination of monoclonal immunoglobulin G1 fragments, *Science*, **229**, 81–83.

BRETT, S., BAXTER, G., COOPER, H., JOHNSTON, J.M., TITE, J. and RAPSON, N., 1996, Repopulation of blood lymphocyte sub-populations in rheumatoid arthritis patients treated with the depleting humanised monoclonal antibody, CAMPATH-1H, *Immunol.*, **88**, 13–19.

BRIDEY, F., PHILIPOTTEAU, C., DREYFUS, M. and SIMONNEAU, G., 1989, Plasma D-dimer and pulmonary embolism, *Lancet*, **I**, 791–792.

BRINKMANN, U., PAI, L.H., FITZGERALD, D.J., WILLINGHAM, M. and PASTAN, I., 1991, B3(Fv)-PE38KDEL, a single chain immunotoxin that causes regression of a human carcinoma in mice, *Proc. Natl Acad. Sci. (USA)*, **88**, 8616–8620.

BRODEUR, B.R. and TSANG, P.S., 1986, High yield monoclonal antibody production in ascites, *J. Immunol. Methods*, **86**, 239–241.

BRONSTEIN, I., VOYTA, J.C., THORPE, G.H.G., KRICKA, L.J. and ARMSTRONG, G., 1989, Chemiluminescent assay of alkaline phosphatase applied in an ultrasensitive immunoassay of thyrotropin, *Clin. Chem.*, **35**, 1441–1446.

BROOKS, P.C., MONTGOMERY, A.M.P., ROSENFELD, M., REISFELD, R.A., HU, T., KLIER, G. and CHERESH, D.A., 1994, Integrin $\alpha_v\beta_3$ antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels, *Cell*, 79, 1157–1164.

BROWN, B.A., COMEAU, R.D., JONES, P.L., LIBERATORE, F.A., NEACY, W.P., SANDS, H. and GALLAGHER, B.M., 1987, Pharmacokinetics of the monoclonal antibody B72.3 and its fragments labelled with either ^{125}I or ^{111}In, *Cancer Res.*, 47, 1149–1154.

BROWN, M.E., RENNER, G., FIELD, R.P. and HASSELL, T., 1992, Process development for the production of recombinant antibodies using the glutamine synthetase (GS) system, *Cytotechnol.*, **9**, 231–236.

BROWN, P.M., TAGARI, P., ROWAN, K.R., YU, V.L., O'NEILL, G.P., MIDDAUGH, C.R., SANYAL, G., FORD-HUTCHINSON, A.W. and NICHOLSON, D.W., 1995, Epitope-labelled soluble human interleukin-5 receptors. Affinity cross-link labelling, IL-5 binding and biological activity, *J. Biol. Chem.*, **270**, 29236–29243.

BROWN, P.S., 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, Prolongation of primate renal allograft survival by anti-Tac, an anti-human IL-2 receptor monoclonal antibody, *Proc. Natl. Acad. Sci. (USA)*, **88**, 2663–2667.

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.

BUCHEGGER, F., MACH, J.P., PELEGRIN, A., GILLET, M., VOGEL, C.A., BUCLIN, T., RYSER, J.E., DELALOYE, B. and DELALOYE, A.B., 1995, Radiolabeled chimeric anti-CEA monoclonal antibody compared with the original mouse monoclonal antibody for surgically treated colorectal carcinoma, *J. Nucl. Med.*, **36**, 420–429.

BUCHEGGER, F., PELEGRIN, A., DELALOYE, B., BISCHOFF-DELALOYE, A. and MACH, J.P., 1990, Iodine-131 labelled Mab $F(ab')_2$ fragments are more effective 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.

BUCHEGGER, F., PELEGRIN, A., HARDMAN, N., HEUSSER, C., LUKAS, J., DOLCI, W. and MACH, J.P., 1992, Different behaviour of mouse–human chimeric antibody $F(ab')_2$ fragments of IgG1, IgG2 and IgG4 subclass *in vivo*, *Int. J. Cancer*, **50**, 416–422.

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

BUCKLEY, D.T.J., ROBINS, A.R. and DURRANT, L.G., 1995, Clinical evidence that the human monoclonal anti-idiotypic antibody 105AD7 delays tumor growth by stimulating anti-tumour T-cell responses, *Hum. Antibod. Hybridomas*, **6**, 68–72.

BUIST, M.R., KENEMANS, P., DEN HOLLANDER, W., VERMORKEN, J.B., MOLTHOFF, C.J.M., BURGER, C.W., HELMERHORST, T.J.M., BAAK, J.P.A. and ROOS, J.C., 1993, Kinetics and tissue distribution of the radiolabelled chimeric monoclonal antibody MOv18 IgG and $F(ab')_2$ fragments in ovarian carcinoma patients, *Cancer Res.*, **53**, 5413–5418.

BUIST, M.R., KENEMANS, P., VAN KAMP, G.J. and HAISMA, H.J., 1995, Minor human antibody response to a mouse and chimeric monoclonal antibody after a single i.v. infusion in ovarian carcinoma patients: a comparison of five assays, *Cancer Immunol. Immunother.*, **40**, 24–30.

BURROWS, F.J., DERBYSHIRE, E.J., TAZZARI, P.L., AMLOT, P., GAZDAR, A.F., KING, S.W., LETARTE, M., VITETTA, E.S. and THORPE, P.E., 1995, Up-regulation of endoglin on vascular endothelial cells in human solid tumours: implications for diagnosis and therapy, *Clin. Cancer Res.*, **1**, 1623–1634.

BURROWS, F.J. and THORPE, P.E., 1993, Eradication of large solid tumours in mice with an immunotoxin directed against tumour vasculature, *Proc. Natl Acad. Sci. (USA)*, **90**, 8996–9000.

BURTON, D.R. and BARBAS, C.F., III, 1994, Human antibodies from combinatorial libraries, *Adv. Immunol.*, **57**, 191–280.

BYWATER, R., ERIKSSON, G. and OTTOSON, T., 1983, Desorption of immunoglobulins from protein A-Sepharose CL-4B under mild conditions, *J. Immunol. Methods*, **64**, 1–6.

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.

CALIFF, R.M. for the EPIC investigators, 1994, Use of a monoclonal antibody directed against the platelet glycoprotein IIb/IIIa receptor in high risk angioplasty, *N. Engl. J. Med.*, **330**, 956–1007.

CALVIN, J., BURLING, K., BLOW, C., BARNES, I. and PRICE, C.P., 1986, Evaluation of fluorescence excitation transfer immunoassay for measurement of specific proteins, *J. Immunol. Methods*, **86**, 249–256.

CAMERA, L., KINUYA, S., GARMESTANI, K., WU, C., BRECHBIEL, M.W., PAI, L.H., MCMURRY, T.J., GANSOW, O.A., PASTAN, I., PAIK, C.H. and CARRASQUILLO, J.A., 1994, Evaluation of the serum stability and *in vivo* biodistribution of CHX-DTPA and other ligands for yttrium labeling of monoclonal antibodies, *J. Nucl. Med.*, **35**, 882–889.

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 CH2 domain and is modulated by the hinge region, *J. Exp. Med.*, **173**, 1483–1491.

CAPON, D.J., CHAMOW, S.M., MORDENTI, J., MARSTERS, S.A., GREGORY, T., MITSUYA, H., BYRN, R.A., LUCAS, C., WURM, F.M., GROOPMAN, J.E., BRODER, S. and SMITH, D.H., 1989, Designing CD4 immunoadhesin for AIDS therapy, *Nature*, **337**, 525–531.

CARAYANNOPOULOS, L., MAX, E.E. and CAPRA, J.D., 1994, Recombinant human IgA expressed in insect cells, *Proc. Natl Acad. Sci. (USA)*, **91**, 8348–8352.

CARLSSON, M., HEDIN, A., INGANAS, M., HARFAST, B. and BLOMBERG, F., 1985, Purification of in vitro produced mouse monoclonal antibodies. A two-step procedure using cation exchange chromatography and gel filtration, *J. Immunol. Methods*, **79**, 89–98.

CARLSSON, R., MARTENSSON, C., KALLIOMAKI, S., OHLIN, M. and BORREBAECK, C.A.K., 1992, Human peripheral blood lymphocytes transplanted into scid mice constitute an *in vivo* culture system exhibiting several parameters found in a normal humoral immune response and are a source of immunocytes for the production of human monoclonal antibodies, *J. Immunol.*, **148**, 1065–1071.

CARON, P.C., CO, M.S., BULL, M.K., AVDALOVIC, N.K., QUEEN, C. and SCHEINBERG, D.A., 1992, Biological and immunological features of humanized M195 (anti-CD33) monoclonal antibodies, *Cancer Res.*, **52**, 6761–6767.

CARON, P.C., JURCIC, J.G., SCOTT, A.M., FINN, R.D., DIVGI, C.R., GRAHAM, M.C., JUREIDINI, I.M., SGOUROS, G., TYSON, D., OLD, L.J., LARSON, S.M. and SCHEINBERG, D.A., 1994, A phase 1B trial of humanized monoclonal antibody M195 (anti-CD33) in myeloid leukemia: specific targeting without immunogenicity, *Blood*, **83**, 1760–1768.

CARRIER, A., DUCANCEL, F., SETTIAWAN, N.B., CATTOLICO, L., MAILLERE, B., LEONETTI, M., DREVET, P., MENEZ, A. and BOULAIN, J.C., 1995, Recombinant antibody–alkaline phosphatase conjugates for diagnosis of human IgGs: application to anti-HBsAg detection, *J. Immunol. Methods*, **181**, 177–186.

CARRIO, I., BERNA, L., BALLESTER, M., ESTORCH, M., OBRADOR, D., CLADELLAS, M., ABADAL, L. and GINJAUNE, M., 1988, Indium-111 antimyosin scintigraphy to assess myocardial damage in patients with suspected myocarditis and cardiac rejection, *J. Nucl. Med.*, **29**, 1893–1900.

CARRIO, I., ESTORCH, M., BERNA, L., LOPEZ-POUSA, J., TABERNO, J. and TORRES, G., 1995, Indium-111-antimyosin and iodine-123-MIBG studies in early assessment of doxorubicin cardiotoxicity, *J. Nucl. Med.*, **36**, 2044–2049.

CARROLL, W.L., MENDEL, E. and LEVY, S., 1988, Hybridoma fusion cell lines contain an aberrant kappa transcript, *Mol. Immunol.*, **25**, 991.

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/Technol.*, **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 SHEPHARD, H.M., 1992b, Humanization of an anti-p185HER2 antibody for human cancer therapy, *Proc. Natl Acad. Sci. (USA)*, **89**, 4285–4289.

CARTNER, A.M., CONRY, R.M., SAFAVY, A., KHAZAELI, M.B., SUMEREL, L.A. and LOBUGLIO, A.F., 1993, An animal model to predict the immunogenicity of murine V regions in humans, *Hum. Antibod. Hybridomas*, **4**, 174–180.

CASADEI, J., POWELL, M.J. and KENTEN, J.H., 1990, Expression and secretion of aequorin as a chimeric antibody by means of a mammalian expression vector, *Proc. Natl Acad. Sci. (USA)*, **87**, 2047–2051.

CASEY, J.L., KEEP, P.A., CHESTER, K.A., ROBSON, L., HAWKINS, R.E. and BEGENT, R.H.J., 1995, Purification of bacterially expressed single chain Fv antibodies for clinical applications using metal chelate chromatography, *J. Immunol. Methods*, **179**, 105–116.

CASEY, J.L., KING, D.J., CHAPLIN, L.C., HAINES, A.M.R., PEDLEY, R.B., MOUNTAIN, A., YARRANTON, G.T. and BEGENT, R.H.J., 1996, Preparation, characterisation and tumour targeting of cross-linked divalent and trivalent anti-tumour Fab′ fragments, *Br. J. Cancer*, **74**, 1397–1405.

CATTANEO, A. and NEUBERGER, M.S., 1987, Polymeric immunoglobulin M is secreted by transfectants of non-lymphoid cells in the absence of immunoglobulin J chain, *EMBO J.*, **6**, 2753–2758.

CHAN, D.W. (ed.), 1996, *Immunoassay Automation: an Updated Guide to Systems*, Academic Press, San Diego.

CHAPLIN, L.C., 1996, Streamline rProtein A for expanded bed adsorption of mabs, *Pharmacia Downstream*, **22**, 22–23.

CHAPMAN, A.P., CHAPLIN, L.C., SPITALI, M., WEIR, N., SEHDEV, M., LAWSON, A.D.G. and KING, D.J., 1996, Purification of recombinant Fab′ fragments using Streamline A in expanded or batch mode. Presented at *1st International Conference on Expanded Bed Adsorption*, Cambridge.

CHARI, R.V.J., JACKEL, K.A., BOURRET, L.A., DERR, S.M., TADAYONI, B.M., MATTOCKS, K.M., SHAH, S.A., LIU, C., BLATTLER, W.A. and GOLDMACHER, V.S., 1995, Enhancement of the selectivity and antitumour efficacy of a CC-1065 analogue through immunoconjugate formation, *Cancer Res.*, **55**, 4079–4084.

CHARI, R.V.J., 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 anticancer drugs, *Cancer Res.*, **52**, 127–131.

CHARNOW, S.M. and ASHKENAZI, A., 1996, Immunoadhesins: principles and applications, *Trends Biotechnol.*, **14**, 52–60.

CHEN, F. and EPSTEIN, A.L., 1988, Preparative separation of IgM monoclonal antibody from mouse ascites and purification of F(ab′)2 fragments – the use of Abx and Superose columns, *Antibod. Immunoconj. Radiopharm.*, **1**, 333–341.

CHEN, S.Y., ZANI, C., KHOURI, Y. and MARASCO, W.A., 1995, Design of a genetic immunotoxin to eliminate toxin immunogenicity, *Gene Therapy*, **2**, 116–123.

CHIANG, C.M. and ROEDER, R.G., 1993, Expression and purification of general transcription factors by FLAG epitope tagging and peptide elution, *Peptide Res.*, **6**, 62–64.

CHRISTOFIDES, N.D. and SHEEHAN, C.P., 1995, Enhanced chemiluminescence labeled-antibody immunoassay (Amerlite-MAB™) for free thyroxine: design, development and technical validation, *Clin. Chem.*, **41**, 17–23.

CLACKSON, T., HOOGENBOOM, H.R., GRIFFITHS, A.D. and WINTER, G., 1991, Making antibody fragments in phage display libraries, *Nature*, **352**, 624–628.

CLARK, P.M.S. and PRICE, C.P., 1986, Enzyme amplified immunoassays: a new ultrasensitive assay of thyrotropin evaluated, *Clin. Chem.*, **32**, 88–92.

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.

CO, M.S., SCHEINBERG, D.A., AVDALOVIC, N.M., MCGRAW, K., VASQUEZ, M., CARON, P.C. and QUEEN, C., 1993, Genetically engineered deglycosylation of the variable domain increases the affinity of an anti-CD33 monoclonal antibody, *Mol. Immunol.*, **30**, 1361–1367.

COCKETT, M.I., 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.

COLBERT, D.L., GALLACHER, G. and MAINWARING-BURTON, R.W., 1985, Single reagent polarization fluoroimmunoassay for amphetamine in urine, *Clin. Chem.*, **31**, 1193–1195.

COLE, W.C., DENARDO, S.J., MEARES, C.F., MCCALL, M.J., DENARDO, G.L., EPSTEIN, A.L., O'BRIEN, H.A. and MOI, M.K., 1987, Comparative serum stability of radiochelates for antibody radiopharmaceuticals, *J. Nucl. Med.*, **28**, 83–90.

COLMAN, A., 1996, Production of proteins in the milk of transgenic livestock: problems, solutions and successes, *Am. J. Clin. Nutrition*, **63**, 639S–645S.

COLOMA, M.J. and MORRISON, S.L., 1997, Design and production of novel tetravalent bispecific antibodies, *Nature Biotechnol.*, **15**, 159–163.

CONNETT, J.M., ANDERSON, C.J., GUO, L.W., SCHWARZ, S.W., ZINN, K.R., ROGERS, B.E., SIEGAL, B.A., PHILPOTT, G.W. and WELCH, M.J., 1996, Radioimmunotherapy with a ^{64}Cu-labeled monoclonal antibody: a comparison with ^{67}Cu, *Proc. Natl Acad. Sci. (USA)*, **93**, 6814–6818.

COOK, A.G. and WOOD, P.J., 1994, Chemical synthesis of bispecific monoclonal antibodies: potential advantages in immunoassay systems, *J. Immunol. Methods*, **171**, 227–237.

COOK, C.J., 1997, Real-time measurements of corticosteroids in conscious animals using an antibody-based electrode, *Nature Biotechnol.*, **15**, 467–471.

COOK, D.B. and SELF, C.H., 1993, Determination of one thousandth of an attomole (1 zeptomole) of alkaline phosphatase: application in an immunoassay of proinsulin, *Clin. Chem.*, **39**, 965–971.

COOPER, L.J.N., SHIKHMAN, A.R., GLASS, D.D., KANGISSER, D., CUNNINGHAM, M.W. and GREENSPAN, N.S., 1993, Role of heavy chain constant domains in antibody–antigen interaction, *J. Immunol.*, **150**, 2231–2242.

COVELL, D.G., BARBET, J., HOLTON, O.D., BLACK, C.D.V., PARKER, R.J. and WEINSTEIN, J.N., 1986, Pharmacokinetics of monoclonal immunoglobulin G1 F(ab′)$_2$ and F(ab′) in mice, *Cancer Res.*, **46**, 3969–3978.

COX, J.P., JANKOWSKI, K.J., KATAKY, R., PARKER, D., BEELEY, N.R.A., BOYCE, B.A., EATON, M.A.W., MILLAR, K., MILLICAN, A.T., HARRISON, A. and WALKER, C., 1989, Synthesis of a kinetically stable yttrium-90 labelled macrocycle–antibody conjugate, *J. Chem. Soc. Chem. Commun.*, **1989**, 797–798.

CRAIG, A.S., HELPS, I.A., JANKOWSKI, K.J., PARKER, D., BEELEY, N.R.A., BOYCE, B.A., EATON, M.A.W., MILLICAN, T.A., MILLAR, K., PHIPPS, A., RHIND, S.K., HARRISON, A. and WALKER, C., 1989, Towards tumour imaging with indium-111 labelled macrocycle antibody conjugates, *J. Chem. Soc. Chem. Commun.*, **12**, 794–796.

CRIPPA, F., BOLIS, G., SEREGNI, E., GAVONI, N., SCARFONE, G., FERRARIS, C., BURAGGI, G.L. and BOMBARDIERI, E., 1995, Single-dose intraperitoneal radioimmunotherapy with the murine monoclonal antibody I-131 Mov18, *Eur. J. Cancer*, **31A**, 686–690.

CROWE, J.S., HALL, V.S., SMITH, M.A., COOPER, H.J. and TITE, J.P., 1992, Humanized monoclonal antibody CAMPATH-1: myeloma cell expression of genomic constructs, nucleotide sequence of cDNA constructs and comparison of effector mechanisms of myeloma and Chinese hamster ovary cell-derived material, *Clin. Exp. Immunol.*, **87**, 105–110.

CUMBER, A.J., WARD, E.S., WINTER, G., PARNELL, G.D. and WAWRZYNCZAK, E.J., 1992, Comparative stabilities in vitro and in vivo of a recombinant mouse antibody FvCys and a bisFvCys conjugate, *J. Immunol.*, **149**, 120–126.

CZUCZMAN, M.S., GRILLO-LOPEZ, A.J., JONAS, C., GORDON, L., SALEH, M., WHITE, C.A., VARNS, C. and ROGERS, J., 1995, IDEC-C2B8 and CHOP chemotherapy of low-grade lymphoma, *Blood*, **86**, Suppl. 1, 55A.

DANIELS, P.B., FLETCHER, J.E., O'NEILL, P.M., STAFFORD, C.G., BACARESE-HAMILTON, T. and ROBINSON, G.A., 1995, A comparison of three fluorophores for use in an optical biosensor for the measurement of prostate-specific antigen in whole blood, *Sensors Actuators*, **B26**, 447–451.

DANIELSSON, L., MOLLER, S.A. and BORREBAECK, C.A.K., 1987, Effect of cytokines on specific in vitro immunization of human peripheral B lymphocytes against T-cell dependent antigens, *Immunology*, **61**, 51–55.

DARVEAU, A., CHEVRIER, M.C., NERON, S., DELAGE, R. and LEMIEUX, R., 1993, Efficient preparation of human monoclonal antibody-secreting heterohybridomas using peripheral B lymphocytes cultured in the CD40 system, *J. Immunol. Methods*, **159**, 139–143.

DAS, C., KULKARNI, P.V., CONSTANTINESCU, A., ANTICH, P., BLATTNER, F.R. and TUCKER, P.W., 1992, Recombinant antibody–metallothionein: design and evaluation for radioimmunoimaging, *Proc. Natl Acad. Sci. (USA)*, **89**, 9749–9753.

DATTA, P., HINZ, V. and KLEE, G., 1996, Superior specificity of monoclonal based assay for digoxin, *Clin. Biochem.*, **29**, 541–547.

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, I.W., 1992, Rapid immunoassays, *Anal. Proc.*, **29**, 459–460.

DAVIES, J. and RIECHMANN, L., 1995, Antibody VH domains as small recognition units, *Bio/Technol.*, **13**, 475–479.

DAVIS, A.C., ROUX, K.H. and SHULMAN, M.J., 1988, On the structure of polymeric IgM, *Eur. J. Immunol.*, **18**, 1001–1008.

DEBINSKI, W. and PASTAN, I., 1995, Recombinant Fab′ C242-Pseudomonas exotoxin, but not the whole antibody based immunotoxin causes regression of a human colorectal tumor xenograft, *Clin. Cancer Res.*, **1**, 1015–1022.

DEFAUCAL, P., PELTIER, P., PLANCHON, B., DUPAS, B., TONZE, M.D., BARON, D., SCIABLE, T., BERGER, H.J. and CHATAL, J.F., 1991, Evaluation of indium-111 labelled antifibrin monoclonal antibody for the diagnosis of venous thrombotic disease, *J. Nucl. Med.*, **32**, 785–791.

DEISENHOFER, J., 1981, Crystallographic refinement and atomic models of a human Fc fragment and its complex with fragment B of protein A from *Staphylococcus aureus* at 2.9 and 2.8 Å resolution, *Biochem.*, **20**, 2361–2370.

DE LALLA, C., TAMBORINI, E., LONGHI, R., TRESOLDI, E., MANONI, M., SICCARDI, A.G., AROSIO, P. and SIDOLI, A., 1996, Human recombinant antibody fragment specific for a rye grass pollen allergen, *Mol. Immunol.*, **33**, 1049–1058.

DELGADO, C., PEDLEY, R.B., HERRAEZ, A., BODEN, R., BODEN, J.A., KEEP, P.A., CHESTER, K.A., FISHER, D., BEGENT, R.H.J. and FRANCIS, G.E., 1996, Enhanced tumour specificity of an anti-carcinoembrionic antigen Fab′ fragment by poly(ethylene glycol) (PEG) modification, *Br. J. Cancer*, **73**, 175–182.

DEMANET, C., BRISSINCK, J., DE JONGE, J. and THIELEMANS, K., 1996, Bispecific antibody-mediated immunotherapy of the BCL1 lymphoma: increased efficacy with multiple injections and CD28 induced costimulation, *Blood*, **10**, 4390–4398.

DEMIDEN, A., HANNA, N., HARIHARAN, H. and BONAVIDA, B., 1995, Chimeric anti-CD20 antibody (IDEC-C2B8) is apoptopic and sensitizes drug resistant human B cell lymphomas and AIDS related lymphomas to the cytotoxic effect of CDDP, VP-16 and toxins, *FASEB J.*, **9**, A206.

DENARDO, S.J., O'GRADY, L.F., RICHMAN, C.M. and DENARDO, G.L., 1994, Overview of radio-immunotherapy in advanced breast cancer using 131-I chimeric L6, *Adv. Exp. Med. Biol.*, **353**, 203–211.

DENARDO, D.A., DENARDO, G.L., YUAN, A., SHEN, S., DENARDO, S.J., MACEY, D.J., LAMBORN, K.R., MAHE, M., GROCH, M.W. and ERWIN, W.D., 1996a, Prediction of radiation doses from therapy using tracer studies with iodine-131-labeled antibodies, *J. Nucl. Med.*, **37**, 1970–1975.

DENARDO, G.L., MIRICK, G.R., KROGER, L.A., O'DONNELL, R.T., MEARES, C.F. and DENARDO, S.J., 1996b, Antibody responses to macrocycles in lymphoma, *J. Nucl. Med.*, **37**, 451–456.

DENEVE, M., DELOOSE, M., JACOBS, A., VAN HOUDT, H., KALUZA, B., WEIDLE, U., VAN MONTAGU, M. and DEPICKER, A., 1993, Assembly of an antibody and its derived antibody fragment in *Nicotiana* and *Arabidopsis*, *Transgenic Res.*, **2**, 227–237.

DERRICK, J.P. and WIGLEY, D.B., 1992, Crystal structure of a streptococcal protein G domain bound to a Fab fragment, *Nature*, **359**, 752–754.

DESHPANDE, S.V., DENARDO, S.J., KUKIS, D.L., MOI, M.K., MCCALL, M.J., DENARDO, G.L. and MEARES, C.F., 1990, Yttrium-90 labelled monoclonal antibody for therapy: labelling by a new macrocyclic bifunctional chelating agent, *J. Nucl. Med.*, **31**, 473–479.

DESMYTER, A., TRANSUE, T.R., GHAHROUDI, M.A., THI, M.H., POORTMANS, F., HAMERS, R., MUYLDERMANS, S. and WYNS, L., 1996, Crystal structure of a camel single-domain VH antibody fragment in complex with lysozyme, *Nature Struct. Biol.*, **3**, 808–811.

DESPLANCQ, D., KING, D.J., LAWSON, A.D.G. and MOUNTAIN, A., 1994, Multimerization behaviour of single chain Fv variants for the tumour binding antibody B72.3, *Protein Engin.*, 7, 1027–1033.

DIAMANDIS, E.P., 1988, Immunoassays with time-resolved fluorescence spectroscopy: principles and applications, *Clin. Biochem.*, **21**, 139–150.

DIAMANDIS, E., 1991, Multiple labeling and time-resolvable fluorophores, *Clin. Chem.*, 37, 1486–1491.

DITZEL, H.J., BINLEY, J.M., MOORE, J.P., SODROSKI, J., SULLIVAN, N., SAWYER, L.S.W., HENDRY, R.M., YANG, W.P., BARBAS, C.F., III, and BURTON, D.R., 1995, Neutralizing recombinant human antibodies to a conformational V2 and CD4-binding site-sensitive epitope of HIV-1 gp120 isolated by using an epitope masking procedure, *J. Immunol.*, **154**, 893–906.

DOERR, R.J., ABDEL-NABI, H., KRAG, D. and MITCHELL, E., 1991, Radiolabelled antibody imaging in the management of colorectal carcinoma, *Ann. Surgery*, **214**, 118–124.

DOHLSTEIN, M., ABRAHMSEN, L., BJORK, P., LANDO, P.A., HEDLUND, G., FORSBERG, G., BRODIN, T., GASCOIGNE, N.R.J., FORBERG, C., LIND, P. and KALLAND, T., 1994, Monoclonal antibody–superantigen fusion proteins: tumor-specific agents for T-cell based tumor therapy, *Proc. Natl Acad. Sci. (USA)*, **91**, 8945–8949.

DOHLSTEIN, M., HANSSON, J., OHLSSON, L., LITTON, M. and KALLAND, T., 1995, Antibody-targeted superantigens are potent inducers of tumor-infiltrating lymphocytes *in vivo*, *Proc. Natl Acad. Sci. (USA)*, **92**, 9791–9795.

DONOHOE, P.J., MACARDLE, P.J. and ZOLA, H., 1995, Making and using conventional mouse monoclonal antibodies, *Monoclonal Antibodies, the Second generation* (Zola, H., ed.), pp. 15–42, Bios Scientific Publishers, Oxford.

DORAI, H., MCCARTNEY, J.E., HUDZIAK, R.M., TAI, M.S., LAMINET, A.A., HOUSTON, L.L., HUSTON, J.S. and OPPERMANN, H., 1994, Mammalian cell expression of single-chain Fv (sFv) antibody proteins and their C-terminal fusions with interleukin-2 and other effector domains, *Biol Technol.*, **12**, 890–897.

DORAI, H. and MOORE, G.P., 1987, The effect of dihydrofolate reductase-mediated gene amplification on the expression of transfected immunoglobulin genes, *J. Immunol.*, **139**, 4232–4241.

DOUGALL, W.C., PETERSON, N.C. and GREENE, M.I., 1994, Antibody-structure-based design of pharmacological agents, *Trends Biotechnol.*, **12**, 372–379.

DOUGLAS, A.S. and MONTEITH, C.A., 1994, Improvements to immunoassays by use of covalent binding assay plates, *Clin. Chem.*, **40**, 1833–1837.

DRAGACCI, S., GLEIZES, E., FREMY, J.M. and CANDLISH, A.A., 1995, Use of immunoaffinity chromatography as a purification step for the determination of aflatoxin M1 in cheeses, *Food Additives Contaminants*, **12**, 59–65.

DUAN, L., BAGASRA, O., LAUGHLIN, M.A., OAKES, J.W. and POMERANTZ, R.J., 1994, Potent inhibition of human immunodeficiency virus type 1 replication by an intracellular anti-rev single-chain antibody, *Proc. Natl Acad. Sci. (USA)*, **91**, 5075–5079.

DUCANCEL, F., GILLET, D., CARRIER, A., LAJEUNESSE, E., MENEZ, A. and BOULAIN, J.C., 1993, Recombinant colorimetric antibodies: construction and characterization of a bifunctional $F(ab)_2$/alkaline phosphatase conjugate produced in *Escherichia coli*, *Bio/Technol.*, **11**, 601–605.

DUCHOSAL, M.A., EMING, S.A., FISCHER, P., LETURCQ, D., BARBAS, C.F., III, MCCONAHEY, P.J., CAOTHEIN, 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.

DUENAS, M., VAZQUEZ, J., AYALA, M., SODRLIND, E., OHLIN, M., PEREZ, L., BORREBAECK, C.A.K. and GAVILONDO, J.V., 1994, Intra- and extracellular expression of an scFv antibody fragment in *E. coli*: effect of bacterial strains and pathway engineering using GroES/L chaperonins, *BioTechniques*, **16**, 476–483.

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.

DYER, M.J.S., HALE, G., HAYHOE, F.G.J. and WALDMANN, H., 1989, Effects of CAMPATH-1 antibodies *in vivo* in patients with lymphoid malignancies: influence of antibody isotype, *Blood*, **73**, 1431–1439.

EARY, J., SCHROFF, R.W., ABRAMS, P.G., FRITZBERG, A.R., MORGAN, A.C., KASINA, S., RENO, J.M., SRINIVASAN, A., WOODHOUSE, C.S., WILBUR, D.S., NATALE, R.B., COLLINS, C., STEHLIN, J.S., MITCHELL, M. and NELP, W.B., 1989, Successful imaging of malignant melanoma with technetium-99m labeled monoclonal antibodies, *J. Nucl. Med.*, **30**, 25–32.

EGAN, R.W., ATHWAL, D., CHOU, C.C., EMTAGE, S., JEHN, C.H., KUNG, T.T., MAUSER, P.J., MURGOLO, N.J. and BODMER, M.W., 1995, Inhibition of pulmonary eosinophilia and hyperreactivity by antibodies to interleukin-5, *Int. Arch. Allergy Immunol.*, **107**, 321–322.

EISENHUT, M., LEHMANN, W.D., BECKER, W., BEHR, T., ELSER, H., STRITTMATTER, W., STEINSTRASSER, A., BAUM, R.P., VALERIUS, T., REPP, R. and DEO, Y., 1996, Bifunctional NHS-BAT ester for antibody conjugation and stable technetium labeling, *J. Nucl. Med.*, **37**, 362–370.

ELDIN, P., PAUZA, M.E., HIEDA, Y., LIN, G., MUTAUGH, M.P., PENTEL, P.R. and PENNELL, C.A., 1997, High-level secretion of two antibody single chain Fv fragments by *Pichia pastoris*, *J. Immunol. Methods*, **201**, 67–75.

ELLIOTT, M.J., MAINI, R.N., FELDMANN, M., KALDEN, J.R., ANTONI, C., SMOLEN, J.S., LEEB, B., BREEDFELD, F.C., MACFARLANE, J.D., BIJL, H. and WOODY, J.N., 1994, Randomised double-blind comparison of chimeric monoclonal antibody to tumor necrosis factor α (cA2) versus placebo in rheumatoid arthritis, *Lancet*, **344**, 1105–1110.

ELLIS, J.H., BARBER, K.A., TUTT, A., HALE, C., LEWIS, A.P., GLENNIE, M.J., STEVENSON, G.T. and CROWE, J.S., 1995, Engineered anti-CD38 monoclonal antibodies for immunotherapy of multiple myeloma, *J. Immunol.*, **155**, 925–937.

ERNTELL, M., MYHRE, E.B. and KRONVALL, G., 1983, Alternative non-immune IgG $F(ab')_2$ binding to group C and G streptococci, *Scand. J. Immunol.*, **17**, 201–209.

ESHHAR, Z., WAKS, T., GROSS, G. and SCHINDLER, D.G., 1993, Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the γ or ζ subunits of the immunoglobulin and T-cell receptors, *Proc. Natl Acad. Sci. (USA)*, **90**, 720–724.

ESKELAND, T. and CHRISTENSEN, T.B., 1975, IgM molecules with and without J chain in serum and after purification, studied by ultracentrifugation, electrophoresis, and electron microscopy, *Scand. J. Immunol.*, **4**, 217–228.

EVANS, M.J., ROLLINS, S.A., WOLFF, D.W., ROTHER, R.P., NORIN, A.J., THERRIEN, D.M., GRIJALVA, G.A., MUELLER, J.P., NYE, S.H., SQUINTO, S.P. and WILKINS, J.A., 1995, In vitro and in vivo inhibition of complement activity by a single chain Fv fragment recognizing human C5, *Mol. Immunol.*, **32**, 1183–1195.

EXLEY, D. and EKEKE, G.I., 1981, Fluoroimmunoassay for 5-dihydrotestosterone, *J. Steroid Biochem.*, **14**, 1297–1302.

FAGERBERG, J., RAGNHAMMAR, P., LILJEFORS, M., HJELM, A.L., MELLSTEDT, H. and FRODIN, J.E., 1996, Humoral anti-idiotypic and anti-anti-idiotypic immune response in cancer patients treated with monoclonal antibody 17-1A, *Cancer Immunol. Immunother.*, **42**, 81–87.

FAGNANI, R., HAGAN, M.S. and BARTHOLOMEW, R., 1990, Reduction of immunogenicity by covalent modification of murine and rabbit immunoglobulins with oxidized dextrans of low molecular weight, *Cancer Res.*, **50**, 3638–3645.

FALLGREEN-GEBAUER, E., GEBAUER, W., BASTIAN, A., KRATZIN, H.D., EIFFERT, H., ZIMMERMAN, B., KARAS, M. and HILSCHMAN, N., 1993, The covalent linkage of secretory component to IgA. Structure of sIgA, *Biol. Chem. Hoppe-Seyler*, **374**, 1023–1028.

FANGER, M.W. and GUYRE, P.M., 1991, Bispecific antibodies for targeted cellular cytotoxicity, *Trends Biotechnol.*, **9**, 375–380.

FEIT, C., BARTAL, A.H., TAUBER, G., DUMBORT, G. and HIRSHAUT, Y., 1983, An enzyme-linked immunosorbent assay (ELISA) for the detection of monoclonal antibodies recognizing surface antigens expressed on viable cells, *J. Immunol. Methods*, **58**, 301–308.

FELDMANN, M., ELLIOT, M.J., WOODY, J.N. and MAINI, R.N., 1997, Anti-tumor necrosis factor – a therapy of rheumatoid arthritis, *Adv. Immunol.*, **64**, 283–350.

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 Fab′ with specificity for carcinomas and human IL-2, *J. Immunol.*, **146**, 2446–2452.

FERGUSON, R.A., YU, H., KALYVAS, M., ZAMMIT, S. and DIAMANDIS, E.P., 1996, Ultrasensitive detection of prostate-specific antigen by a time-resolved immunofluorometric assay and the immulite immunochemiluminescent third-generation assay: potential application in prostate and breast cancers, *Clin. Chem.*, **42**, 675–684.

FIEDLER, U. and CONRAD, U., 1995, High-level production and long-term storage of engineered antibodies in transgenic tobacco seeds, *Bio/Technol.*, 13, 1090–1093.

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, *Protein Engin.*, **3**, 641–647.

FIELDS, B.A., GOLDBAUM, F.A., YSERN, X., POLJAK, R. and MARIUZZA, R.A., 1995, Molecular basis of antigen mimicry by an anti-idiotope, *Nature*, **374**, 739–742.

FISCH, I., KUNZI, G., ROSE, K. and OFFORD, R.E., 1992, Site-specific modification of a fragment of a chimeric monoclonal antibody using reverse proteolysis, *Bioconj. Chem.*, **3**, 147–153.

FISHWILD, D.M., O'DONNELL, S.L., BENGOECHEA, T., HUDSON, D.V., HARDING, F., BERNHARD, S.L., JONES, D., KAY, R.M., HIGGINS, K.M., SCHRAMM, S.R. and LONBERG, N., 1996, High-avidity human IgGk monoclonal antibodies from a novel strain of minilocus transgenic mice, *Nature Biotechnol.*, **14**, 845–851.

FOOTE, J. and WINTER, G., 1992, Antibody framework residues affecting the conformation of the hypervaraiable loops, *J. Mol. Biol.*, **224**, 487–499.

FOSANG, A.J., LAST, K. and MACIEWICZ, R.A., 1996, Aggrecan is degraded by matrix metalloproteinases in human arthritis, *J. Clin. Invest*, **98**, 2292–2299.

FOWELL, S.A. and CHASE, H.A., 1986, A comparison of some activated matrices for preparation of immunosorbents, *J. Biotechnol.*, **4**, 355–368.

FRANKEN, E., TEUSCHEL, U. and HAIN, R., 1997, Recombinant proteins from transgenic plants, *Curr. Opin. Biotechnol.*, **8**, 411–416.

FRENCH, R.R., HAMBLIN, T.J., BELL, A.J., TUTT, A.L. and GLENNIE, M., 1995, Treatment of B-cell lymphoma with combination of bispecific antibodies and saporin, *Lancet*, **346**, 223–224.

FRIEDMAN, P.N., CHACE, D.F., TRAIL, P.A. and SIEGALL, C.B., 1993, Antitumor activity of the single-chain immunotoxin BR96 sFv-PE40 against established breast and lung tumor xenografts, *J. Immunol.*, **150**, 3054–3061.

FRITSCH, G., STIMPFL, M., KURZ, M., LEITNER, A., PRINTZ, D., BUCHINGER, P., HOECKER, P. and GADNER, H., 1995, Characterization of hematopoietic stem cells, *Ann. NY Acad. Sci.*, **770**, 42–52.

FRITZBERG, A.R., ABRAMS, P.G., BEUMIER, P.L., KASINA, S., MORGAN, A.C., RAO, T.N., RENO, J.M., SANDERSON, J.A., SRINIVASAN, A., WILBUR, D.S. and VANDERHEYDEN, J.L., 1988, Specific and stable labeling of antibodies with technetium-99m with a diamide dithiolate chelating agent, *Proc. Natl Acad. Sci. (USA)*, **85**, 4025–4029.

GADINA, M., NEWTON, D.L., RYBAK, S.M., WU, Y.N. and YOULE, R.J., 1994, Humanized immunotoxins, *Ther. Immunol.*, **1**, 59–64.

GANDECHA, A., OWEN, M.R.L., COCKBURN, W. and WHITELAM, G.C., 1994, Antigen detection using recombinant bifunctional single-chain Fv fusion proteins synthesised in *Escherichia coli*, *Protein Exp. Purific.*, **5**, 385–390.

GARKAVIJ, M., TENNVALL, J., STRAND, S.E., SJOGREN, H.O., JIANCHENG, C., NILSSON, R. and ISAKSSON, M., 1997, Extracorporeal whole-blood immunoadsorption enhances radioimmunotargeting of iodine-125-labeled BR96–biotin monoclonal antibody, *J. Nucl. Med.*, **38**, 895–901.

GARRARD, L.J., YANG, M., O'CONNELL, M.P., KELLEY, R.F. and HENNER, D.J., 1991, Fab assembly and enrichment in a monovalent phage display system, *Bio/Technol.*, **9**, 1373–1377.

GEORGE, A.J.T., JAMAR, F., TAI, M.S., HEELAN, B.T., ADAMS, G.P., MCCARTNEY, J.E., HOUSTON, L.L., WEINER, L.M., OPPERMANN, H., PETERS, A.M. and HUSTON, J.S., 1995, Radiometal labeling of recombinant proteins by a genetically engineered minimal chelation site, *Proc. Natl Acad. Sci. (USA)*, **92**, 8358–8362.

GHETIE, M.A., PODAR, E.M., ILGEN, A., GORDON, B.E., UHR, J.W. and VITETTA, E.S., 1997a, Homodimerization of tumor-reactive monoclonal antibodies markedly increases their ability to induce growth arrest or apoptosis of tumor cells, *Proc. Natl Acad. Sci. (USA)*, **94**, 7509–7514.

GHETIE, V., HUBBARD, J.G., KIM, J.K., TSEN, M.F., LEE, Y. and WARD, E.S., 1996, Abnormally short serum half lives of IgG in β2-microglobulin deficient mice, *Eur. J. Immunol.*, **26**, 690–696.

GHETIE, V., POPOV, S., BORVAK, J., RADU, C., MATESOI, D., MEDESAN, C., OBER, R.J. and WARD, E.S., 1997b, Increasing the serum persistence of an IgG fragment by random mutagenesis, *Nature Biotechnol.*, **15**, 637–640.

GHETIE, V. and VITETTA, E., 1994, Immunotoxins in the therapy of cancer: from bench to clinic, *Pharmac. Ther.*, **63**, 209–234.

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

GILLIES, S.D., LO, K.M. and WESLOWSKI, J., 1989b, High level expression of chimeric antibodies using adapted cDNA variable region cassettes, *J. Immunol. Methods*, **125**, 191–202.

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.

GILLIES, S.D., YOUNG, D., LO, K.M. and ROBERTS, S., 1993, Biological activity and *in vivo* clearance of antitumour antibody/cytokine fusion proteins, *Bioconj. Chem.*, **4**, 230–235.

GINSBERG, J.S., WELLS, P.S., BRILL-EDWARDS, P., DONOVAN, D., PANJU, A., VAN BEEK, J.R. and PATEL, A., 1995, Application of a novel and rapid whole blood assay for D-Dimer in patients with clinically suspected pulmonary embolism, *Thrombosis Haemostasis*, **73**, 35–38.

GIOVAGNOLI, M.R. and VECCHIONE, A., 1996, Immunocytochemistry of cytological specimens as a diagnostic and prognostic tool, *Anticancer Res.*, **16**, 2225–2232.

GLENNIE, M.J., MCBRIDE, H.M., WORTH, A.T. and STEVENSON, G.T., 1987, Preparation and performance of bispecific F(ab′γ)$_2$ antibody containing thioether-linked Fab′γ fragments, *J. Immunol.*, **139**, 2367–2375.

GLOCKSHUBER, R., MALIA, M., PFITZINGER, I. and PLUKTHUN, A., 1990, A comparison of strategies to stabilise immunoglobulin Fv fragments, *Biochem.*, **29**, 1362–1367.

GOLDBERG, M.E. and DJAVADI-OHANIANCE, L., 1993, Methods for measurement of antibody/antigen affinity based on ELISA and RIA, *Curr. Opin. Immunol.*, **5**, 278–281.

GOLDENBERG, D.M., GOLDENBERG, H., SHARKEY, R.M., HIGGINBOTHAM-FORD, E., LEE, R.E., SWAYNE, L.C., BURGER, K.A., TSAIN, D., HOROWITZ, J.A., HALL, T.C., PINSKY, C.M. and HANSEN, H.J., 1990, Clinical studies of radioimmunodetection with carcinoembryonic antigen monoclonal antibody fragments labelled with ^{123}I or ^{99m}Tc, *Cancer Res.*, **50**, 909–921.

GOODMAN, G.E., HELLSTROM, I., YELTON, D.E., MURRAY, J.L., O'HARA, S., MEAKER, E., ZEIGLER, L., PALAZOLLO, P., NICAISE, C., USAKEWICZ, J. and HELLSTROM, K.E., 1993, Phase I trial of chimeric (human–mouse) monoclonal antibody L6 in patients with non-small-cell lung, colon, and breast cancer, *Cancer Immunol. Immunother.*, **36**, 267–273.

GOODWIN, D.A., MEARES, C.F., MCCALL, M.J., MCTIGUE, M. and CHAOVAPONG, W., 1988, Pretargeted immunoscintigraphy of murine tumours with indium-111 labeled bifunctional haptens, *J. Nucl. Med.*, **29**, 226–234.

GOODWIN, D.A., MEARES, C.F., WATANABE, N., MCTIGUE, M., CHAOVAPONG, W., RANSONE, C.M., RENN, O., GREINER, D.P., KUKIS, D.L. and KRONENBERGER, S.I., 1994, Pharmacokinetics or pretargeted monoclonal antibody 2D12.5 and ^{88}Y-janus-2-(*p*-nitrobenzyl)-1,4,7,10-tetraazacyclododecanetetraacetic acid (DOTA) in BALB/c mice with KHJ mouse adenocarcinoma: a model for radioimmunotherapy, *Cancer Res.*, **54**, 5937–5946.

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

GORMAN, S.C., CLARK, M.E., ROUTLEDGE, E.G., COBBOLD, S.P. and WALDMANN, H., 1991, Reshaping a therapeutic CD4 antibody, *Proc. Natl Acad. Sci. (USA)*, **88**, 4181–4185.

GOSHORN, S.C., SVENSSON, H.P., KERR, D.E., SOMERVILLE, J.E., SENTER, P. and FELL, H.P., 1993, Genetic construction, expression and characterization of a single-chain anti-carcinoma antibody fused to β-lactamase, *Cancer Res.*, **53**, 2123–2127.

GRAHAM, B.M., PORTER, A.J. and HARRIS, W.J., 1995, Cloning, expression and characterization of a single-chain antibody fragment to the herbicide paraquat, *J. Chem. Technol. Biotechnol.*, **63**, 279–289.

GREEN, L.L., HARDY, M.C., MAYNARD-CURRIE, C.E., TSUDA, H., LOUIE, D.M., MENDEZ, M.J., ABDERRAHIM, H., NOGUCHI, M., SMITH, D.H., ZENG, Y., DAVID, N.E., SASAI, H., GARZA, D., BRENNER, D.G., HALES, J.F., MCGUINNESS, R.P., CAPON, D.J., KLAPHOLZ, S. and JAKOBOVITS, A., 1994, Antigen-specific human monoclonal antibodies from mice engineered with human Ig heavy and light chain YACs, *Nature Genetics*, **7**, 13–21.

GREENMAN, J., JONES, E., WRIGHT, M.D. and BARCLAY, A.N., 1996, The use of intracellular single-chain antibody fragments to inhibit specifically the expression of cell surface molecules, *J. Immunol. Methods*, **194**, 169–180.

GREENWOOD, J., CLARK, M. and WALDMANN, H., 1993, Structural motifs involved in human IgG antibody effector functions, *Eur. J. Immunol.*, **23**, 1098–1104.

GRIFFITHS, A.D., MALMQVIST, M., MARKS, J.D., BYE, J.M., EMBLETON, M.J., MCCAFFERTY, J., BAIER, M., HOLLIGER, K.P., GORICK, B.D., HUGHES-JONES, N.C., HOOGENBOOM, H.R. and WINTER, G., 1993, Human anti-self antibodies with high specificity from phage display libraries, *EMBO J.*, **12**, 725–734.

GRIFFITHS, A.D., WILLIAMS, S.C., HARTLEY, O., TOMLINSON, I.M., WATERHOUSE, P., CROSBY, W.L., KONTERMANN, R.E., JONES, P.T., LOW, N.M., ALLISON, T.J., PROSPERO, T.D., HOOGENBOOM, H.R., NISSIM, A., COX, J.P.L., HARRISON, J.L., ZACCOLO, M., GHERARDI, E. and WINTER, G., 1994, Isolation of high affinity human antibodies directly from large synthetic repertoires, *EMBO J.*, **13**, 3245–3260.

GRUBER, M., SCHODIN, B.A., WILSON, E.R. and KRANZ, D.M., 1994, Efficient tumour cell lysis mediated by a bispecific single chain antibody expressed in *Escherichia coli*, *J. Immunol.*, **152**, 5368–5374.

GUAN, X.M., KOBILKA, T.S. and KOBILKA, B.K., 1992, Enhancement of membrane insertion and function in a type IIIb membrane protein following introduction of a cleavable signal peptide, *J. Biol. Chem.*, **267**, 21995–21998.

GUDDAT, L.W., SHAN, L., ANCHIN, J.M. and EDMUNDSON, A.B., 1994, Local and transmitted conformational changes on complexation of an anti-sweetener Fab, *J. Mol. Biol.*, **236**, 247–274.

GURTLER, L., 1996, Difficulties and strategies of HIV diagnosis, *Lancet*, **348**, 176–179.

GUSTAFSSON, B. and HINKULA, J., 1994, Antibody production of a human EBV-transformed B cell line and its heterohybridoma and trioma cell line descendants in different culture systems, *Human Antibod. Hybridomas*, **5**, 98–104.

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.

HALE, G., DYER, M., 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.

HANK, J.A., SURFUS, J.E., GAN, J., JAEGER, P., GILLIES, S.D., REISFELD, R.A. and SONDEL, P.M., 1996, Activation of human effector cells by a tumor reactive recombinant anti-ganglioside GD2 interleukin-2 fusion protein (ch14.18-IL2), *Clin. Cancer Res.*, **2**, 1951–1959.

HANSSON, J., OHLSSON, L., PERSSON, R., ANDERSSON, G., ILBACK, N.G., LITTON, M.J., KALLAND, T. and DOHLSTEIN, M., 1997, Genetically engineered superantigens as tolerable antitumor agents, *Proc. Natl Acad. Sci. (USA)*, **94**, 2489–2494.

HARLOW, E. and LANE, D., 1988, *Antibodies: a Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

HARRIS, L.J., LARSON, S.B., HASEL, K.W. and MCPHERSON, A., 1997, Refined structure of an intact IgG2a monoclonal antibody, *Biochem.*, **36**, 1581–1597.

HARRISON, A., WALKER, C.A., PARKER, D., JANKOWSKI, K.J., COX, J.P.L., CRAIG, A.S., SANSOM, J.M., BEELEY, N.R.A., BOYCE, R.A., CHAPLIN, L.C., EATON, M.A.W., FARNSWORTH, A.P.H., MILLAR, K., MILLICAN, A.T., RANDALL, A.M., RHIND, S.K., SECHER, D.S. and TURNER, A., 1991, The *in vivo* release of ^{90}Y from cyclic and acyclic ligand–antibody conjugates, *Nucl. Med. Biol.*, **18**, 469–476.

HARTMANN, F., HORAK, E.M., GARMESTANI, K., WU, C., BRECHBIEL, M.W., KOZAK, R.W., TSO, J., KOSTEINY, S.A., GANSOW, O.A., NELSON, D.L. and WALDMANN, T.A., 1994, Radioimmunotherapy of nude mice bearing a human interleukin 2 receptor α-expressing lymphoma utilizing the α-emitting radionuclide-conjugated monoclonal antibody ^{212}Bi–anti-Tac, *Cancer Res.*, **54**, 4362–4370.

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

HAUNSCHILD, J., FARO, H.P., PACK, P. and PLUCKTHUN, A., 1995, Pharmacokinetic properties of bivalent miniantibodies and comparison to other immunoglobulin forms, *Antibod. Immunoconj. Radiopharm.*, **8**, 111–128.

HEMMINKI, A., HOFFREN, A.M., TAKKINEN, K., VEHNIAINEN, M., MAKINEN, M.L., PETTERSSON, K., TELEMAN, O., SODERLUND, H. and TEERI, T.T., 1995, Introduction of lysine residues on the light chain constant domain improves the labelling properties of a recombinant Fab′ fragment, *Protein Engin.*, **8**, 185–191.

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.

HENDERSON, D.R., FRIEDMAN, S.B., HARRIS, J.D., MANNING, W.B. and ZOCCOLO, M.A., 1986, CEDIA™, a new homogeneous immunoassay system, *Clin. Chem.*, **32**, 1637–1641.

HENDRICKSON, E.R., HATFIELD-TRUBY, T.M., JOERGER, R.D., MAJARIAN, W.R. and EBERSOLE, R.C., 1995, High sensitivity multianalyte immunoassay using covalent DNA labelled antibodies and polymerase chain reaction, *Nucl. Acids Res.*, **23**, 522–529.

HERLYN, D., SOMASUNDARAM, R., LI, W. and MARAYUMA, H., 1996, Anti-idiotype cancer vaccines: past and future, *Cancer Immunol. Immunother.*, **43**, 65–76.

HIATT, A., 1990, Antibodies produced in plants, *Nature*, **344**, 469–470.

HIATT, A., CAFFERKEY, R. and BOWDISH, K., 1989, Production of antibodies in transgenic plants, *Nature*, **342**, 76–78.

HINKLE, G.H., MOJZISIK, C.M., LOESCH, J.A., HILL, T.L., THURSTON, M.O., SAMPSEL, J., OLSEN, J. and MARTIN, E.W., 1991, The evolution of the radioimmunoguided surgery system: an innovative technique for the intraoperative detection of tumour, *Antibody Immunoconj. Radiopharm.*, **4**, 339–358.

HINMAN, L.M., HAMANN, P.R., WALLACE, R., MENENDEZ, A.T., DURR, F.E. and UPESLACIS, J., 1993, Preparation and characterization of monoclonal antibody conjugates of the calicheamicins: a novel and potent family of antitumor antibiotics, *Cancer Res.*, **53**, 3336–3342.

HIRD, V., MARAVEYAS, A., SNOOK, D., DHOKIA, B., SOUTTER, W.P., MEARES, C., STEWART, J.S., MASON, P., LAMBERT, H.E. and EPENETOS, A.A., 1993, Adjuvant therapy of ovarian cancer with radioactive monoclonal antibody, *Br. J. Cancer*, **68**, 403–406.

HIRD, V., VERHOEYEN, M., BADLEY, R.A., PRICE, D., SNOOK, D., KOSMAS, C., GOODEN, C., BAMIAS, A., MEARES, C., LAVENDAR, J.P. and EPENETOS, A.A., 1991, Tumour localisation

with a radioactively labelled reshaped human monoclonal antibody, *Br. J. Cancer*, **64**, 911–914.

HNATOWICH, D.J., CHILDS, R.L., LANTEIGNE, D. and NAJAFI, A., 1983, The preparation of DTPA coupled antibodies radiolabelled with metallic radionuclides; an improved method, *J. Immunol. Methods*, **65**, 911–914.

HNATOWICH, D.J., MARDIROSSIAN, G., RUSCKOWSKI, M., FOGARASI, M., VIRZI, F. and WINNARD, P., 1993, Directly and indirectly technetium-99m-labeled antibodies – a comparison of *in vitro* and *in vivo* properties, *J. Nucl. Med.*, **34**, 109–119.

HNATOWICH, D.J., VIRZI, F. and RUCHOWSKI, M., 1987, Investigation of avidin and biotin for imaging applications, *J. Nucl. Med.*, **28**, 1294–1302.

HOLLIGER, P., BRISSINCK, J., WILLIAMS, R.L., THIELEMANS, K. and WINTER, G., 1996, Specific killing of lymphoma cells by cytotoxic T-cells mediated by a bispecific diabody, *Protein Engin.*, **9**, 299–305.

HOLLIGER, P., PROSPERO, T. and WINTER, G., 1993, 'Diabodies': small bivalent and bispecific antibody fragments, *Proc. Natl Acad. Sci. (USA)*, **90**, 6444–6448.

HOLLIGER, P., WING, M., POUND, J.D., BOHLEN, H. and WINTER, G., 1997, Retargeting serum immunoglobulin with bispecific diabodies, *Nature Biotechnol.*, **15**, 632–636.

HOLLIS, G.F. and MARK, G.E., 1995, *Homologous recombination antibody expression system for murine cells*, International patent application, WO95/17516.

HOLVOET, P., LAROCHE, Y., LIJNEN, H.R., VAN HOEF, B., BROUWERS, E., DE COCK, F., LAUWEREYS, M., GANSEMANS, Y. and COLLEN, D., 1991, Biochemical characterization of single-chain chimeric plasminogen activators consisting of single-chain Fv fragment of a fibrin-specific antibody and single-chain urokinase, *Eur. J. Biochem.*, **210**, 945–952.

HOLWILL, I., GILL, A., HARRISON, J., HOARE, M. and LOWE, P.A., 1996, Rapid analysis of biosensor data using the initial rate determination and its application to bioprocess monitoring, *Process Control Quality*, **8**, 133–145.

HOOGENBOOM, H.R., GRIFFITHS, A.D., JOHNSON, K.S., CHISWELL, D.J., HUDSON, P. and WINTER, G., 1991, Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains, *Nucl. Acids Res.*, **19**, 4133–4137.

HOPP, T.P., GALLIS, B. and PRICKETT, K.S., 1996, Metal-binding properties of a calcium dependent monoclonal antibody, *Mol. Immunol.*, **33**, 601–608.

HOPP, T.P., PRICKETT, K.S., PRICE, V., LIBBY, R.T., MARCH, C.J., CERRETTI, P., URDAL, D.L. and CONLON, P.J., 1988, A short polypeptide marker sequence useful for recombinant protein identification and purification, *Bio/Technol.*, **6**, 1204–1210.

HORI, A., SASADA, R., MATSUTANI, E., NAITO, K., SAKURA, Y., FUJITA, T. and KOZAI, Y., 1991, Suppression of solid tumour growth by immunoneutralising monoclonal antibody against human basic fibroblast growth factor, *Cancer Res.*, **51**, 6180–6184.

HORN, U., STRITTMATTER, W., KREBBER, A., KNUPFER, U., KUJAU, M., WENDEROTH, R., MULLER, K., MATZKU, S., PLUCKTHUN, A. and RIESENBERG, D., 1996, High volumetric yields of functional dimeric miniantibodies in *Escherichia coli* using an optimised expression vector and high cell density fermentation under non-limited growth conditions, *Appl. Microbiol. Biotechnol.*, **46**, 524–532.

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.

HOULDEN, B.A., WIDACKI, S.M. and BLUESTONE, J.A., 1991, Signal transduction through class I MHC by a monoclonal antibody that detects multiple murine and human class I molecules, *J. Immunol.*, **146**, 425–430.

HU, P., GLASKY, M.S., YUN, A., ALAUDDIN, M.M., HORNICK, J.L., KHAWLI, L.A. and EPSTEIN, A.L., 1995, A human–mouse chimeric lym-1 monoclonal antibody with specificity for human lymphomas expressed in a baculovirus system, *Hum. Antibod. Hybridomas*, **6**, 57–67.

HU, S., SHIVELY, L., RAUBITSCHEK, A., SHERMAN, M., WILLIAMS, L.E., WONG, J.Y.C., SHIVELY, J.E. and WU, A.M., 1996, Minibody: a novel engineered anti-carcinoembryonic antigen anti-

body fragment (single-chain Fv-CH3) which exhibits rapid, high-level targeting of xenografts, *Cancer Res.*, **56**, 3055–3061.

HUANG, X., MOLEMA, G., KING, S., WATKINS, L., EDGINGTON, T.S. and THORPE, P.E., 1997, Tumor infarction in mice by antibody-directed targeting of tissue factor to tumor vasculature, *Science*, **275**, 547–550.

HUMPHREYS, D.P., WEIR, N., LAWSON, A., MOUNTAIN, A. and LUND, P.A., 1996, Co-expression of human protein disulphide isomerase (PDI) can increase the yield of an antibody Fab′ fragment expressed in *Escherichia coli*, *FEBS Lett.*, **380**, 194–197.

HUSAIN, M. and BIENARZ, C., 1994, Fc site-specific labeling of immunoglobulins with calf intestinal alkaline phosphatase, *Bioconjugate Chem.*, **5**, 482–490.

HUSE, W.D., SASTRY, L., IVERSON, S.A., KANG, A.S., ALTING-MEES, M., BURTON, D.R., BENKOVIC, S.J. and LERNER, R., 1989, Generation of a large combinatorial library of the immunoglobulin repertoire in the phage lambda, *Science*, **246**, 1275–1281.

HUSTON, J.S., LEVINSON, D., MUDGETT-HUNTER, M., TAI, M.S., NOVOTNY, J., MARGOLIES, M.N., RIDGE, R.J., BRUCCOLERI, R.E., HABER, E., CREA, R. and OPPERMANN, 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 γ1), *Cancer Res.*, **51**, 181–189.

ILIADES, P., KORTT, A.A. and HUDSON, P.J., 1997, Triabodies: single chain Fv fragments without a linker form trivalent trimers, *FEBS Lett.*, **409**, 437–441.

INBAR, D., HOCHMAN, J. and GIVOL, D., 1972, Localization of antibody-combining sites within the variable portions of heavy and light chains, *Proc. Natl. Acad. Sci. (USA)*, **69**, 2569–2662.

IND, T.E., GRANOWSKA, M., BRITTON, K.E., MORRIS, G., LOWE, D.G., HUDSON, C.N. and SHEPHARD, J.H., 1994, Preoperative radioimmunodetection of ovarian carcinoma using a hand-held gamma detection probe, *Br. J. Cancer*, **70**, 1263–1266.

IP, C.C., MILLER, W.J., SILBERKLANG, M., MARK, G.E., ELLIS, R.W., HUANG, L., GLUSHKA, J., VAN HALBEEK, H., ZHU, J. and ALHADEFF, J.A., 1994, Structural characterisation of the N-glycans of a humanised anti-CD18 murine immunoglobulin G, *Arch. Biochem. Biophys.*, **308**, 387–399.

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.

ISSEKUTZ, A.C., AYER, L., MIYASAKA, M. and ISSEKUTZ, T.B., 1996, Treatment of established adjuvant arthritis in rats with monoclonal antibody to CD18 and very late activation antigen-4 integrins suppresses neutrophil and T-lymphocyte migration to the joints and improves clinical disease, *Immunol.*, **88**, 569–576.

JACKSON, J.R., SATHE, G., ROSENBERG, M. and SWEET, R., 1995, In vitro antibody maturation, improvement of a high affinity neutralizing antibody against IL-1β, *J. Immunol.*, **154**, 3310–3319.

JACKSON, L.R., TRUDEL, L.J., FOX, J.G. and LIPMAN, N.S., 1996, Evaluation of hollow fibre bioreactors as an alternative to murine ascites production for small scale monoclonal antibody production, *J. Immunol. Methods*, **189**, 217–231.

JACOBO-MOLINA, A., DING, J., NANNI, R.G., CLARK, A.D., LU, X., TANTILLO, C., WILLIAMS, R.L., KAMER, G., FERRIS, A.L., CLARK, P., HIZI, A., HUGHES, S.H. and ARNOLD, E., 1993, Crystal structure of human immunodeficiency virus type 1 reverse transcriptase complexed with double-stranded DNA at 3.0 Å resolution shows bent DNA, *Proc. Natl Acad. Sci. (USA)*, **90**, 6320–6324.

JAHN, G. and PLACHTER, B., 1993, Diagnostics of persistent viruses: human cytomegalovirus as an example, *Intervirology*, **35**, 60–72.

JANDA, K.D., LO, L.-C., LO, C.-H.L., SIM, M.-M., WANG, R., WONG, C.H. and LERNER, R.A., 1997, Chemical selection for catalysts in combinatorial antibody libraries, *Science*, **275**, 945–948.

JESPERS, L.S., ROBERTS, A., MAHLER, S.M., WINTER, G. and HOOGENBOOM, H.R., 1994, Guiding the selection of human antibodies from phage display repertoires to a single epitope of an antigen, *Bio/Technol.*, **12**, 899–903.

JOHNSON, D.A., BARTON, R.L., FIX, D.V., SCOTT, W.L. and GUTOWSKI, M.C., 1991a, Induction of immunogenicity of monoclonal antibodies by conjugation with drugs, *Cancer Res.*, **51**, 5774–5776.

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., 1991b, Radioimmunoadsorption of KC-4G3 antibody in peripheral blood: implications for radioimmunotherapy, *Antibod. Immunoconj. Radiopharm.*, **4**, 885–893.

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.

JOST, C.R., TITUS, J.A., KURUCZ, I. and SEGAL, D.M., 1996, A single-chain bispecific Fv2 molecule produced in mammalian cells redirects lysis by activated CTL, *Mol. Immunol.*, **33**, 211–219.

JUNGHANS, R.P., DOBBS, D., BRECHBIEL, M.W., MIRZADEH, S., RAUBITSCHEK, A.A., GANSOW, O.A. and WALDMANN, T.A., 1993, Pharmacokinetics and bioactivity of 1,4,7,10-tetraazacylododecane *N,N′,N″,N‴*-tetraacetic acid (DOTA)–bismuth-conjugated anti-tac antibody for α-emitter (^{212}Bi) therapy, *Cancer Res.*, **53**, 5683–5689.

JUNGHANS, R.P., WALDMANN, T.A., LANDOLFI, N.F., AVDALOVIC, N.M., SCHNEIDER, W.P. and QUEEN, C., 1990, 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.

JURCIC, J.G., CARON, P.C., NIKULA, T.K., PAPADOPOULOS, E.B., FINN, R.D., GANSOW, O.A., MILLER, W.H., GEERLINGS, M.W., WARRELL, R.P., LARSON, S.M. and SCHEINBERG, D.A., 1995, Radiolabelled anti-CD33 monoclonal antibody M195 for myeloid leukemias, *Cancer Res.*, **55**, 5908s–5910s.

JUWEID, M., SHARKEY, R.M., ALAVI, A., SWAYNE, L.C., HERSKOVIC, T., HANLEY, D., RUBIN, A.D., PEREIRA, M. and GOLDENBERG, D.M., 1997, Regression of advanced refractory ovarian cancer treated with iodine-131-labeled anti-CEA monoclonal antibody, *J. Nucl. Med.*, **38**, 257–260.

JUWEID, M., SHARKEY, R.M., BEHR, T., SWAYNE, L.C., DUNN, R., SIEGAL, J. and GOLDENBERG, D.M., 1996, Radioimmunotherapy of patients with small-volume tumours using iodine-131-labeled anti-CEA monoclonal antibody NP-4 F(ab′)$_2$, *J. Nucl. Med.*, **37**, 1504–1510.

KABAT, E.A., WU, T.T., PERRY, H.M., GOTTESMAN, K.S. and FOELLER, C., 1991, *Sequences of Proteins of Immunological Interest*, 5th edn, US Department of Health and Human Services, Public Health Service, National Institutes of Health (NIH Publication 91-3242), Washington, DC.

KAKU, S., KAWASAKI, T., SAKAI, Y., TANIUCHI, Y., YANO, S., SUZUKI, K., TERAZAKI, C., KAWAMURA, K., MASUHO, Y., SATOH, N., TAKENAKA, T., YANAGI, K. and OHSHIMA, N., 1995, Antithrombotic effect of a humanized anti-GPIIb/IIIa monoclonal antibody, YM207, in a photochemically induced thrombosis model in monkeys, *Eur. J. Pharmacol.*, **279**, 115–121.

KALAB, T. and SKLADEL, P., 1995, A disposable amperometric immunosensor for 2,4-dichlorophenoxyacetic acid, *Anal. Chim. Acta*, **304**, 361–368.

KALOFONOS, H.P., KOSMAS, C., HIRD, V., SNOOK, D.E. and EPENETOS, A.A., 1994, Targeting of tumours with murine and reshaped human monoclonal antibodies against placental alkaline phosphatase: immunlocalisation, pharmacokinetics and immune response, *Eur. J. Cancer*, **30A**, 1842–1850.

KAMINSKI, M.S., ZASADNY, K.R., FRANCIS, I.R., FENNER, M.C., ROSS, C.W., MILIK, A.W., ESTES, J., TUCK, M., REGAN, D., FISHERE, S., GLENN, S.D. and WAHL, R.L., 1996, Iodine-131-anti-B1 radioimmunotherapy for B-cell lymphoma, *J. Clin. Oncol.*, **14**, 1974–1981.

KARPOVSKI, B., TITUS, J.A., STEPHANY, D.A. and SEGAL, D.M., 1984, Production of target-specific effector cells using hetero-cross-linked aggregates containing anti-target cell and anti-Fcγ receptor antibodies, *J. Exp. Med.*, **160**, 1686–1701.

KASINA, S., RAO, T.L., SRINAVASAN, A., SANDERSON, J.A., FITZNER, J.N., RENO, J.M., BEAUMIER, P.L. and FRITZBERG, A.R., 1991, Development and biologic evaluation of a kit for preformed chelate technetium-99m radiolabeling of an antibody fab fragment using a diamide dimercaptide chelating agent, *J. Nucl. Med.*, 32, 1445–1451.

KAUFMANN, S.H., EWING, C.M. and SHAPER, J.H., 1987, The erasable western blot, *Anal. Biochem.*, **161**, 89–95.

KAVANAUGH, A.F., DAVIS, L.S., NICHOLS, L.A., NORRIS, S.H., ROTHLEIN, R., SCHARSCHMIDT, L.A. and LIPSKY, P.E., 1994, Treatment of refractory rheumatoid athritis with a monoclonal antibody to intercellular adhesion molecule 1, *Arthritis Rheum.*, **37**, 992–999.

KEELAN, E.T.M., HARRISON, A.A., CHAPMAN, P.T., BINNS, R.M., PETERS, A.M. and HASKARD, D.O., 1994, Imaging vascular endothelial activation: an approach using radiolabelled monoclonal antibodies against the endothelial cell adhesion molecule E-selectin, *J. Nucl. Med.*, 35, 276–281.

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 humanised anti-p185HER2 antibody Fab fragments, *Biochem.*, **31**, 5434–5441.

KENT, S.J., KARLIK, S.J., CANNON, C., HINES, D.K., YEDNOCK, T.A., FRITZ, L.C. and HORNER, H.C., 1995, a monoclonal antibody to α4 integrin suppresses and reverses experimental allergic encephalomyelitis, *J. Neuroimmunol.*, **58**, 1–10.

KHAW, B.A., KLIBANOV, A., O'DONNELL, S.M., SAITO, T., NOSSIFF, N., SLINKIN, M.A., NEWELL, J.B., STRAUSS, H.W. and TORCHILIN, V.P., 1991, Gamma imaging with negatively charge-modified monoclonal antibody: modification with synthetic polymers, *J. Nucl. Med.*, **32**, 1742–1751.

KHAW, B.A., YASUDA, T., GOLD, H.K., LEINBACH, R.C., JOHNS, J.A., KANKE, M., BARLAI-KOVACH, M., STRAUSS, H.W. and HABER, E., 1987, Acute myocardial infarct imaging with 111-indium labeled monoclonal antimyosin Fab, *J. Nucl. Med.*, **28**, 1671–1678.

KHAZAELI, M.B., CONRY, R.M. and LOBUGLIO, A.F., 1994, Human immune response to monoclonal antibodies, *J. Immunotherapy*, **15**, 42–52.

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, D. and LOBUGLIO, A.F., 1991, Pharmacokinetics and immune response of ^{131}I-chimeric mouse/human B72.3 (human γ4) monoclonal antibody in humans, *Cancer Res.*, **51**, 5461–5466.

KIM, H.C., MCMILLAN, C.W., WHITE, G.C., BERGMAN, G.E., HORTON, M.W. and SAIDI, P., 1992, Purified factor IX using monoclonal immunoaffinity technique, *Blood*, **79**, 568–575.

KIM, J.K., LI, B., WINER, J., ARMANINI, M., GILLET, N., PHILLIPS, H.S. and FERRARA, N., 1993, Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo, *Nature*, **362**, 841–844.

KIM, J.K., TSEN, M.F., GHETIE, V. and WARD, E.S., 1994, Identifying residues that influence plasma clearance of murine IgG1 fragments by site-directed mutagenesis, *Eur. J. Immunol.*, **24**, 542–548.

KING, D.J., ADAIR, J.R., ANGAL, S., LOW, D.C., PROUDFOOT, K.A., LLOYD, J.C., BODMER, M.W. and YARRANTON, G.T., 1992a, Expression, purification and characterization of a mouse–human chimeric antibody and chimeric Fab′ fragment, *Biochem. J.*, **281**, 317–323.

KING, D.J., ANTONIW, P., OWENS, R.J., ADAIR, J.R., HAINES, A.M.R., FARNSWORTH, A.P.H., FINNEY, H., LAWSON, A.D.G., LYONS, A., BAKER, T.S., BALDOCK, D., MACKINTOSH, J., GOFTON, C., YARRANTON, G.T., MCWILLIAMS, W., SHOCHAT, D., LEICHNER, P., WELT, S., OLD, L.J. and MOUNTAIN, A., 1995, Preparation and preclinical evaluation of humanised A33 immunoconjugates for radioimmunotherapy, *Br. J. Cancer*, **72**, 1364–1372.

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., 1993, Expression, purification and characterization of B72.3 Fv fragments, *Biochem. J.*, **290**, 723–729.

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., BODEN, R., BEGENT, R.H.J.

and YARRANTON, G.T., 1992b, Tumor localization of engineered antibody fragments, *Antibod. Immunoconj. Radiopharm.*, **5**, 159–170.

KING, D.J., TURNER, A., FARNSWORTH, A.P.H., ADAIR, J.R., OWENS, R.J., PEDLEY, R.B., BALDOCK, D., PROUDFOOT, K.A., LAWSON, A.D.G., BEELEY, N.R.A., MILLAR, K., MILLICAN, T.A., BOYCE, B., ANTONIW, P., MOUNTAIN, A., BEGENT, R.H.J., SHOCHAT, D. and YARRANTON, G.T., 1994, Improved tumour targeting with chemically cross-linked recombinant antibody fragments, *Cancer Res.*, **54**, 6176–6185.

KINNE, R.W., BECKER, W., KOSCHECK, T., KUHLMANN, J., SHARKEY, R.M., BEHR, T., PALOMBO-KINNE, E., GOLDENBERG, D.M., WOLF, F. and EMMRICH, F., 1995, Rat adjuvant arthritis: imaging with technetium-99m-anti-CD4 Fab′ fragments, *J. Nucl. Med.*, **36**, 2268–2275.

KIPRIYANOV, S.M., LITTLE, M., KROPSHOFER, H., BREITLING, F., GOTTER, S. and DUBEL, S., 1996, Affinity enhancement of a recombinant antibody: formation of complexes with multiple valency by a single-chain Fv fragment–core streptavidin fusion, *Protein Engin.*, **9**, 203–211.

KIPRIYANOV, S.M., MOLDENHAUER, G. and LITTLE, M., 1997a, High level production of soluble single chain antibodies in small scale *Escherichia coli* cultures, *J. Immunol. Methods*, **200**, 69–77.

KIPRIYANOV, S.M., MOLDENHAUER, G., MARTIN, A.C.R., KUPRIYANOVA, O.A. and LITTLE, M., 1997b, Two amino acid mutations in an anti-human CD3 single chain Fv antibody fragment that affect the yield on bacterial secretion but not the affinity, *Protein Engin.*, **10**, 445–453.

KIRCHER,. V. and PARLAR, H., 1996, Determination of delta 9-tetrahydrocannabinol from human saliva by tandem immunoaffinity chromatography–high-performance liquid chromatography, *J. Chromatogr.*, **677**, 245–255.

KITAMURA, K., TAKAHASHI, T., YAMAGUCHI, T., NOGUCHI, A., NOGUCHI, A., TAKASHINA, K., TSURUMI, T., 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.

KLEYMANN, G., OSTERMEIER, C., HEITMANN, K., HAASE, W. and MICHEL, H., 1995, Use of antibody fragments (Fv) in immunohistochemistry, *J. Histochem. Cytochem.*, **43**, 607–614.

KNAPPIK, A., KREBBER, C. and PLUCKTHUN, A., 1993, The effect of folding catalysts on the *in vivo* folding process of different antibody fragments expressed in *Escherichia coli*, *BioTechnol.*, **11**, 77–83.

KNAPPIK, A. and PLUCKTHUN, A., 1994, An improved affinity tag based on the FLAG epitope for detection and purification of recombinant antibody fragments, *Biotechniques*, **17**, 754–761.

KNAPPIK, A. and PLUCKTHUN, A., 1995, Engineered turns of a recombinant antibody improve its *in vivo* folding, *Protein Engin.*, **8**, 81–89.

KNIGHT, D.M., WAGNER, C., JORDAN, R., MCALEER, M.F., DERITA, R., FASS, D.N., COLLER, B.S., WEISMAN, H.F. and GHRAYEB, J., 1995, The immunogenicity of the 7E3 murine monoclonal Fab antibody fragment variable region is dramatically reduced in humans by substitution of human for murine constant regions, *Mol. Immunol.*, **32**, 1271–1281.

KNIGHT, L.C., RADCLIFFE, R., MAURER, A.H., RODWELL, J.D. and ALVAREZ, V.L., 1994, Thrombus imaging with technetium-99m synthetic peptides based upon the binding domain of a monoclonal antibody to activated platelets, *J. Nucl. Med.*, **35**, 282–288.

KNOX, S.J., GORIS, M.L., TRISLER, K., NEGRIN, R., DAVIS, T., LILES, T.M., GRILLO-LOPEZ, A., CHINN, P., VARNS, C., NING, S.C., FOWLER, S., DEB, N., BECKER, M., MARQUEZ, C. and LEVY, R., 1996, Yttrium-90 labeled anti-CD20 monoclonal antibody therapy of recurrent B-cell lymphoma, *Clin. Cancer Res.*, **2**, 457–470.

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, *Human Antibod. Hybridomas*, **1**, 15–22.

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

KONIG, B. and GRATZEL, M., 1993, Detection of viruses and bacteria with piezoelectric immunosensors, *Anal. Lett.*, **26**, 1567–1585.

KONTERMANN, R.E., WING, M.G. and WINTER, G., 1997, Complement recruitment using bispecific diabodies, *Nature Biotechnol.*, **15**, 629–631.

KOSMAS, K., SNOOK, D., GOODEN, C.S., COURTENAY-LUCK, N.J., MCCALL, M.J., MEARES, C. and EPENETOS, A.A., 1992, Development of humoral immune responses against a macrocyclic chelating agent (DOTA) in cancer patients receiving radioimmunoconjugates for imaging and therapy, *Cancer Res.*, **52**, 904–911.

KOSTELNY, S.A., COLE, M.S. and TSO, J.Y., 1992, Formation of a bispecific antibody by the use of leucine zippers, *J. Immunol.*, **148**, 1547–1553.

KOVARI, L.C., MOMANY, C. and ROSSMAN, M.G., 1995, The use of antibody fragments for crystallization and structure determinations, *Structure*, **3**, 1291–1293.

KOZBOR, D., LAGARDE, A. and RODER, J.C., 1982, Human hybridomas constructed with antigen specific Epstein-Barr virus-transformed cell lines, *Proc. Natl Acad. Sci. (USA)*, **79**, 6651–6655.

KRANENBORG, M.H.G.C., OYEN, W.J.G., CORSTENS, F.H.M., OOSTERWIJK, E., VAN DER MEER, J.W.M. and BOERMAN, O.C., 1997, Rapid imaging of experimental infection with technetium-99m-DTPA after anti-DTPA monoclonal antibody priming, *J. Nucl. Med.*, **38**, 901–906.

KRETZSCHMAR, T., AOUSTIN, L., ZINGEL, O., MARANGI, M., VONACH, B., TOWBIN, H. and GEISER, M., 1996, High-level expression in insect cells and purification of secreted monomeric single-chain Fv antibodies, *J. Immunol. Methods*, **195**, 93–101.

KRICKA, L.J., 1993, Ultrasensitive immunoassay techniques, *Clin. Biochem.*, **26**, 325–331.

KROUWELS, F.H., NOCKER, R.E.T., SNOEK, M., LUTTER, R., VAN DER ZEE, J.S., WELLER, F.R., JANSEN, H.M. and OUT, T.A., 1997, Immunocytochemical and flow cytofluorimetric detection of intracellular IL-4, IL-5 and IFN-γ: applications using blood and airway derived cells, *J. Immunol. Methods*, **203**, 89–101.

KUROKAWA, T., IWASA, S. and KAKINUMA, A., 1989, Enhanced fibrinolysis by a bispecific monoclonal antibody reactive to fibrin and tissue plasminogen activator, *Bio/Technol.*, **7**, 1163–1167.

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*, European patent application EP0403156.

KURUCZ, I., TITUS, J.A., JOST, C.R. and SEGAL, D.M., 1995, Correct disulphide pairing and efficient refolding of detergent solubilised single-chain Fv proteins from bacterial inclusion bodies, *Mol. Immunol.*, **32**, 1443–1452.

LAMBERT, J.M., GOLDMACHER, V.S., COLLINSON, A.R., NADLER, L.M. and BLATTLER, W.A., 1991, An immunotoxin prepared with blocked ricin: a natural plant toxin adapted for therapeutic use, *Cancer Res.*, **51**, 6236–6242.

LANDRY, D.W., ZHAO, K., YANG, C.X.Q., GLICKMAN, M. and GEORGIADIS, T.M., 1993, Antibody catalysed degradation of cocaine, *Science*, **259**, 1899–1901.

LANE, D.M., EAGLE, K.F., BEGENT, R.H.J., HOPE-STONE, L.D., GREEN, A.J., CASEY, J.L., KEEP, P.A., KELLY, A.M.B., LEDERMAN, J.A., GLASER, M.G. and HILSON, A.J.W., 1994, Radioimmunotherapy of metastatic colorectal tumours with iodine-131-labelled antibody to carcinoembryonic antigen: phase I/II study with comparative biodistribution of intact and $F(ab')_2$ antibodies, *Br. J. Cancer*, **70**, 521–525.

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

LAROCHE, Y., DEMAEYER, M., STASSEN, J.M., GANSEMANS, Y., DEMARSIN, E., MATTHYSSENS, G., COLLEN, D. and HOLVOET, P., 1991, Characterization of a recombinant single-chain molecule comprising the variable domains of a monoclonal antibody specific for human fibrin fragment D-dimer, *J. Biol. Chem.*, **266**, 16343–16349.

LARSON, S.M., 1995, Improving the balance between treatment and diagnosis: a role for radioimmunodetection, *Cancer Res.*, **55**, 5756s–5758s.

LAUKKANEN, M.J., ORELLANA, A. and KEINANEN, K., 1995, Use of genetically engineered lipid-tagged antibody to generate functional europium chelate loaded liposomes. Application in fluoroimmunoassay, *J. Immunol. Methods*, **185**, 95–102.

LAUKKANEN, M.J., TEERI, T.T. and KEINANEN, K., 1993, Lipid-tagged antibodies: bacterial expression and characterization of a lipoprotein–single chain antibody fusion protein, *Protein Engin.*, **6**, 449–454.

LAW, S.J., MILLER, T., PIRAN, U., KLUKAS, C., CHANG, S. and UNGER, J., 1989, Novel poly-substituted aryl acridinium esters and their use in immunoassay, *J. Biolumin. Chemilumin.*, 4, 88–98.

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.

LEDOUSSAL, J.M., CHETANNEAU, A., GRUAZ-GUYON, A., MARTIN, M., GAUTHEROT, E., LEHUR, P.A., CHATAL, J.F., DELAAGE, M. and BARBET, J., 1993, Bispecific monoclonal antibody mediated targeting of an indium-111-labeled DTPA dimer to primary colorectal tumors, *J. Nucl. Med.*, **34**, 1662–1671.

LEDOUSSAL, J.M., MARTIN, M., GAUTHEROT, E., DELAAGE, M. and BARBET, J., 1989, *In vitro* and *in vivo* targeting of radiolabeled monovalent and divalent haptens with dual specificity monoclonal antibody conjugates, *J. Nucl. Med.*, **30**, 1358–1366.

LEGER, O.J.P., YEDNOCK, T.A., TANNER, L., HORNER, H.C., HINES, D.K., KEEN, S., SALDANHA, J., JONES, S.T., FROTZ, L.C. and BENDIG, M.M., 1997, Humanization of a mouse antibody against human alpha-4 integrin: a potential therapeutic for the treatment of multiple sclerosis, *Human Antibodies*, **8**, 3–16.

LEONETTI, J.P., MACHY, P., DEGOLS, G., LEBLEU, B. and LESERMAN, L., 1990, Antibody-targeted liposomes containing oligodeoxyribonucleotides complementary to viral RNA selectively inhibit viral replication, *Proc. Natl Acad. Sci. (USA)*, **87**, 2448–2451.

LEUNG, S., LOSMAN, M.J., GOVIDAN, S.V., GRIFFITHS, G.L., GOLDENBERG, D.M. and HANSEN, H.J., 1995, Engineering a unique glycosylation site for site-specific conjugation of haptens to antibody fragments, *J. Immunol.*, **154**, 5919–5926.

LEVI, M., SALLBERG, M., RUDEN, U., HERLYN, D., MARUYAMA, H., WIGZELL, H., MARKS, J. and WAHREN, B., 1993, A complementarity-determining region synthetic peptide acts as a miniantibody and neutralises human immunodeficiency virus type 1 in vitro, *Proc. Natl Acad. Sci. (USA)*, **90**, 4374–4378.

LEVIN, R., MHASHILKAR, A.M., DORFMAN, T., BUKOVSKY, A., ZANI, C., BAGLEY, J., HINKULA, J., NIEDRIG, M., ALBERT, J., WAHREN, B., GOTTLINGER, H.G. and MARASCO, W.A., 1997, Inhibition of early and late events of the HIV-1 replication cycle by cytoplasmic Fab intrabodies against the matrix protein p17, *Mol. Med.*, **3**, 96–110.

LEVY, R., WEISMAN, M., WIESENHUTTER, C., YOCUM, D., SCHNITZER, T., GOLDMAN, A., SCHIFF, M., BREEDVELD, F., SOLINGER, A., MACDONALD, B. and LIPANI, J., 1996, Results of a placebo-controlled multicenter trial using a primatised non-depleting anti-CD4 monoclonal antibody in the treatment of rheumatoid arthritis, *Arthritis Rheum.*, **39**, S122, abstract 574.

LIEBER, M., 1996, Immunoglobulin diversity: rearranging by cutting and repairing, *Curr. Biol.*, 6, 134–136.

LIN, L. and PUTNAM, F.W., 1978, Cold pepsin digestion, a novel method to produce the Fv fragment from human immunoglobulin M, *Proc. Natl Acad. Sci. (USA)*, **75**, 2649–2653.

LINDHOFER, H., MOCIKAT, R., STEIPE, B. and THIERFELDER, S., 1995, Preferential species-restricted heavy/light chain pairing in rat/mouse quadromas, *J. Immunol.*, **155**, 219–225.

LIU, A.H., CREADON, G. and WYSLOCKI, 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., 1987, Expression of a mouse:human immunoglobulin heavy-chain cDNA in lymphoid cells, *Gene*, **54**, 33–40.

LIU, C., TADAYONI, B.M., BOURRET, L.A., MATTOCKS, K.M., DERR, S.M., WIDDISON, W.C., KEDERSHA, N.L., ARINIELLO, P.D., GOLDMACHER, V.S., LAMBERT, J.M., BLATTLER, W.A.

and CHARI, R.V.J., 1996, Eradication of large colon tumour xenografts by targeted delivery of maytansinoids, *Proc. Natl Acad. Sci. (USA)*, **93**, 8618–8623.

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

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.

LOCKWOOD, C.M., THIRU, S., ISAACS, J.D., HALE, G. and WALDMANN, H., 1993, Long term remission of intractable systemic vasculitis with monoclonal antibody therapy, *Lancet*, **341**, 1620–1622.

LONBERG, N., TAYLOR, L.D., HARDING, F.A., TROUNSTINE, M., HIGGINS, K.M., SCHRAMM, S.R., KUO, C.C., MASHAYEKH, R., WYMORE, K., MCCABE, J.G., MUNOZ-O'REGAN, D., O'DONNELL, S.L., LAPACHET, E.S.G., BENGOCHEA, T., FISHWILD, D.M., CARMACK, C.E., KAY, R.M. and HUSZAR, D., 1994, Antigen-specific human antibodies from mice comprising four distinct genetic modifications, *Nature*, **368**, 856–859.

LOVGREN, T., MERIO, L., MITRUMEN, K., MAKINEN, M.L., MAKELA, M., BLOMBERG, K., PALENIUS, T. and PETTERSSON, K., 1996, One-step all-in-one dry reagent immunoassays with fluorescent europium chelate label and time resolved fluorometry, *Clin. Chem.*, **42**, 410–415.

LOVQUIST, A., SUNDIN, A., AHLSTROM, H., CARLSSON, J. and LUNDQVIST, H., 1997, Pharmacokinetics and experimental PET imaging of a bromine-76-labeled monoclonal anti-CEA antibody, *J. Nucl. Med.*, **38**, 395–401.

LUND, J., TAKAHASHI, N., NAKAGAWA, H., GOODALL, M., BENTLEY, T., HINDLEY, S.A., TYLER, R. and JEFFERIS, R., 1993, Control of IgG/Fc glycosylation: a comparison of oligosaccharides from chimeric human/mouse and mouse subclass immunoglobulin Gs, *Mol. Immunol.*, **30**, 741–748.

LUND, J., TAKAHASHI, N., POUND, J.D., GOODALL, M. and JEFFERIS, R., 1996, Multiple interactions of IgG with its core oligosaccharide can modulate recognition by complement and human FcγRI and influence the synthesis of its oligosaccharide chains, *J. Immunol.*, **157**, 4963–4969.

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.

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, *Protein Engin.*, **3**, 703–708.

MA, J.K. and HEIN, M.B., 1995, Immunotherapeutic potential of antibodies produced in plants, *Trends Biotechnol.*, **13**, 522–527.

MA, J.K., HIATT, A., HEIN, M., VINE, N.D., WANG, F., STABILA, P., VAN DOLLEWEERD, C., MOSTOV, K. and LEHNER, T., 1995, Generation and assembly of secretory antibodies in plants, *Science*, **268**, 716–719.

MCCAFFERTY, J., GRIFFITHS, A.D., WINTER, G. and CHISWELL, D.J., 1990, Phage antibodies: filamentous phage displaying antibody variable domains, *Nature*, **348**, 552–554.

MCCLOSKEY, N., TURNER, M.W., STEFFNER, P., OWENS, R. and GOLDBLATT, D., 1996, Human constant regions influence the antibody binding characteristics of mouse–human chimeric IgG subclasses, *Immunology*, **88**, 169–173.

MACGLASHAN, D.W., BOCHNER, B.S., ADELMAN, D.C., JARDIEU, P.M., TOGIAS, A., MCKENZIE-WHITE, J., STERBINSKY, S.A., HAMILTON, R.G. and LICHTENSTEIN, L.M., 1997, Down-regulation of FceRI expression on human basophils during *in vivo* treatment of atopic patients with anti-IgE antibody, *J. Immunol*, **158**, 1438–1445.

MCGUINNESS, B.T., WALTER, G., FITZGERALD, K., SCHULER, P., MAHONEY, W., DUNCAN, A.R. and HOOGENBOOM, H.R., 1996, Phage diabody repertoires for selection of large numbers of bispecific antibody fragments, *Nature Biotechnol.*, **14**, 1149–1154.

MACIEJEWSKI, J.P., WIECHOLD, F.F., YOUNG, N.S., CARA, A., ZELLA, D., REITZ, M.S. and GALLO, R.C., 1995, Intracelluar expression of antibody fragments against HIV reverse transcriptase prevents HIV infection *in vitro*, *Nature Med.*, **1**, 667–673.

MACK, M., REITHMULLER, G. and KUFER, P., 1995, A small bispecific antibody construct expressed as a functional single-chain molecule with high tumour cell cytotoxicity, *Proc. Natl Acad. Sci. (USA)*, **92**, 7021–7025.

MACKENZIE, C.R., CLARK, I.D., EVANS, S.V., HILL, I.E., MACMANUS, J.P., DUBUC, G., BUNDLE, D.R., NARANG, S.A., YOUNG, N.M. and SZABO, A.G., 1995, Bifunctional fusion proteins consisting of a single-chain antibody and an engineered lanthanide binding protein, *Immunotechnology*, **1**, 139–150.

MACKENZIE, C.R., SHARMA, V., BRUMMELL, D., BILOUS, D., DUBUC, G., SADOWSKA, J., YOUNG, N.M., BUNDLE, D.R. and NARANG, S.A., 1994, Effect of Cλ–Cκ domain switching on Fab activity and yield in *Escherichia coli*: synthesis and expression of genes encoding two anti-carbohydrate Fabs, *Bio/Technol.*, **12**, 390–395.

MACLEAN, J.A., SU, Z., COLVIN, R.B. and WONG, J.T., 1995, Anti-CD3:anti-IL-2 receptor–bispecific mab-mediated immunomodulation, *J. Immunol.*, **155**, 3674–3682.

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

MAERZ, H., HAHN, S.O., MAASSEN, A., MEISEL, H., ROGGENBUCK, D., SATO, T., TANZMANN, H., EMMRICH, F. and MARX, U., 1996, Improved removal of viruslike particles from purified monoclonal antibody IgM preparation via virus filtration, *Nature Biotech.*, **14**, 651–652.

MAGNANI, P., PAGANELLI, G., SONGINI, C., SAMUEL, A., SUDATI, F., SICCARDI, A.G. and FAZIO, F., 1996, Pretargeted immunoscintigraphy in patients with medullary thyroid carcinoma, *Br. J. Cancer*, **74**, 825–831.

MAIORELLA, B.L., WINKELHAKE, J., YOUNG, J., MOYER, B., BAUER, R., HORA, M.J., THOMSON, J., PATEL, T. and PAREKH, R., 1993, Effect of culture conditions on IgM antibody structure, pharmacokinetics and activity, *Bio/Technol.*, **11**, 387–392.

MALLENDER, W.D. and VOSS, E.W., 1994, Construction, expression and activity of a bivalent bispecific single-chain antibody, *J. Biol. Chem.*, **269**, 199–206.

MALMBORG, A.C. and BORREBAECK, C.A.K., 1995, BIAcore as a tool in antibody engineering, *J. Immunol. Methods*, **183**, 7–13.

MALMQVIST, M., 1993, Biospecific interaction analysis using biosensor technology, *Nature*, **361**, 186–187.

MALONEY, D.G., GRILLO-LOPEZ, A.J., WHITE, C.A., BODKIN, D., SCHILDER, R.J., NEIDHART, J.A., JANAKIRAMAN, N., FOON, K.A., LILES, T.M., DALLAIRE, B.K., WEY, K., ROYSTON, I., DAVIS, T. and LEVY, R., 1997, IDEC-C2B8 (Rituximab) anti-CD20 monoclonal antibody therapy in patients with relapsed low-grade non-Hodgkin's lymphoma, *Blood*, **90**, 2188–2195.

MALONEY, D.G., LILES, T.M., CZERWINSKI, D.K., WALDICHUK, C., GRILLO-LOPEZ, A. and LEVY, R., 1994, Phase I clinical trial using escalating single-dose infusion of chimeric anti-CD20 monoclonal antibody (IDEC-C2B8) in patients with recurrent B-cell lymphoma, *Blood*, **84**, 2457–2466.

MARASCO, W.A., HASELTINE, W.A. and CHEN, S.Y., 1993, Design, intracellular expression and activity of a human anti-human immunodeficiency virus type 1 gp120 single-chain antibody, *Proc. Natl Acad. Sci. (USA)*, **90**, 7889–7893.

MARES, A., DE BOEVER, J., OSHER, J., QUIROGA, S., BARNARD, G. and KOHEN, F., 1995, A direct non-competitive idiometric enzyme immunoassay for serum oestradiol, *J. Immunol. Methods*, **181**, 83–90.

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

MARIANI, M., CAMAGNA, M., TARDITI, L. and SECCAMANI, E., 1991, A new enzymatic method to obtain high-yield F(ab)$_2$ suitable for clinical use from mouse IgG1, *Mol. Immunol.*, **28**, 69–77.

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

MARKS, J.D., TRISTREM, M., KARPAS, A. and WINTER, G., 1991, Oligonucleotide primers for polymerase chain reaction amplification of human immunoglobulin variable genes and design of family specific oligonucleotide probes, *Eur. J. Immunol.*, **21**, 985–991.

MARQUET, P.Y., DAVIER, A., SAPIN, R., BRIDGI, B., MURATET, J.P., HARTMANN, D.J., PAOLUCCI, F. and PAU, B., 1996, Highly sensitive immunoradiometric assay for serum thyroglobulin with minimal interference from autoantibodies, *Clin. Chem.*, **42**, 258–262.

MARSHALL, D., PEDLEY, R.B., BODEN, J.A., BODEN, R. and BEGENT, R.H.J., 1994, Clearance of circulating radio-antibodies using streptavidin or second antibodies in a xenograft model, *Br. J. Cancer*, **69**, 502–507.

MARTIN, F., VOLPARI, C., STEINKUHLER, C., DIMASI, N., BRUNETTI, M., BIASIOL, G., ALTAMURA, S., CORTESE, R., DEFRANCESCO, R. and SOLLAZZO, M., 1997, Affinity selection of a camelized VH domain antibody inhibitor of hepatitis C virus NS3 protease, *Protein Engin.*, **10**, 607–614.

MARTIN, P.J., NELSON, B.J., APPELBAUM, F.R., ANASETTI, C., DEEG, H.J., HANSEN, J.A., MCDONALD, G.B., NASH, R.A., SULLIVAN, K.M., WITHERSPOON, R.P., SCANNON, P.J., FRIEDMANN, N. and STORB, R., 1996, Evaluation of a CD5 specific immunotoxin for treatment of acute graft versus host disease after allogenic marrow transplantation, *Blood*, **88**, 824–830.

MASAT, L., WABL, M. and JOHNSON, J.P., 1994, A simpler sort of antibody, *Proc. Natl Acad. Sci. (USA)*, **91**, 893–896.

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

MEIJS, W.E., HAISMA, H.J., KLOK, R.P., VAN GOG, F.B., KIEVIT, E., PINEDO, H.M. and HERSCHEID, J.D.M., 1997, Zirconium-labeled monoclonal antibodies and their distribution in tumor-bearing nude mice, *J. Nucl. Med.*, **38**, 112–118.

MELTON, R.G. and SHERWOOD, R.F., 1996, Antibody–enzyme conjugates for cancer therapy, *J. Natl Cancer Inst.*, **88**, 153–165.

MENDEZ, M.J., GREEN, L.L., CORVALAN, J.R., JIA, X.C., MAYNARD-CURRIE, C.E., YANG, X.D., GALLO, M.L., LOUIE, D.M., LEE, D.V., ERICKSON, K.L., LUNA, J., ROY, C.M., ABDERRAHIM, H., KIRSCHENBAUM, F., NOGUCHI, M., SMITH, D.H., FUKUSHIMA, A., HALES, J.F., FINER, M.H., DAVIS, C.G., ZSEBO, K.M. and JAKOBOVITS, A., 1997, Functional transplant of megabase human immunoglobulin loci recapitulates human antibody responses in mice, *Nature Genet.*, **15**, 146–156.

MEREDITH, R.F., KHAZAELI, M.B., LIU, T.P., PLOTT, G., WHEELER, R.H., RUSSELL, C., COLCHER, D., SCHLOM, J., SHOCHAT, D. and LOBUGLIO, A.F., 1992a, Dose fractionation of radiolabelled antibodies in patients with metastatic colon cancer, *J. Nucl. Med.*, **33**, 1648–1653.

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., SHOCHAT, D., WHEELER, R.H. and LOBUGLIO, A.F., 1992b, Phase I trial of iodine-131–chimeric B72.3 (human IgG4) in metastatic colorectal cancer, *J. Nucl. Med.*, **33**, 23–32.

MEREDITH, R.F., KHAZAELI, M.B., PLOTT, W.E., SPENCER, S.A., WHEELER, R.H., BRADY, L.W., WOO, D.V. and LOBUGLIO, A.F., 1995, Initial clinical evaluation of iodine-125-labeled chimeric 17-1A for metastatic colon cancer, *J. Nucl. Med.*, **36**, 2229–2233.

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,k 17-1A monoclonal antibody, *J. Nucl. Med.*, **32**, 1162–1168.

MEREDITH, R.F., PARTRIDGE, E.E., ALVAREZ, R.D., KHAZAELI, M.B., PLOTT, G., RUSSELL, C.D., WHEELER, R.H., LIU, T., GRIZZLE, W.E., SCHLOM, J. and LOBUGLIO, A.F., 1996, Intraperitoneal radioimmunotherapy of ovarian cancer with lutetium-177-CC49, *J. Nucl. Med.*, **37**, 1491–1496.

MERWIN, J.R., CARMICHAEL, E.P., NOELL, G.S., DEROME, M.E., THOMAS, W.L., ROBERT, N., SPITALNY, G. and CHIOU, H.C., 1995, CD5-mediated specific delivery of DNA to T lymphocytes: compartmentalization augmented by adenovirus, *J. Immunol. Methods*, **186**, 257–266.

MEUSEL, M., RENNEBERG, R., SPENER, F. and SCHMITZ, G., 1995, Development of a heterogeneous amperometric immunosensor for the determination of apolipoprotein E in serum, *Biosens. Bioelectronics*, **10**, 577–586.

MEYER, K.B. and NEUBERGER, M.S., 1989, The immunoglobulin kappa locus contains a second stronger B-cell specific enhancer which is located downstream of the constant region, *EMBO J.*, **8**, 1959–1964.

MHASHILKAR, A.M., BAGLEY, J., CHEN, S.Y., SZILVAY, A.M., HELLAND, D.G. and MARASCO, W.A., 1995, Inhibition of HIV-1 tat-mediated LTR transactivation and HIV-1 infection by anti-tat single-chain intrabodies, *EMBO J.*, **14**, 1542–1551.

MHATRE, R., NASHABEH, W., SCHMALZING, D., YAO, X., FUCHS, M., WHITNEY, D. and REGNIER, F., 1995, Purification of antibody Fab fragments by cation-exchange chromatography and pH gradient elution, *J. Chromatogr.*, **707**, 225–231.

MILENIC, D.E., ESTEBAN, J.M. and COLCHER, D., 1989, Comparisons of methods for the generation of immunoreactive fragments of a monoclonal antibody (B72.3) reactive with human carcinomas, *J. Immunol. Methods*, **120**, 71–83.

MILENIC, D.E., YOKOTA, T., FILPULA, 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.

MILES, L.E.M. and HALES, C.N., 1968, Labelled antibodies and immunological assay systems, *Nature*, **219**, 186–189.

MILSTEIN, C. and CUELLO, A.C., 1983, Hybrid hybridomas and their use in immunohistochemistry, *Nature*, **305**, 537–540.

MIURA, N., HIGOBASHI, H., SAKAI, G., TAKEYASHU, A., UDA, T. and YAMAZOE, N., 1993, Piezoelectric crystal immunosensor for sensitive detection of metamphetamine (stimulant drug) in human urine, *Sensors Actuators B Chem.*, **13**, 188–191.

MIYASHITA, H., KARAKI, Y., KIKUCHI, M. and FUJII, I., 1993, Prodrug activation via catalytic antibodies, *Proc. Natl Acad. Sci. (USA)*, **90**, 5337–5340.

MOI, M.K., MEARES, C.F., MCCALL, M.J., COLE, W.C. and DENARDO, S.J., 1985, Copper chelates as probes of biological systems: stable copper complexes with a macrocyclic chelating agent, *Anal. Biochem.*, **148**, 249–253.

MOLLOY, P., BRYDON, L., PORTER, A.J. and HARRIS, W.J., 1995, Separation and concentration of bacteria with immobilized antibody fragments, *J. Appl. Bact.*, **78**, 359–365.

MONFARDINI, C., KIEBER-EMMONS, T., VONFELDT, J.M., O'MALLEY, B., ROSENBAUM, H., GODILLOT, A.P., KAUSHANSKY, K., BROWN, C.B., VOET, D., MCCALLUS, D.E., WEINER, D.B. and WILLIAMS, W.V., 1995, Recombinant antibodies in bioactive peptide design, *J. Biol. Chem.*, **270**, 6628–6638.

MORELOCK, M.M., ROTHLEIN, R., BRIGHT, S.M., ROBINSON, M.K., GRAHAM, E.T., SABO, J.P., OWENS, R., KING, D.J., NORRIS, S.H., SCHER, D.S., WRIGHT, J.L. and ADAIR, J.R., 1994, Isotype choice for chimeric antibodies affects binding properties, *J. Biol. Chem.*, **269**, 13048–13055.

MORGAN, A., JONES, N.D., NESBITT, A.M., CHAPLIN, L., BODMER, M. and EMTAGE, J.S., 1995, The N-terminal end of the C_H2 domain of chimeric human IgG1 anti-HLA-DR is necessary for C1q, FcγRI and FcγRIII binding, *Immunology*, **86**, 319–324.

MORGUET, A.J., SANDROCK, D., STILLE-SIEGENER, M. and FIGULLA, H.R., 1995, Indium-111-antimyosin Fab imaging to demonstrate myocardial involvement in systemic lupus erythematosus, *J. Nucl. Med.*, **36**, 1432–1435.

MORPHY, J.R., PARKER, D., ALEXANDER, R., BAINS, A., CARNE, A.F., EATON, M.A.W., HARRISON, A., MILLICAN, A., PHIPPS, A., RHIND, S.K., TITMAS, R. and WEATHERBY, D., 1988, Anti-

body labelling with functionalised cyclam macrocycles, *J. Chem. Soc. Chem. Commun.*, 156–158.

MORPHY, J.R., PARKER, D., KATAKY, R., HARRISON, A., EATON, M.A.W., MILLICAN, A., PHIPPS, A. and WALKER, C., 1989, Towards tumour targeting with copper-radiolabelled macrocycle–antibody conjugates, *J. Chem Soc. Chem. Commun.*, 792–794.

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.

MORTON, H.C., ATKIN, J.D., OWENS, R.J. and WOOF, J.M., 1993, Purification and characterization of chimeric human IgA1 and IgA2 expressed in COS and Chinese hamster ovary cells, *J. Immunol.*, **151**, 4743–4752.

MOSIER, D.E., GULIZIA, R.J., BAIRD, S.M. and WILSON, D.B., 1988, Transfer of a functional immune system to mice with severe combined immunodeficiency, *Nature*, **335**, 256–259.

MUELLER, B.M., REISFELD, R.A. and GILLIES, S.D., 1990, Serum half-life and tumor localization of a chimeric antibody deleted of the CH2 domain and directed against the disialoganglioside GD2, *Proc. Natl Acad. Sci. (USA)*, **87**, 5702–5705.

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

MURRAY, J.L., MACEY, D.J., KASI, L.P., RIEGER, P., CUNNINGHAM, J., BHADKAMKAR, V., ZHANG, H.Z., SCHLOM, J., ROSENBLUM, M.G. and PODOLOFF, D.A., 1994, Phase II radioimmunotherapy trial with ^{131}I-CC49 in colorectal cancer, *Cancer*, **73**, 1057–1066.

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

NARULA, J., PETROV, A., PAK, C.K.Y., DITLOW, C., CHEN, F. and KHAW, B.A., 1995, Noninvasive localisation of experimental atherosclerotic lesions with Tc-99m labeled chimeric Z2D3 antibody fab′, *J. Nucl. Med.*, **36**, 138P–139P.

NATANSON, C., HOFFMAN, W.D., SUFFREDINI, A.F., EICHAKER, P.Q. and DANNER, R.L., 1994, Selected treatment strategies for septic shock based on proposed mechanisms of pathogenesis, *Ann. Intern. Med.*, **120**, 771–783.

NAVARRO-TEULON, I., PERALDI-ROUX, S., BERNARDI, T., MARIN, M., PIECHACZYK, M., SHIRE, D., PAU, B. and BIARD-PIECHACZYK, M., 1995, Expression in *E. coli* of soluble and M13 phage-displayed forms of a single-chain antibody fragment specific for digoxin: assessment in a novel drug immunoassay, *Immunotechnol.*, **1**, 41–52.

NEBLOCK, D.S., CHANG, C.H., MASCELLI, M.A., FLEEK, M., STUMPO, L., CULLEN, M.M. and DADDONA, P.E., 1992, Conjugation and evaluation of 7E3xP4B6, a chemically cross-linked bispecific F(ab′)$_2$ antibody which inhibits platelet aggregation and localises tissue plasminogen activator to the platelet surface, *Bioconj. Chem.*, **3**, 126–131.

NEDELMAN, M.A., SHEALY, D.J., BOULIN, R., BRUNT, E., SEASHOLTZ, J.I., ALLEN, I.E., MCCARTNEY, J.E., WARREN, F.D., OPPERMANN, H., PANG, R.H.L., BERGER, H.J. and WEISMAN, H.F., 1993, Rapid infarct imaging with a technetium-99m-labeled antimyosin recombinant single-chain Fv: evaluation in a canine model of acute myocardial infarction, *J. Nucl. Med.*, **34**, 234–241.

NERI, D., PETRUL, H., WINTER, G., LIGHT, Y., MARAIS, R., BRITTON, K.E. and CREIGHTON, A.M., 1996, Radioactive labeling of antibody fragments by phosphorylation using human casein kinase II and γ-^{32}P-ATP, *Nature Biotechnol.*, **14**, 485–490.

NEUBERGER, M., WILLIAMS, G.T. and FOX, R.O., 1984, Recombinant antibodies possessing novel effector functions, *Nature*, **312**, 604–608.

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.

NEVENS, J.R., MALLIA, A.K., WENDT, M.W. and SMITH, P.K., 1992, Affinity chromatographic purification of immunoglobulin M antibodies utilizing immobilized mannan binding protein, *J. Chromatogr.*, **597**, 247–256.

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/Technol.*, **10**, 1455–1460.

NEWTON, D.L., NICHOLLS, P.J., RYBAK, S.M. and YOULE, R.J., 1994, Expression and characterization of recombinant human eosinophil-derived neurotoxin and eosinophil-derived neurotoxin–anti-transferrin receptor sFv, *J. Biol. Chem.*, **269**, 26739–26745.

NEWTON, D.L., XUE, Y., OLSON, K.A., FETT, J.W. and RYBAK, S.M., 1996, Angiogenin single-chain immunofusions: influence of peptide linkers and spacers between fusion protein domains, *Biochem.*, **35**, 545–553.

NICOLAOU, K.C., SMITH, A.L. and YUE, E.W., 1993, Chemistry and biology of natural and designed enediynes, *Proc. Natl Acad. Sci. (USA)*, **90**, 5881–5888.

NIEBA, L., HONEGGER, A., KREBBER, C. and PLUCKTHUN, A., 1997, Disrupting the hydrophobic patches at the antibody variable/constant domain interface: improved *in vivo* folding and physical chacterization of an engineered scFv fragment, *Protein Engin.*, **10**, 435–444.

NIERODA, C.A., MILENIC, D.E., CARRASQUILLO, J.A., SCHLOM, J. and GREINER, J.W., 1995, Improved tumor radioimmunodetection using a single-chain Fv and γ-interferon: potential clinical applications for radioimmunoguided surgery and γ scanning, *Cancer Res.*, **55**, 2858–2865.

NILSON, B.H.K., LOGDBERG, L., KASTERN, W., BJORCK, L. and AKERSTROM, B., 1993, Purification of antibodies using protein L-binding framework structures in the light chain variable domain, *J. Immunol. Methods*, **164**, 33–40.

NISHINAKA, S., SUZUKI, T., MATSUDA, H. and MURATA, M., 1991, A new cell line for the production of chicken monoclonal antibody by hybridoma technology, *J. Immunol. Methods*, **139**, 217–222.

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.

NORMAN, T.J., PARKER, D., ROYLE, L., HARRISON, A., ANTONIW, P. and KING, D.J., 1995a, Improved tumour targeting with recombinant antibody–macrocycle conjugates, *J. Chem. Soc. Chem. Commun.*, 1877–1878.

NORMAN, T.J., PARKER, D., SMITH, F.C. and KING, D.J., 1995b, Towards selective DNA targeting: synthesis of an antibody–macrocycle–intercalator conjugate, *J. Chem. Soc. Chem. Commun.*, 1879–1880.

NORRGREN, K., STRAND, S.E., NILSSON, R., LINDGREN, L. and LILLIEHORN, P., 1991, Evaluation of extracorporeal immunoadsorption for reduction of the blood background in diagnostic and therapeutic applications of radiolabelled monoclonal antibodies, *Antibody Immunoconj. Radiopharm.*, **4**, 907–914.

NYYSSONEN, E., PENTTILA, M., HARKKI, A., SALOHEIME, A., KNOWLES, J.K.C. and KERANEN, S., 1993, Efficient production of antibody fragments by the filamentous fungus *Trichoderma reesei*, *Bio/Technol.*, **11**, 591–595.

O'HARE, M., BROWN, A.N., HUSSAIN, K., GEBHARDT, A., WATSON, G., ROBERTS, L.M., VITETTA, E.S., THORPE, P.E. and LORD, J.M., 1990, Cytotoxicity of a recombinant ricin–A chain fusion protein containing a proteolytically cleavable spacer sequence, *FEBS Lett.*, **273**, 200–204.

OHLIN, M., OWMAN, H., MACH, M. and BORREBAECK, C.A.K., 1996, Light chain shuffling of a high affinity antibody results in a drift in epitope recognition, *Mol. Immunol.*, **33**, 47–56.

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.

ORLANDI, R., GUSSOW, D., 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.

ORNATOWSKA, M. and GLASEL, J.A., 1991, Direct production of Fv fragments from a family of monoclonal IgGs by papain digestion, *Mol. Immunol.*, **28**, 383–391.

O'SHANESSY, D.J., 1990, Hydrazido-derivatized supports in affinity chromatography, *J. Chromatogr.*, **510**, 13–21.

OSTER, Z.H., SRIVASTA, S.C., SOM, P., MEINKEN, G.E., SCUDDER, L.E., YAMAMOTO, K., ATKINS, H.L., BRILL, A.B. and COLLER, B.S., 1985, Thrombus radioimmunoscintigraphy, an approach using monoclonal antiplatelet antibody, *Proc. Natl. Acad. Sci. (USA)*, **82**, 3465–3468.

OSTERMEIER, C., IWATA, S., LUDWIG, B. and MICHEL, H., 1995, Fv fragment mediated crystallization of the membrane protein bacterial cytochrome c oxidase, *Nature Struct. Biol.*, **2**, 842–846.

OWEN, M., GANDECHA, A., COCKBURN, B. and WHITELAM G., 1992, Synthesis of a functional anti-phytochrome single-chain Fv protein in transgenic tobacco, *Bio/Technol.*, **10**, 790–794.

OWENS, R.J. and ROBINSON, M.K., 1995, *Antibodies against E-selectin*, International Patent Application WO95/26403.

PACK, P., KUJAU, M., SCHROEWCKH, V., KNUPFER, U., WENDEROTH, R., RIESENBERG, D. and PLUCKTHUN, A., 1993, Improved bivalent miniantibodies with identical avidity as whole antibodies, produced by high cell density fermentation of *Escherichia coli*, *Bio/Technol.*, **11**, 1271–1277.

PACK, P., MULLER, K., ZAHN, R. and PLUCKTHUN, A., 1995, Tetravalent miniantibodies with high avidity assembling in *Escherichia coli*, *J. Mol. Biol.*, **246**, 28–34.

PACK, P. and PLUCKTHUN, A., 1992, Miniantibodies: use of amphipathic helices to produce functional, flexibly linked dimeric Fv fragments with high avidity in *Escherichia coli*, *Biochem.*, **31**, 1579–1584.

PACKARD, B., EDIDIN, M. and KOMORIYA, A., 1986, Site-directed labeling of a monoclonal antibody: targeting to a disulphide bond, *Biochem.*, **25**, 3548–3552.

PADDOCK, S., DEVRIES, P., BUTH, E. and CARROLL, S., 1994, Production of monoclonal antibodies by genetic immunization, *Biotechniques*, **16**, 616–620.

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., 1994, Anatomy of the antibody molecule, *Mol. Immunol.*, **31**, 169–217.

PAGANELLI, G., MAGNANI, P., ZITO, F., VILLA, E., SUDATI, F., LOPALCO, L., ROSSETTI, C., MALCOVATI, M., CHIOLERIO, F., SECCAMANI, E., SICCARDI, A.G. and FAZIO, F., 1991, Three step monoclonal antibody tumor targeting in carcinoembryonic antigen positive patients, *Cancer Res.*, **51**, 5960–5966.

PAGE, M.J. and SYDENHAM, M.A., 1991, High level expression of the humanised antibody CAMPATH–1H in chinese hamster ovary cells, *Bio/Technol.*, **9**, 65–68.

PAI, L.H., WITTES, R., SETSER, A., WILLINGHAM, M.C. and PASTAN, I., 1996, Treatment of advanced solid tumors with immunotoxin LMB-1: an antibody linked to *Pseudomonas* exotoxin, *Nature Med.*, **2**, 350–353.

PAK, K.Y., NEDELMAN, M.A., KANKE, M., KHAW, B.A., MATTIS, J.A., STRAUSS, H.W., DEAN, R.T. and BERGER, H.J., 1992, An instant kit method for labeling antimyosin Fab′ with technetium-99m: evaluation in an experimental myocardial infarct model, *J. Nucl. Med.*, **33**, 144–149.

PARHAM, P., 1986, Preparation and purification of active fragments from mouse monoclonal antibodies, *Immunochemistry* (Weir, D.M., ed.), pp.14.1–14.23, Blackwell Scientific Publications, Oxford.

PARK, J.W., HONG, K., CARTER, P., ASGARI, H., GUO, L.Y., KELLER, G.A., WIRTH, C., SHALABY, R., KOTTS, C., WOOD, W.I., PAPAHADJOPOULOS, D. and BENZ, C.C., 1995, Development of anti-$p185^{HER2}$ immunoliposomes for cancer therapy, *Proc. Natl Acad. Sci. (USA)*, **92**, 1327–1331.

PARK, L.S., FRIEND, D., PRICE, V., ANDERSON, D., SINGER, J., PRICKETT, K.S. and URDAL, D.L., 1989, Heterogeneity in human interleukin 3 receptors: a subclass that binds human granulocyte/macrophage colony stimulating factor, *J. Biol. Chem.*, **264**, 5420–5427.

PARKINSON, S., 1995, Production of monoclonal antibodies in the milk of transgenic animals, *Exploiting Transgenic Technology*, IBC symposium, San Diego, November.

PASTAN, I.H., ARCHER, G.E., MCLENDON, R.E., FRIEDMAN, H.S., FUCHS, H.E., WANG, Q.C., PAI, L.H., HERNDON, J. and BIGNER, D.D., 1995, Intrathecal administration of single-chain immunotoxin, LMB-7 [B3(Fv)-PE38], produces cures of carcinomatous meningitis in a rat model, *Proc. Natl Acad. Sci. (USA)*, **92**, 2765–2769.

PEAKMAN, T.C., WORDEN, J., HARRIS, R.H., COOPER, H., TITE, J., PAGE, M.J., GEWERT, D.R., BARTHOLEMEW, M., CROWE, J.S. and BRETT, S., 1994, Comparison of expression of a humanised monoclonal antibody in mouse NS0 myeloma cells and chinese hamster ovary cells, *Hum. Antibod. Hybridomas*, **5**, 65–74.

PEDLEY, R.B., BODEN, J.A., BODEN, R., BEGENT, R.H.J., TURNER, A., HAINES, A.M.R. and KING, D.J., 1994, The potential for enhanced tumour localization by poly(ethylene glycol) modification of anti-CEA antibody, *Br. J. Cancer*, **70**, 1126–1130.

PEDLEY, R.B., BODEN, J., BODEN, R., DALE, R. and BEGENT, R.H.J., 1993, Comparative radioimmunotherapy using intact or $F(ab')_2$ fragments of ^{131}I anti-CEA antibody in a colonic xenograft model, *Br. J. Cancer*, **68**, 69–73.

PEDLEY, R.B., DALE, R., BODEN, J.A., BEGENT, R.H.J., KEEP, P. and GREEN, A., 1989, The effect of second antibody clearance on the distribution and dosimetry of radiolabelled anti-CEA antibody in a human colonic tumour xenograft model, *Int. J. Cancer*, **43**, 713–718.

PEI, X.Y., HOLLIGER, P., MURZIN, A.G. and WILLIAMS, R.L., 1997, The 2.0 Å resolution crystal structure of a trimeric antibody fragment with noncognate VH–VL domain pairs shows a rearrangement of VH CDR3, *Proc. Natl Acad. Sci. (USA)*, **94**, 9637–9642.

PELTIER, P., CURTET, C., CHATAL, J.F., LEDOUSSAL, J.M., DANIEL, G., AILLET, G., GRUAZ-GUYON, A., BARBET, J. and DELAAGE, M., 1993, Radioimmunodetection of medullary thyroid cancer using a bispecific anti-CEA/anti-indium-DTPA antibody and an indium-111-labeled DTPA dimer, *J. Nucl. Med.*, **34**, 1267–1273.

PERISIC, O., WEBB, P.A., HOLLIGER, P., WINTER, G. and WILLIAMS, R.L., 1994, Crystal structure of a diabody, a bivalent antibody fragment, *Structure*, **2**, 1217–1226.

PETTERSSON, S., COOK, G.P., BRUGGEMANN, M., WILLIAMS, G.T. and NEUBERGER, M.S., 1990, A second B cell-specific enhancer 3′ of the immunoglobulin heavy chain locus, *Nature*, **344**, 165–168.

PHILLIPS, T.M., 1989, High-performance immunoaffinity chromatography, *Adv. Chromatogr.*, **29**, 133–173.

PHILPOTT, G.W., SCHWARZ, S.W., ANDERSON, C.J., DEHDASHTI, F., CONNETT, J.M., ZINN, K.R., MEARES, C.F., CUTLER, P.D., WELCH, M.J. and SIEGAL, B.A., 1995, RadioimmunoPET: detection of colorectal carcinoma with positron-emitting copper-64-labeled monoclonal antibody, *J. Nucl. Med.*, **36**, 1818–1824.

PIETERSZ, G.A., ROWLAND, A., SMYTH, M.J. and MCKENZIE, I.F.C., 1994, Chemoimmunoconjugates for the treatment of cancer, *Adv. Immunol.*, **56**, 301–387.

PILSON, R.S., LEVIN, W., DESAI, B., REIK, L.M., LIN, P., KORKMAZ-DUFFY, E., CAMPBELL, E., TSO, J.Y., KERWIN, J.A. and HAKIMI, J., 1997, Bispecific humanised anti-IL-2 receptor αβ antibodies inhibitory for both IL-2 and IL-15 mediated proliferation, *J. Immunol.*, **159**, 1543–1556.

PINCUS, S.H. and MCCLURE, J., 1993, Soluble CD4 enhances the activity of immunotoxins directed against gp41 of the human immunodeficiency virus, *Proc. Natl Acad. Sci. (USA)*, **90**, 332–336.

POLAK, J.M. and PRIESTLEY, J.V., eds, 1992, *Electron Microscopic Immunocytochemistry*, Oxford University Press, Oxford.

POLJAK, R.J., AMZEL, L.M., CHEN, B.L., PHIZACKERLY, R.P. and SAUL, F., 1973, Three-dimensional structure of the Fab′ fragment of a human immunoglobulin at 2.8 Å resolution, *Proc. Natl Acad. Sci. (USA)*, **71**, 3440–3444.

POLLACK, S.J., JACOBS, J.W. and SCHULZ, P.G., 1986, Selective chemical catalysis by an antibody, *Science*, **234**, 1570–1573.

PONCET, P., PANCZAK, A., GOUPY, C., GUSTAFSSON, K., BLANPIED, C., CHAVANEL, G., HIRSCH, R. and HIRSCH, F., 1996, Antifection, an antibody-mediated method to introduce genes into lymphoid cells in vitro and in vivo, *Gene Therapy*, **3**, 731–738.

POTTER, K.N., LI, Y. and CAPRA, J.D., 1996, Staphylococcal protein A simultaneously interacts with framework region 1, complementarity determining region 2 and framework region 3 on human VH3 encoded Igs, *J. Immunol.*, **157**, 2982–2988.

POU, K., ONG, H., ADAM, A., LAMOTHE, P. and DELAHAUT, P., 1994, Combined immunoextraction approach coupled to a chemiluminescence enzyme immunoassay for the determination of trace levels of salbutamol and clenbuterol in tissue samples, *Analyst*, **119**, 2659–2662.

POUL, M., CERUTTI, M., CHAABIHI, H., TICCHIONI, M., DERAMOUDT, F., BERNARD, A., DEVAUCHELLE, G., KACZOREK, M. and LEFRANC, M.P., 1995, Cassette baculovirus vectors for the production of chimeric, humanized, or human antibodies in insect cells, *Eur. J. Immunol.*, **25**, 2005–2009.

PRESS, O.W., EARY, J.F., APPELBAUM, F.R., MARTIN, P.J., NELP, W.B., GLENN, S., FISHER, D.R., PORTER, B., MATTHEWS, D.C., GOOLEY, T. and BERNSTEIN, I.D., 1995, Phase II trial of ^{131}I-B1 (anti-CD20) antibody therapy with autologous stem cell transplantation for relapsed B cell lymphomas, *Lancet*, **346**, 336–340.

PRESS, O.W., SHAN, D., HOWELL-CLARK, J., EARY, J., APPELBAUM, F.R., MATTHEWS, D., KING, D.J., HAINES, A.M.R., HAMANN, P., HINMAN, L., SHOCHAT, D. and BERNSTEIN, I., 1996, Comparative metabolism and retention of iodine-125, yttrium-90 and indium-111 radioimmunoconjugates by cancer cells, *Cancer Res.*, **56**, 2123–2129.

PRESTA, L.G., LAHR, S.J., SHIELDS, R.L., PORTER, J.P., GORMAN, C.M., FENDLY, B.M. and JARDIEU, P.M., 1993, Humanization of an antibody directed against IgE, *J. Immunol.*, **151**, 2623–2632.

PRESTA, L.G., SHIELDS, R.L., O'CONNELL, L., LAHR, S.J., PORTER, J.P., GORMAN, C.M. and JARDIEU, P.M., 1994, The binding site on human immunoglobulin E for its high affinity receptor, *J. Biol. Chem.*, **269**, 26368–26373.

PRISYAZHNOY, V.S., FUSEK, M. and ALAKHOV, Y., 1988, Synthesis of high-capacity immunoaffinity sorbents with oriented immobilized immunoglobulins or their Fab′ fragments for isolation of proteins, *J. Chromatogr.*, **424**, 243–253.

PRONGAY, A.J., SMITH, T.J., ROSSMAN, M.G., EHRLICH, L.S., CARTER, C.A. and MCCLURE, J., 1990, Preparation and crystallization of a human immunodeficiency virus p24–Fab complex, *Proc. Natl Acad. Sci. (USA)*, **87**, 9980–9984.

PROUDFOOT, K.A., TORRANCE, C., LAWSON, A.D.G. and KING, D.J., 1992, Purification of recombinant chimeric B72.3 Fab′ and F(ab′)2 using Streptococcal protein G, *Protein Expression Purification*, **3**, 368–373.

PULITO, V.L., ROBERTS, V.A., ADAIR, J.R., ROTHERMEL, A.L., COLLINS, A.M., VARGA, S.S., MARTOCELLO, C., BODMER, M., JOLLIFFE, L.K. and ZIVIN, R.A., 1996, Humanization and molecular modelling of the anti-CD4 monoclonal antibody OKT4A, *J. Immunol.*, **156**, 2840–2850.

QUADRI, S.M., LAI, J., MOHAMMADPOR, H., VRIESENDORP, H.M. and WILLIAMS, J.R., 1993, Assessment of radiolabelled stabilized F(ab′)$_2$ fragments of monoclonal antiferritin in nude mouse model, *J. Nucl. Med.*, **34**, 2152–2159.

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.

QUESNIAUX, V.F., 1991, Monoclonal antibody technology for cyclosporin monitoring, *Clin. Biochem.*, **24**, 37–42.

QUEZADO, Z.M.N., BANKS, S.M. and NATANSON, C., 1995, New strategies for combatting sepsis: the magic bullets missed the mark but the search continues, *Trends Biotechnol.*, **13**, 56–63.

RAAG, R. and WHITLOW, M., 1995, Single-chain Fvs, *FASEB J.*, **9**, 73–80.

RAJEWSKY, K., 1996, Clonal selection and learning in the immune system, *Nature*, **381**, 751–758.

RAMAN-SURI, C., JAIN, P.K. and MISHRA, G.C., 1995, Development of a piezoelectric crystal based microgravimetric immunoassay for determination of insulin concentration, *J. Biotechnol.*, **39**, 27–34.

RANKIN, E.C.C., CHOY, E.H.S., KASSIMOS, D., KINGSLEY, G.H., SOPWITH, A.M., ISENBERG, D.A. and PANAYI, G.S., 1995, The therapeutic effects of an engineered human anti-tumour necrosis factor alpha antibody (CDP571) in rheumatoid arthritis, *Br. J. Rheumatol.*, **34**, 334–342.

REA, D.W. and ULTEE, M.E., 1993, A novel method for controlling the pepsin digestion of antibodies, *J. Immunol. Methods*, **157**, 165–173.

REARDAN, D.T., MEARES, C.F., GOODWIN, D.A., MCTIGUE, M., DAVID, G.S., STONE, M.R., LEUNG, J.P., BARTHOLOMEW, R.M. and FRINCKE, J.M., 1985, Antibodies against metal chelates, *Nature*, **316**, 265–267.

REES, A.R., STAUNTON, D., WEBSTER, D.M., SEARLE, S.J., HENRY, A.H. and PEDERSEN, J.T., 1994, Antibody design: beyond the natural limits, *Trends Biotechnol.*, **12**, 199–206.

REFF, M.E., CARNER, K., CHAMBERS, K.S., CHINN, P.C., LEONARD, J.E., RAAB, R., NEWMAN, R.A., HANNA, N. and ANDERSON, D.R., 1994, Depletion of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20, *Blood*, **83**, 435–445.

REIST, C.J., ARCHER, G.E., KURPAD, S.N., WIKSTRAND, C.J., VAIDANATHAN, G., WILLINGHAM, M.C., MOSCATELLO, D.K., WONG, A.J., BIGNER, D.D. and ZALUTSKY, M.R., 1995, Tumor-specific anti-epidermal growth factor receptor variant III monoclonal antibodies: use of the tyramine–cellobiose radioiodination method enhances cellular retention and uptake in tumour xenografts, *Cancer Res.*, **55**, 4375–4482.

REIST, C.J., GARG, P.K., ALSTON, K.L., BIGNER, D.D. and ZALUTSKY, M.R., 1996, Radioiodination of internalising monoclonal antibodies using *N*-succinimidyl-5-iodo-3-pyridine carbonate, *Cancer Res.*, **56**, 4970–4977.

REITER, Y., BRINKMANN, U., KREITMAN, R.J., JUNG, S.H., LEE, B. and PASTAN, I., 1994, Stabilization of the Fv fragments in recombinant immunotoxins by disulfide bonds engineered into conserved framework regions, *Biochem.*, **33**, 5451–5459.

REITER, Y. and PASTAN, I., 1996, Antibody engineering of recombinant Fv immunotoxins for improved targeting of cancer: disulfide stabilized Fv immunotoxins, *Clin. Cancer Res.*, **2**, 245–252.

REITER, Y., WRIGHT, A.F., TONGE, D.W. and PASTAN, I., 1996, Recombinant single-chain and disulfide-stabilized Fv-immunotoxins that cause complete regression of a human colon cancer xenograft in nude mice, *Int. J. Cancer*, **67**, 113–123.

RENNER, C., JUNG, W., SAHIN, U., DENFIELD, R., POHL, C., TRUMPER, L., HARTMANN, F., DIEHL, V., VAN LIER, R. and PFREUNDSCHUH, M., 1994, Cure of xenografted human tumors by bispecific monoclonal antibodies and human T cells, *Science*, **264**, 833–835.

RENNER, C. and PFREUNDSCHUH, M., 1995, Tumor therapy by immune recruitment with bispecific antibodies, *Immunol. Rev.*, **145**, 179–209.

RETTIG, W.J., GARIN-CHESA, P., HEALEY, J.H., SU, S.L., JAFFE, E.A. and OLD, L.J., 1992, Identification of endosialin, a cell surface glycoprotein of vascular endothelial cells in human cancer, *Proc. Natl Acad. Sci. (USA)*, **89**, 10832–10836.

RHEINNECKER, M., HARDT, C., ILAG, L.L., KUFER, P., GRUBER, R., HOESS, A., LUPAS, A., ROTTENBERGER, C., PLUCKTHUN, A. and PACK, P., 1996, Multivalent antibody fragments with high functional affinity for a tumor-associated antigen, *J. Immunol.*, **157**, 2989–2997.

RHIND, S.K., KING, D.J., BODEN, J., BODEN, R., 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.

RICHARDSON, J.H., SODROSKI, J.G., WALDMANN, T.A. and MARASCO, W.A., 1995, Phenotypic knockout of the high-affinity human interleukin-2 receptor by intracellular single-chain antibodies against the alpha subunit of the receptor, *Proc. Natl Acad. Sci. (USA)*, **92**, 3137–3141.

RIDDER, R., SCHMITZ, R., LEGAY, F. and GRAM, H., 1995, Generation of rabbit monoclonal antibody fragments from a combinatorial phage display library and their production in the yeast *Pichia pastoris*, *Bio/Technol.*, **13**, 255–260.

RIDGEWAY, J.B.B., PRESTA, L.G. and CARTER, P., 1996, Knobs-into-holes engineering of antibody CH3 domains for heavy chain heterodimerization, *Protein Engin.*, **9**, 617–621.

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

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

RIECHMANN, L., WEILL, M. and CAVANAGH, J., 1992, Improving the antigen affinity of an antibody Fv-fragment by protein design, *J. Mol. Biol.*, **224**, 913–918.

RIETHMULLER, G., SCHNEIDER-GADICKE, E., SCHLIMOK, G., SCHMIEGEL, W., RAAB, R., HOFFKEN, K., GRUBER, R., PICHLMAIER, H., HIRCHE, H., PICHLMAYR, R., BUGGISCH, P. and WITTE, J., 1994, Randomised trial of monoclonal antibody for adjuvant therapy of resected Dukes' C colorectal carcinoma, *Lancet*, **343**, 1177–1183.

RIGL, T., RIVERA, H.N., PATEL, M.T., BALL, R.T., STULTS, N.L. and SMITH, D.F., 1995, Bioluminescence immunoassays for human endocrine hormones based on aqualite, a calcium activated photoprotein, *Clin. Chem.*, **41**, 1363–1364.

RINI, J.M., SCHULZE-GAHMEN, U. and WILSON, I.A., 1992, Structural evidence for induced fit as a mechanism for antibody–antigen recognition, *Science*, **255**, 959–965.

ROBERTS, S., CHEETHAM, J.C. and REES, A.R., 1987, Generation of an antibody with enhanced affinity and specificity for its antigen by protein engineering, *Nature*, **328**, 731–734.

RODRIGUES, M.L., PRESTA, L.G., KOTTS, C.E., WIRTH, C., MORDENTI, J., OSAKA, G., WONG, W.L.T., NUIJENS, A., BLACKBURN, B. and CARTER, P., 1995, Development of a humanized disulfide-stabilized anti-p185^{HER2} Fv-β-lactamase fusion protein for activation of a cephalosporin doxorubicin prodrug, *Cancer Res.*, **55**, 63–70.

RODRIGUES, M.L., SNEDCOR, B., CHEN, C., WONG, W.L.T., GARG, S., BLANK, G.S., MANEVAL, D. and CARTER, P., 1993, Engineering Fab′ fragments for efficient F(ab′)$_2$ formation in *Escherichia coli* and for improved *in vivo* stability, *J. Immunol.*, **151**, 6954–6961.

RODWELL, J., ALVAREZ, V., LEE, C., LOPES, A., GOERS, J., KING, H., POWSNER, H. and MCKEARN, T., 1986, Site-specific covalent modification of mAbs: *in vitro* and *in vivo* evaluations, *Proc. Natl Acad. Sci. (USA)*, **83**, 2632–2636.

ROES, J. and RAJEWSKY, K., 1993, Immunoglobulin D (IgD)-deficient mice reveal an auxiliary receptor function for IgD in antigen-mediated recruitment of B cells, *J. Exp. Med.*, **177**, 45–55.

ROGUSKA, M.A., PEDERSEN, J.T., KEDDY, C.A., HENRY, A.H., SEARLE, S.J., LAMBERT, J.M., GOLDMACHER, V.S., BLATTER, W.A., REES, A.R. and GUILD, B.C., 1994, Humanization of murine monoclonal antibodies through variable domain resurfacing, *Proc. Natl Acad. Sci. (USA)*, **91**, 969–973.

RONDELLI, D., ANDREWS, R.G., HANSEN, J.A., RYNCARZ, R., FAERBER, M.A. and ANASETTI, C., 1996, Alloantigen presenting function of normal human CD34+ hematopoietic cells, *Blood*, **88**, 2619–2675.

RONG, H., DEFTOS, L.J., JI, H. and BUCHT, E., 1997, Two-site immunofluorometric assay of intact salmon calcitonin with improved sensitivity, *Clin. Chem.*, **43**, 71–75.

RONGEN, H.A.H., VAN DER HORST, H.M., OOSTERHOUT, A.J.M., BULT, A. and VAN BENNEKOM, W.P., 1997, Application of xanthine oxidase-catalyzed luminol chemiluminescence in a mouse interleukin-5 immunoassay, *J. Immunol. Methods*, **197**, 161–169.

ROSS, A.H., HERLYN, D. and KOPROWSKI, H., 1987, Purification of monoclonal antibodies from ascites using ABx liquid chromatography column, *J. Immunol. Methods*, **102**, 227–231.

ROUSSEAUX, J., ROUSSEAUX-PREVOST, R. and BAZIN, H., 1983, Optimal conditions for the preparation of Fab and F(ab′)$_2$ fragments from monoclonal IgG of different rat IgG subclasses, *J. Immunol. Methods*, **64**, 141–146.

ROUX, P., JEANTEUR, P. and PIECHACZYK, M., 1989, A versatile and potentially general approach to the targeting of specific cell types by retroviruses, *Proc. Natl Acad. Sci. (USA)*, **86**, 9079–9083.

RUBIN, R.H., BALTIMORE, D., CHEN, B.K., WILKINSON, R.A. and FISCHMAN, A.J., 1996, *In vivo* tissue distribution of CD4 lymphocytes in mice determined by radioimmunoscintigraphy with an ^{111}In-labeled anti-CD4 monoclonal antibody, *Proc. Natl Acad. Sci. (USA)*, **93**, 7460–7463.

RUBIN, R.H., FISCHMAN, A.J., CALLAHAN, R.J., KHAW, B.A., KEECH, F., AHMAD, M., WILKINSON, R. and STRAUSS, H.W., 1989, ^{111}In labeled nonspecific immunoglobulin scanning in the detection of focal infection, *N. Engl. J. Med.*, **321**, 935–940.

RUNGE, M.S., BODE, C., MATSUEDA, G.R. and HABER, E., 1988, Conjugation to an antifibrin monoclonal antibody enhances the fibrinolytic potency of tissue plasminogen activator *in vitro*, *Biochem.*, **27**, 1153–1157.

RUNGE, M.S., QUERTERMOUS, T., ZAVODNY, P.J., LOVE, T.W., BODE, C., FREITAG, M., SHAW, S.Y., HUANG, P.L., CHOU, C.C., MULLINS, D., SCHNEE, J.M., SAVARD, C.E., ROTHENBERG, M.E., NEWELL, J.B., MATSUEDA, G.R. and HABER, E., 1991, A recombinant chimeric plasminogen activator with high affinity for fibrin has increased thrombolytic potency *in vitro* and *in vivo*, *Proc. Natl Acad. Sci. (USA)*, **88**, 10337–10341.

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.

SAGARA, H., MATSUDA, H., WADA, N., YAGITA, H., FUKUDA, T., OKUMURA, K., MAKINO, S. and RA, C., 1997, A monoclonal antibody against very late activation antigen-4 inhibits eosinophil accumulation and late asthmatic response in a guinea pig model of asthma, *Int. Arch. Allergy Immunol.*, **112**, 287–294.

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. and DAVIS, G.L., 1986, A genetically engineered murine/chimeric antibody retains specificity for human tumour-associated antigen, *J. Immunol.*, **137**, 1066–1074.

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., 1992, Phase I trial of the chimeric anti-GD2 monoclonal antibody ch14.18 in patients with malignant melanoma, *Hum. Antibod. Hybridomas*, **3**, 19–24.

SANDHU, J., SHPITZ, B., GALLINGER, S. and HOZUMI, N., 1994, Human primary immune response in SCID mice engrafted with human peripheral blood lymphocytes, *J. Immunol.*, **152**, 3806–3813.

SANO, T., SMITH, C.L. and CANTOR, C.R., 1992, Immuno-PCR: very sensitive antigen detection by means of specific antibody–DNA conjugates, *Science*, **258**, 120–122.

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.

SASSO, D.E., GIONFRIDDO, M.A., THRALL, R.S., SYRBU, S.I., SMILOWITZ, H.M. and WEINER, R.E., 1996, Biodistribution of indium-111-labeled antibody directed against intercellular adhesion molecule-1, *J. Nucl. Med.*, **37**, 656–661.

SATO, H., WATANABE, K., AZUMA, J., KIDAKA, T. and HORI, M., 1989, Specific removal of IgE by therapeutic immunoadsorption system, *J. Immunol. Methods*, **118**, 161–168.

SATO, K., TSUCHIYA, M., SALDANHA, J., KOISHIHARA, Y., OHSUGI, Y., KISHIMOTO, T. and BENDIG, M.M., 1994, Humanization of a mouse anti-human interleukin-6 receptor antibody comparing two methods for selecting human framework regions, *Mol. Immunol.*, **31**, 371–381.

SAUER-ERICKSSON, A.E., KLEYWEGT, G.J., UHLEN, M. and JONES, T.A., 1995, Crystal structure of the C2 fragment of Streptococcal protein G in complex with the Fc domain of human IgG, *Structure*, **3**, 265–278.

SAWYER, J.R., SCHLOM, J. and KAHMIRI, S.V.S., 1994, The effects of induction conditions on production of a soluble anti-tumor sFv in *Escherichia coli*, *Protein Engin.*, **7**, 1401–1406.

SCHIABLE, T., DEWOODY, K., WEISMAN, H., LINE, B., KEENAN, A. and ALAVI, A., 1992, Accurate diagnosis of acute deep vein thrombosis with technetium99m antifibrin scintigraphy: final phase III results, *J. Nucl. Med.*, **33** (suppl.), 848.

SCHIER, R., MCCALL, A., ADAMS, G.P., MARSHALL, K.W., MERRITT, H., YIM, M., CRAWFORD, R.S., WEINER, L.M., MARKS, C. and MARKS, J.D., 1996, Isolation of picomolar affinity anti-c-erb-B2 single-chain Fv by molecular evolution of the complementarity determining regions in the center of the antibody binding site, *J. Mol. Biol.*, **263**, 551–567.

SCHMIDT, T.G.M. and SKERRA, A., 1994, One-step affinity purification of bacterially produced proteins by means of the strep-tag and immobilized recombinant core streptavidin, *J. Chromatogr.*, **676**, 337–343.

SCHOTT, M.E., FRAZIER, K.A., POLLOCK, D.K. and VERBANAC, K.M., 1993, Preparation, characterization, and *in vivo* biodistribution properties of synthetically cross-linked multivalent antitumour antibody fragments, *Bioconjugate Chem.*, 4, 153–165.

SCHOTT, M.E., MILENIC, D.E., YOKOTA, T., WHITLOW, M., WOOD, J.F., FORDYCE, W.A., CHENG, R.C. and SCHLOM, J., 1992, Differential metabolic patterns of iodinated versus radiometal chelated anticarcinoma single-chain Fv molecules, *Cancer Res.*, **52**, 6413–6417.

SCHRAPPE, M., BUMOL, T.F., APELGREN, L.D., BRIGGS, S.L., KOPPEL, G.A., MARKOWITZ, D.D., MUELLER, B.M. and REISFELD, R.A., 1992, Long-term growth suppression of human glioma xenografts by chemoimmunoconjugates of 4-desacetylvinblastine-3-carboxyhydrazide and monoclonal antibody 9.2.27, *Cancer Res.*, **52**, 3838–3844.

SCHRIER, D.M., STEMMER, S.M., JOHNSON, T., KASLIWAL, R., LEAR, J., MATTHES, S., TAFFS, S., DUFTON, C., GLENN, S.D., BUTCHKO, G., CERIANI, R.L., ROVIRA, D., BUNN, P., SHPALL, E.J., BEARMAN, S.I., PURDY, M., CAGNONI, P. and JONES, R.B., 1995, High-dose ^{90}Y Mx-DTPA-BrE3 and autologous stem cell support (AHSCS) for the treatment of advanced breast cancer: a phase 1 trial, *Cancer Res.*, **55**, 5921s–5924s.

SCHULTZ, P.G. and LERNER, R.A., 1995, From molecular diversity to catalysis: lessons from the immune system, *Science*, **269**, 1835–1842.

SCHUMACHER, J., KLIVENYI, G., MATYS, R., KIRCHGEBNER, H., HAUSER, H., MAIER-BORST, W. and MATZKU, S., 1990, Uptake of indium-111 in the liver of mice following administration of indium-111 DTPA-labeled monoclonal antibodies, *J. Nucl. Med.*, **31**, 1084–1093.

SCHUSTER, J.M., GARG, P.K., BIGNER, D.D. and ZALUTSKY, M.R., 1991, Improved therapeutic efficacy of a monoclonal antibody radioiodinated using *N*-succinimidyl-3-(tri-n-butylstannyl) benzoate, *Cancer Res.*, **51**, 4164–4169.

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, *Br. J. Cancer*, **53**, 377–384.

SEIDMAN, J.G. and LEDER, P., 1978, The arrangement and rearrangement of antibody genes, *Nature*, **276**, 790–795.

SELF, C.H., DESSI, J.L. and WINGER, L.A., 1994, High-performance assays of small molecules: enhanced sensitivity, rapidity and convenience demonstrated with a non-competitive immunometric anti-immune complex assay system for digoxin, *Clin. Chem.*, **40**, 2035–2041.

SEON, B.K., MATSUNO, F., HARUTA, Y., KONDO, M. and BARCOS, M., 1997, Long-lasting complete inhibition of human solid tumors in SCID mice by targeting endothelial cells of tumor vasculature with anti-human endoglin immunotoxin, *Clin. Cancer Res.*, **3**, 1031–1044.

SHARKEY, R.M., JUWEID, M., SHEVITZ, J., BEHR, T., DUNN, R., SWAYNE, L.C., WONG, G.Y., BLUMENTHAL, R.D., GRIFFITHS, G.L., SIEGAL, J.A., LEUNG, S., HANSEN, H.J. and GOLDENBERG, D.M., 1995, Evaluation of a complementarity-determining region-grafted (humanized) anti-carcinoembryonic antigen monoclonal antibody in preclinical and clinical studies, *Cancer Res.*, Suppl. **55**, 5935s–5945s.

SHARMA, S.K., BAGSHAWE, K.D., BURKE, P.J., BODEN, R.W. and ROGERS, G.T., 1990, Inactivation and clearance of an anti-CEA carboxypeptidase G2 conjugate in blood after localisation in a xenograft model, *Br. J. Cancer*, **61**, 659–662.

SHARON, J. and GIVOL, D., 1976, Preparation of Fv fragment from the mouse myeloma XRPC-25 immunoglobulin possessing anti-dinitrophenyl activity, *Biochem.*, **15**, 1591–1594.

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 human IgG subclasses, *J. Natl Cancer Inst.*, **80**, 1553–1559.

SHIH, L.B., GOLDENBERG, D.M., XUAN, H., LU, H., SHARKEY, R.M. and HALL, T.C., 1991, Anthracycline immunoconjugates prepared by a site-specific linkage via an amino-dextran intermediate carrier, *Cancer Res.*, **51**, 4192–4198.

SHIN, S.U., FRIDEN, P., MORAN, M. and MORRISON, S.L., 1994, Functional properties of antibody insulin-like growth factor fusion proteins, *J. Biol. Chem.*, **269**, 4979–4985.

SHIN, S.U., FRIDEN, P., MORAN, M., OLSON, T., KANG, Y., PARDRIDGE, W.M. and MORRISON, S.L., 1995, Transferrin–antibody fusion proteins are effective in brain targeting, *Proc. Natl Acad. Sci. (USA)*, **92**, 2820–2824.

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, *Human Antibod. Hybrid.*, **3**, 65–74.

SHIN, S.U., WU, D., RAMANTHAN, R., PARDRIDGE, W.M. and MORRISON, S.L., 1997, Functional and pharmacokinetic properties of antibody–avidin fusion proteins, *J. Immunol.*, **158**, 4797–4804.

SHOPES, B., 1992, A genetically engineered human IgG mutant with enhanced cytolytic activity, *J. Immunol.*, **148**, 2918–2922.

SIEGALL, C.B., LIGGITT, D., CHACE, D., MIXAN, B., SUGAI, J., DAVIDSON, T. and STEINITZ, M., 1997, Characterization of vascular leak syndrome induced by the toxin component of *Pseudomonas* exotoxin-based immunotoxins and its potential inhibition with non-steroidal anti-inflammatory drugs, *Clin. Cancer Res.*, **3**, 339–345.

SIMMONS, L.C. and YANSURA, D.G., 1996, Translational level is a critical factor for the secretion of heterologous proteins in *Escherichia coli*, *Nature Biotech.*, **14**, 629–634.

SIMON, T. and RAJEWSKY, K., 1992, A functional antibody mutant with an insertion in the framework region 3 loop of the VH domain: implications for antibody engineering, *Protein Engin.*, **5**, 229–234.

SIMS, M.J., HASSAL, D.G., BRETT, S., ROWAN, W., LOCKYER, M.J., ANGEL, A., LEWIS, A.P., HALE, G., WALDMANN, H. and CROWE, J.S., 1993, A humanized CD18 antibody can block function without cell destruction, *J. Immunol.*, **151**, 2296–2308.

SINGER, I.I., KAWKA, D.W., DEMARTINO, J.A., DAUGHERTY, B.L., ELLISTON, K.O., ALVES, K., BUSH, B.L., CAMERON, P.M., CUCA, G.C., DAVIES, P., FORREST, M.J., KAZAZIS, D.M., LAW, M.F., LENNY, A.B., MACINTYRE, D.E., MEURER, R., PADLAN, E.A., PANDYA, S., SCHMIDT, J.A., SEAMANS, T.C., SCOTT, S., SILBERKLANG, M., WILLIAMSON, A.R. and MARK, G.E., 1993, Optimal humanization of 1B4, an anti-CD18 murine monoclonal antibody is achieved by correct choice of human V-region framework sequences, *J. Immunol.*, **150**, 2844–2857.

SISSON, T.H. and CASTOR, C.W., 1990, An improved method for immobilising IgG antibodies to protein A agarose, *J. Immunol. Methods*, **127**, 215–220.

SIVOLAPENKO, G.B., DOULI, V., PECTASIDES, D., SKARLOS, D., SIRMALIS, G., HUSSAIN, R., COOK, J., COURTNEY-LUCK, N.S., MERKOURI, E., KONSTANTINIDES, K. and EPENETOS, A.A., 1995, Breast cancer imaging with radiolabelled peptide from complementarity-determining region of antitmour antibody, *Lancet*, **346**, 1662–1666.

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 technique, *Bio/Technol.*, **9**, 273–278.

SKERRA, A. and PLUCKTHUN, A., 1988, Assembly of a functional immunoglobulin Fv fragment in *Escherichia coli*, *Science*, **240**, 1038–1041.

SKERRA, A. and PLUCKTHUN, A., 1991, Secretion and *in vivo* folding of the Fab fragment of the antibody McPC603 in *Escherichia coli*: influence of disulphides and cis-prolines, *Protein Engin.*, **4**, 971–979.

SLAMON, D.J., CLARK, G.M., WONG, S.G., LEVIN, W.J., ULLRICH, A. and MCQUIRE, W.L., 1987, Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene, *Science*, **235**, 177–182.

SMITH, G.P., 1985, Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface, *Science*, **228**, 1315–1317.

SMITH, R.I.F., COLOMA, M.J. and MORRISON, S.L., 1995, Addition of a μ-tailpiece to IgG results in polymeric antibodies with enhanced effector functions including complement mediated cytolysis by IgG4, *J. Immunol.*, **154**, 2226–2236.

SMITH, R.I.F. and MORRISON, S.L., 1994, Recombinant polymeric IgG: an approach to engineering more potent antibodies, *Bio/Technol.*, **12**, 683–688.

SONGSIVILAI, S., CLISSOLD, P.M. and LACHMAN, P.J., 1989, A novel strategy for producing chimeric bispecific antibodies by gene transfection, *Biochem. Biophys. Res. Commun.*, **164**, 271–276.

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

SPADA, S. and PLUCKTHUN, A., 1997, Selectively infective phage (SIP) technology: a novel method for *in vivo* selection of interacting protein–ligand pairs, *Nature Med.*, **3**, 694–696.

SPEIKER-POLET, H., SETHUPATHI, P., YAM, P.C. and KNIGHT, K.L., 1995, Rabbit monoclonal antibodies: generating a fusion partner to produce rabbit–rabbit hybridomas, *Proc. Natl Acad. Sci. (USA)*, **92**, 9348–9352.

SPITZNAGEL, T.M. and CLARK, D.S., 1993, Surface-density and orientation effects on immobilized antibodies and antibody fragments, *Bio/Technol.*, **11**, 825–829.

SPRINGER, T.A., 1994, Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm, *Cell*, **76**, 301–314.

STACK, W.A., MANN, S.D., ROY, A.J., HEATH, P., SOPWITH, M., FREEMAN, J., HOLMES, G., LONG, R., FORBES, A., KAMM, M.A. and HAWKEY, C.J., 1997, Randomised controlled trial of CDP571 antibody to tumour necrosis factor-α in Crohn's disease, *Lancet*, **349**, 521–524.

STANCOVSKI, I., SCHINDLER, D.G., WAKS, T., YARDEN, Y., SELA, M. and ESHHAR, Z., 1993, Targeting of T-lymphocytes to neu/HER2-expressing cells using chimeric single chain Fv receptors, *J. Immunol.*, **151**, 6577–6582.

STANKER, L.H., VANDERLAAN, M. and JUAREZ-SALINAS, H., 1985, One-step purification of mouse monoclonal antibodies from ascites fluid by hydroxylapatite chromatography, *J. Immunol. Methods*, **79**, 157–169.

STANLEY, C.J., JOHANNSSON, A. and SELF, C.H., 1985, Enzyme amplification can enhance both the speed and sensitivity of immunoassays, *J. Immunol. Methods*, **83**, 89–95.

STEIN, R., GOLDENBERG, D.M., THORPE, S.R. and MATTES, M.J., 1997, Advantage of a residualising iodine radiolabel for radioimmunotherapy of xenografts of human non-small cell carcinoma of the lung, *J. Nucl. Med.*, **38**, 391–395.

STEISS, R.G., CARRASQUILLO, J.A., MCCABE, R., BOOKMAN, M.A., REYNOLDS, J.C., LARSON, S.M., SMITH, J.W., CLARK, J.W., DAILEY, V., DEL VECCHIO, S., SHUKE, N., PINSKY, C.M., URBA, W.J., HASPEL, M., PERENTESIS, P., PARIS, B., LONGO, D.L. and HANNA, M.G., JR, 1990, Toxicity, immunogenicity and tumor radioimmunodetecting ability of two human monoclonal antibodies in patients with metastatic colorectal carcinoma, *J. Clin. Oncol.*, **8**, 476–490.

STEPHENS, P. and COCKETT, M., 1989, A versatile set of mammalian expression vectors, *Nucl. Acids Res.*, **17**, 7110.

STEPHENS, S., EMTAGE, S., VETTERLEIN, O., CHAPLIN, L., BEBBINGTON, C., NESBITT, A., SOPWITH, M., ATHWAL, D., NOVAK, C. and BODMER, M., 1995, Comprehensive pharmacokinetics of a humanized antibody and analysis of residual anti-idiotypic responses, *Immunol.*, **85**, 668–674.

STEWART, J.S.W., HIRD, V., SNOOK, D., DHOKIA, B., SIVOLAPENKO, G.B., HOOKER, G., TAYLOR-PAPADIMITRIOU, J., SULLIVAN, M., LAMBERT, H.E., COULTER, C., MASON, W.P., SOUTTER, W.P. and EPENETOS, A.A., 1990, Intraperitoneal yttrium-90 labeled monoclonal antibody in ovarian cancer, *J. Clin. Oncol.*, **8**, 1941–1950.

STEWART, J.S.W., HIRD, V., SNOOK, D., SULLIVAN, M., HOOKER, G., COURTENAY-LUCK, N., SIVOLAPENKO, G., GRIFFITHS, G., MYERS, M.J., LAMBERT, H.E. and EPENETOS, A.A., 1989, Intraperitoneal radioimmunotherapy for ovarian cancer: pharmacokinetics, toxicity and efficacy of I-131 labeled monoclonal antibodies, *Int. J. Radiat. Oncol. Biol. Phys.*, **16**, 405–413.

STRATTON, J.R., CERQUEIRA, M.D., DEWHURST, T.A. and KOHLER, T.R., 1994, Imaging arterial thrombosis: comparison of technetium-99m labeled monoclonal antifibrin antibody and indium-111 labeled platelets, *J. Nucl. Med.*, **35**, 1731–1737.

SU, X., PRESTWOOD, A.K. and MCGRAW, R.A., 1992, Production of recombinant porcine tumor necrosis factor alpha in a novel *E. coli* expression system, *Biotechniques*, **13**, 756–762.

SUITTERS, A.J., FOULKES, R., OPAL, S.M., PALARDY, J.E., EMTAGE, J.S., ROLFE, M., STEPHENS, S., MORGAN, A., HOLT, A.R., CHAPLIN, L.C., SHAW, N.E., NESBITT, A.M. and BODMER, M.W., 1994, Differential effect of isotype on efficacy of anti-tumor necrosis factor α chimeric antibodies in experimental septic shock, *J. Exp. Med.*, **179**, 849–856.

SUN, L.K., FUNG, M.S.C., SUN, W.N.C., SUN, C.R.Y., CHANG, W. and CHANG, T.W., 1995, Human IgA monoclonal antibodies specific for a major ragweed pollen antigen, *Bio/Technol.*, **13**, 779–786.

SUTTON, B.J. and GOULD, H.J., 1993, The human IgE network, *Nature*, **366**, 421–428.

TAI, M.S., MUDGETT-HUNTER, M., LEVINSON, D., WU, G.M., HABER, E., OPPERMANN, H. and HUSTON, J.S., 1990, A bifunctional fusion protein containing Fc binding fragment B of Staphylococcal protein A amino terminal to antidigoxin single-chain Fv, *Biochem.*, **29**, 8024–8030.

TAKAHASHI, H., ODAKA, A., KAWAMINAMI, S., MATSUNAGA, C., KATO, K., SHIMADA, I. and ARATA, Y., 1991, Multinuclear NMR study of the structure of the Fv fragment of anti-dansyl mouse IgG2a antibody, *Biochem.*, **30**, 6611–6619.

TAO, M.H. and MORRISON, S.L., 1989, Studies of aglycosylated chimeric mouse–human IgG, *J. Immunol.*, **143**, 2595–2601.

TAO, M.H., SMITH, R.I.F. and MORRISON, S.L., 1993, Structural features of human immunoglobulin G that determine isotype-specific differences in complement activation, *J. Exp. Med.*, **178**, 661–667.

TAVLADORAKI, P., BENVENUTO, E., TRINCA, S., DEMARTINIS, D., CATTANEO, A. and GALEFFI, P., 1993, Transgenic plants expressing a functional single-chain Fv are specifically protected from virus attack, *Nature*, **366**, 469–472.

TEMPEST, P.R., BREMNER, P., LAMBERT, M., TAYLOR, M., FURZE, L.M., CARR, F.J. and HARRIS, W.J., 1991, Reshaping a human monoclonal antibody to inhibit human respiratory syncytial virus infection in vivo, *Bio/Technol.*, **9**, 266–271.

TERSKIKH, A., COUTY, S., PELEGRIN, A., HARDMAN, N., HUNZIKER, W. and MACH, J.P., 1994, Dimeric recombinant IgA directed against carcino-embryonic antigen, a novel tool for carcinoma localization, *Mol. Immunol.*, **31**, 1313–1319.

THAKUR, M.L., DEFULVIO, J., PARK, C.H., DAMJANOV, A., YAGHSEZIAN, H., JUNGKIND, D., EPSTEIN, A. and MCAFEE, J.G., 1991, Technetium labeled proteins for imaging inflammatory foci, *Nucl. Med. Biol.*, **18**, 605–612.

THAKUR, M.L., MARCUS, C.S., HENNEMAN, P., BUTLER, J., SINOW, R., DIGGLES, L., MINAMI, C., MASON, G., KLEIN, S. and RHODES, B., 1996, Imaging inflammatory diseases with neutrophil-specific technetium-99m-labeled monclonal antibody anti-SSEA-1, *J. Nucl. Med.*, **37**, 1789–1795.

THOMMES, J., HALFAR, M., LENZ, S. and KULA, M.R., 1995, Purification of monoclonal antibodies from whole hybridoma fermentation broth by fluidized bed adsorption, *Biotech. Bioengin.*, **45**, 205–211.

THORPE, G.H.G. and KRICKA, L.J., 1986, Enhanced chemiluminescence reactions catalysed by horseradish peroxidase, *Methods Enzymol.*, **133**, 331–354.

THORPE, P., WALLACE, P., KNOWLES, P., RELF, M., BROWN, N., WATSON, G., BLAKEY, D. and NEWELL, D., 1988, Improved antitumour effects of immunotoxins prepared with deglycosylated ricin A-chain and hindered disulphide linkages, *Cancer Res.*, **48**, 6396–6403.

TINUBU, S.A., HAKIMI, J., KONDAS, J.A., BAILON, P., FAMILLETTI, P.C., SPENCE, C., CRITTENDEN, M.D., PARENTEAU, G.L., DIRBAS, F.M., TSUDO, M., BACHER, J.D., KASTEN-SPORTES, C., MARTINUCCI, J.L., GOLDMAN, C.K., CLARK, R.E. and WALDMANN, T.A., 1994, Humanized antibody directed to the IL-2 receptor b-chain prolongs cardiac allograft survival, *J. Immunol.*, **153**, 4330–4338.

TOMASI, T.B., 1992, The discovery of secretory IgA and the mucosal immune system, *Immunol. Today*, **13**, 416–418.

TOMIZUKA, K., YOSHIDA, H., UEJIMA, H., KUGOH, H., SATO, K., OHGUMA, A., HAYASAKA, M., HANAOKA, K., OSHIMURA, M. and ISHIDA, I., 1997, Functional expression and germline transmission of a human chromosome fragment in chimaeric mice, *Nature Genet.*, **16**, 133–143.

TOM-MOY, M., BAER, R.L., SPIRA-SOLOMON, D. and DOHERTY, T.P., 1995, Atrazine measurements using surface transverse wave devices, *Anal. Chem.*, **67**, 1510–1516.

TORCHILIN, V.P., 1994, Immunoliposomes and PEGylated immunoliposomes: possible use for targeted delivery of imaging agents, *Immunomethods*, **4**, 244–258.

TORNOE, I., TITLESTAD, I.L., KEJLING, K., ERB, K., DITZEL, H.J. and JENSENIUS, J.C., 1997, Pilot scale purification of human monoclonal IgM (COU-1) for clinical trials, *J. Immunol. Methods*, **205**, 11–17.

TRAIL, P.A., WILLNER, D., LASCH, S.J., HENDERSON, A.J., HOFSTEAD, S., CASAZZA, A.M., FIRESTONE, R.A., HELLSTROM, I. and HELLSTROM, K.E., 1993, Cure of xenografted human carcinomas by BR96–doxorubicin immunoconjugates, *Science*, **261**, 212–215.

TRAMONTANO, A., JANDA, K.D. and LERNER, R.A., 1986, Catalytic antibodies, *Science*, **234**, 1566–1570.

TRELOAR, P.H., NKOHKWO, A.T., KANE, J.W., BARBER, D. and VADGAMA, P.M., 1994, Electrochemical immunoassay: simple kinetic determination of alkaline phosphatase enzyme labels in limited and excess reagent systems, *Electroanalysis*, **6**, 561–566.

TRILL, J.J., SHATZMAN, A.R. and GANGULY, S., 1995, Production of monoclonal antibodies in COS and CHO cells, *Curr. Opin. Biotechnol.*, **6**, 553–560.

TSAI, P.K., BURKE, C.J., IRWIN, J.W., BRUNER, M.W., TUNG, J.S., HOLLIS, G.F., MARK, G.E., KESSLER, J.A., BOOTS, L.J., CONLEY, A.J. and MIDDAUGH, C.R., 1995, Enhancing the avidity of a human recombinant anti-HIV-1 monoclonal antibody through oligomerization, *J. Pharm. Sci.*, **84**, 866–870.

TSCHMELITSCH, J., BARENDSWAARD, E., WILLIAMS, C., YAO, T.J., COHEN, A.M., OLD, L.J. and WELT, S., 1997, Enhanced antitumour activity of combination radioimmunotherapy (131I-labeled monoclonal antibody A33) with chemotherapy (fluorouracil), *Cancer Res.*, **57**, 2181–2186.

TSUI, P., TORNETTA, M.A., AMES, R.S., BANKOSKY, B.C., GRIEGO, S., SILVERMAN, C., PORTER, T., MOORE, G. and SWEET, R.A., 1996, Isolation of a neutralising human RSV antibody from a dominant, non-neutralising immune repertoire by epitope-blocked panning, *J. Immunol.*, **157**, 772–780.

TURNER, A., KING, D.J., FARNSWORTH, A.P.H., RHIND, S.K., PEDLEY, R.B., BODEN, J., BODEN, R., MILLICAN, T.A., MILLAR, K., BOYCE, B., BEELEY, N.R.A., EATON, M.A.W. and PARKER, D., 1994, Comparative biodistributions of indium-111-labelled macrocycle chimeric B72.3 antibody conjugates in tumour-bearing mice, *Br. J. Cancer*, **70**, 35–41.

TUTT, A., GREENMAN, J., STEVENSON, G.T. and GLENNIE, M.J., 1991a, Bispecific $F(ab'\gamma)_3$ antibody derivatives for redirecting unprimed cytotoxic T cells, *Eur. J. Immunol.*, **21**, 1351–1358.

TUTT, A., STEVENSON, G.T. and GLENNIE, M.J., 1991b, Trispecific $F(ab')_3$ derivatives that use cooperative signaling via the TCR/CD3 complex and CD2 to activate and redirect resting cytotoxic T cells, *J. Immunol.*, **147**, 60–69.

UEDA, H., TSUMOTO, K., KUBOTA, K., SUZUKI, E., NAGAMUNE, T., NISHIMURA, H., SCHEULER, P.A., WINTER, G., KUMAGAI, I. and MAHONEY, W.C., 1996, Open sandwich ELISA: a novel immunoassay based on the interchain interaction of antibody variable region, *Nature Biotechnol.*, **14**, 1714–1718.

ULTEE, M.E., BRIDGER, G.J., ABRAMS, M.J., LONGLEY, C.B., BURTON, C.A., LARSEN, S.K., HENSON, G.W., PADMANABHAN, S., GAUL, F.E. and SCHWARTZ, D.A., 1997, Tumor imaging with technetium-99m-labeled hydrazinonicotinamide–Fab′ conjugates, *J. Nucl. Med.*, **38**, 133–138.

UTTENREUTHER-FISCHER, M.M., HUANG, C.S. and YU, A.L., 1995, Pharmacokinetics of human–mouse chimeric anti-GD2 mAb ch14.18 in a phase I trial in neuroblastoma patients, *Cancer Immunol. Immunother.*, **41**, 331–338.

VALLERA, D.A., PANOSKALTIS-MORTARI, A., JOST, C., RAMAKRISHNAN, S., EIDE, C.R., KREITMAN, R.J., NICHOLLS, P.J., PENNELL, C. and BLAZAR, B.R., 1996, Anti-graft versus host disease

effect of DT390-anti-CD3 sFv, a single chain Fv fusion immunotoxin specifically targeting the CD3 epsilon moiety of the T cell receptor, *Blood*, **88**, 2342–2353.

VALLERA, D.A., TAYLOR, P.A., PANOSKALTSIS-MORTARI, A. and BLAZAR, B.R., 1995, Therapy of ongoing graft versus host disease induced across the major or minor histocompatibility barrier in mice with anti-CD3 F(ab′)2-ricin toxin A chain immunotoxin, *Blood*, **86**, 4367–4375.

VAN DER LUBBE, P.A., DIJKMANS, B.A., MARKUSSE, H.M., NASSANDER, U. and BREEDVELD, F.C., 1995, A randomised double-blind placebo controlled study of CD4 monoclonal antibody therapy in early rheumatoid arthritis, *Arthritis Rheum.*, **38**, 1097–1106.

VAN DER WEIDE, J., HOMAN, H.C., COZIJNSEN-VAN RHEENAN, E., VIVIE-KIPP, Y., POORTMAN, J. and KRAAIJENHAGEN, R.J., 1992, Nonisotopic binding assay for measuring vitamin B12 and folate in serum, *Clin. Chem.*, **38**, 766–768.

VAN HOF, A.C., MOLTHOFF, C.F.M., DAVIES, Q., PERKINS, A.C., VERHEIJEN, R.H.M., KENEMANS, P., DEN HOLLANDER, W., WILHELM, A.J., BAKER, T.S., SOPWITH, M., FRIER, M., SYMONDS, E.M. and ROOS, J.C., 1996, Biodistribution of 111indium labeled engineered human antibody CTM01 in ovarian cancer patients: influence of protein dose, *Cancer Res.*, **56**, 5179–5185.

VAUGHAN, T.J., WILLIAMS, A.J., PRITCHARD, K., OSBOURN, J.K., POPE, A.R., EARNSHAW, J.C., MCCAFFERTY, J., HODITS, R.A., WILTON, J. and JOHNSON, K.S., 1996, Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library, *Nature Biotechnol.*, **14**, 309–314.

VERHAAR, M.J., KEEP, P.A., HAWKINS, R.E., ROBSON, L., CASEY, J.L., PEDLEY, B., BODEN, J.A., BEGENT, R.H.J. and CHESTER, K.A., 1996, Technetium-99m radiolabelling using a phage derived single-chain Fv with a C-terminal cysteine, *J. Nucl. Med.*, **37**, 868–872.

VERHOEYEN, M., MILSTEIN, C. and WINTER, G., 1988, Reshaping human antibodies: grafting an anti-lysozyme activity, *Science*, **239**, 1534–1536.

VITETTA, E.S. and UHR, J.W., 1994, Monoclonal antibodies as agonists: an expanded role for their use in cancer therapy, *Cancer Res.*, **54**, 5301–5309.

VOGEL, C.A., GALMICHIE, M.C. and BUCHEGGER, F., 1997, Radioimmunotherapy and fractionated radiotherapy of human colon cancer liver metastases in nude mice, *Cancer Res.*, **57**, 447–453.

VOGL, M., PARISCH, M., KLEIN, C. and MULLER, M.M., 1996, Evaluation of the Syva Emit^{R} 2000 digoxin assay on a Hitachi 717 analyzer, *Clin. Biochem.*, **29**, 389–391.

VOLA, R., LOMBARDI, A., TARDITI, L., BJORCK, L. and MARIANI, M., 1995, Recombinant proteins L and LG: efficient tools for purification of murine immunoglobulin G fragments, *J. Chromatogr.*, **668**, 209–218.

VOLLMERS, H.P., WOZNIAK, E., STEPIEN-BOTSCH, E., ZIMMERMAN, U. and MULLER-HERMELINK, H.K., 1996, A rapid method for purification of monoclonal human IgM from mass culture, *Human Antibod. Hybridomas*, **7**, 37–41.

VOSS, A., NIERSBACH, M., HAIN, R., HIRSCH, H.J., LIAO, Y.C., KREUZALER, F. and FISCHER, R., 1995, Reduced virus infectivity in *N. tabacum* secreting a TMV-specific full-size antibody, *Mol. Breed.*, **1**, 39–50.

VUILLEZ, J.P., MORO, D., BRICHON, P.Y., ROUVIER, E., BRAMBILLA, E., BARBET, J., PELTIER, P., MEYER, P., SARRAZIN, R. and BRAMBILLA, C., 1997, Two-step immunoscintigraphy for non-small-cell lung cancer staging using a bispecific anti-CEA/anti-indium-DTPA antibody and an indium-111-labeled DTPA dimer, *J. Nucl. Med.*, **38**, 507–511.

WALDMANN, T.A., WHITE, J.D., CARRASQUILLO, J.A., REYNOLDS, J.C., PAIK, C.H., GANSOW, O.A., BRECHBIEL, M.W., JAFFE, E.S., FLEISHER, T.A., GOLDMAN, C.K., TOP, L.E., BAMFORD, R., ZAKNOEN, S., ROESSLER, E., KSTEN-SPORTES, C., ENGLAND, R., LITOU, H., JOHNSON, J.A., JACKSON-WHITE, T., MANNS, A., HANCHARD, B., JUNGHANS, R.P. and NELSON, D., 1995, Radioimmunotherapy of interleukin-2Rα-expressing adult T-cell leukemia with yttrium-90-labelled anti-Tac, *Blood*, **86**, 4063–4075.

WALKER, W. and GALLAGHER, G., 1994, The *in vivo* production of specific human antibodies by vaccination of human-PBL-SCID mice, *Immunol.*, **83**, 163–170.

WANG, Y., HU, Q., MADRI, J.A., ROLLINS, S.A., CHODERA, A. and MATIS, L.A., 1996, Amelioration of lupus-like autoimmune disease in NZB/WF1 mice after treatment with a blocking

monoclonal antibody specific for complement component C5, *Proc. Natl Acad. Sci. (USA)*, **93**, 8563–8568.

WANG, Y., ROLLINS, S.A., MADRI, J.A. and MATIS, L.A., 1995, Anti-C5 monoclonal antibody therapy prevents collagen-induced arthritis and ameliorates established disease, *Proc. Natl Acad. Sci. (USA)*, **92**, 8955–8959.

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.

WASHBURN, L.C., SUN, T.T.H., LEE, Y.C.C., BYRD, B.L., HOLLOWAY, E.C., CROOK, J.E., STUBBS, J.B., STABIN, M.G., BRECHBIEL, M.W., GANSOW, O.A. and STEPLEWSKI, Z., 1991, Comparison of five bifunctional chelate techniques for ^{90}Y-labeled monoclonal antibody CO17-1A, *Nucl. Med. Biol.*, **18**, 313–321.

WAWRZYNCZAK, E.J., CUMBER, A.J., PARNELL, G.D., JONES, P.T. and WINTER, G., 1992, Blood clearance in the rat of a recombinant mouse monoclonal antibody lacking the N-linked oligosaccharide side-chains of the CH2 domains, *Mol. Immunol.*, **29**, 213–220.

WEBBER, K.O., REITER, Y., BRINKMANN, U., KREITMAN, R. and PASTAN, I., 1995, Preparation and characterization of a disulfide-stabilized Fv fragment of the anti-Tac antibody, comparison with its single-chain analog, *Mol. Immunol.*, **32**, 249–258.

WEBER, R.W., BOUTIN, R.H., NEDELMAN, M.A., LISTER-JAMES, J., and DEAN, R.T., 1990, Enhanced kidney clearance with an ester linked 99m-Tc-radiolabelled antibody Fab′-chelator conjugate, *Bioconjug. Chem.*, **1**, 431–437.

WEEKS, I., BEHESTI, I., MCCAPRA, F., CAMPELL, A.K. and WOODHEAD, J.S., 1983, Acridinium esters as high specific activity labels in immunoassay, *Clin. Chem.*, **29**, 1474–1479.

WEIDEN, P.L., BREITZ, H.B., SEILER, C.A., BJORN, M.J., RATLIFF, B.A., MALLETT, R., BEAUMIER, P.L., APPELBAUM, J.W., FRITZBERG, A.R. and SALK, D., 1993, Rhenium-186-labeled chimeric antibody NR-LU-13: pharmacokinetics, biodistribution and immunogenicity relative to murine analog NR-LU-10, *J. Nucl. Med.*, **34**, 2111–2119.

WEIJTENS, M.E.M., WILLEMSEN, R.A., VALERIO, D., STAM, K. and BOLHUIS, R.L.H., 1996, Single chain Ig/g gene-redirected human T-lymphocytes produce cytokines, specifically lyse tumour cells, and recycle lytic capacity, *J. Immunol.*, **157**, 836–843.

WEINER, L.M., CLARK, J.I., DAVEY, M., LI, W.S., DE PALAZZO, I.G., RING, D.B. and ALPAUGH, R.K., 1995, Phase I trial of 2B1, a bispecific monoclonal antibody targeting c-erb-b2 and FcγRIII, *Cancer Res.*, **55**, 4586–4593.

WEISS, E., CHATALLIER, J. and ORFANOUDAKIS, G., 1994, *In vivo* biotinylated recombinant antibodies: construction, characterization and application of a bifunctional Fab-BCCP fusion protein produced in *E. coli*, *Protein Expression Purification*, **5**, 509–517.

WELLING, G.W., VAN GORKUM, J., DAMHOF, R.A. and DRIJFHOUT, J.W., 1991, A ten residue fragment of an antibody (mini-antibody) directed against lysozyme as a ligand in immunoaffinity chromatography, *J. Chromatogr.*, **548**, 235–242.

WELS, W., HARWERTH, I.M., ZWICKL, M., HARDMAN, N., GRONER, B. and HYNES, N.E., 1992, Construction, bacterial expression and characterization of a bifunctional single-chain antibody–phosphatase fusion protein targeted to the human erbb-2 receptor, *Bio/Technol.*, **10**, 1128–1132.

WELT, S., SCOTT, A.M., DIVGI, C.R., KEMENY, N.E., FINN, R.D., DAGHIGHIAN, F., ST GERMAIN, J., CARSWELL-RICHARDS, E., LARSON, S.M. and OLD, L.J., 1996, Phase I/II study of iodine-125-labelled monoclonal antibody A33 in patients with advanced colon cancer, *J. Clin. Oncol.*, **14**, 1787–1797.

WENDLING, D., RACADOT, E., WIDENES, J. and the FRENCH INVESTIGATORS GROUP, 1996, Randomized, double-blind, placebo controlled multicenter trial of murine anti-CD4 therapy in rheumatoid arthritis, *Arthritis Rheum.*, **39**, S245, abstract 1303.

WENTWORTH, P., DATTA, A., BLAKEY, D., BOYLE, T., PARTRIDGE, L.J. and BLACKBURN, G.M., 1996, Towards antibody-directed 'abzyme' prodrug therapy, ADAPT: carbamate prodrug activation by a catalytic antibody and its *in vitro* application to human tumor cell killing, *Proc. Natl Acad. Sci. (USA)*, **93**, 799–803.

WERLEN, R.C., LANKINEN, M., OFFORD, R.E., SCHUBIGER, P.A., SMITH, A. and ROSE, K., 1996, Preparation of a trivalent antigen binding construct using polyoxime chemistry: improved biodistribution and potential for therapeutic application, *Cancer Res.*, **56**, 809–815.

WERLEN, R.C., LANKINEN, M., ROSE, K., BLAKEY, D., SHUTTLEWORTH, H., MELTON, R. and OFFORD, R.E., 1994, Site-specific conjugation of an enzyme and an antibody fragment, *Bioconj. Chem.*, **5**, 411–417.

WERTHER, W.A., GONZALEZ, T.N., O'CONNOR, S.J., MCCABE, S., CHAN, B., HOTALING, T., CHAMPE, M., FOX, J.A., JARDIEU, P.M., BERMAN, P.W. and PRESTA, L.G., 1996, Humanization of an anti-lymphocyte function associated antigen LFA-1 monoclonal antibody and reengineering of the humanised antibody for binding to rhesus LFA-1, *J. Immunol.*, **157**, 4986–4995.

WHITLOW, M., BELL, B.A., FENG, S.L., FILPULA, D., HARDMAN, K.D., HUBERT, S.L., ROLLENCE, M.L., WOOD, J.F., SCHOTT, M.E., MILENIC, D.E., YOKOTA, T. and SCHLOM, J., 1993, An improved linker for single-chain Fv with reduced aggregation and enhanced proteolytic stability, *Protein Engin.*, **6**, 989–995.

WHITLOW, M. and FILPULA, D., 1991, Single-chain Fv proteins and their fusion proteins, *Methods*, **2**, 97–105.

WHITLOW, M., FILPULA, D., ROLLENCE, M.L., FENG, S.L. and WOOD, J.F., 1994, Multivalent Fv's: characterization of single-chain Fv oligomers and preparation of a bispecific Fv, *Protein Engin.*, **7**, 1017–1026.

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, *Protein Engin.*, **1**, 499–505.

WILKINSON, I., JACKSON, C.A., LANG, G.M., HOLFORD-STREVENS, V. and SEHON, A., 1987, Tolerance induction in mice by conjugates of monoclonal immunoglobulins and monomethoxypolyethylene glycol, *J. Immunol.*, **139**, 326–331.

WILLIAMS, R.O., FELDMANN, M. and MAINI, R.N., 1992, Anti-tumour necrosis factor ameliorates joint disease in murine collagen induced arthritis, *Proc. Natl Acad. Sci. (USA)*, **89**, 9784–9788.

WILLIAMS, W.V., KIEBER-EMMONS, T., VONFELDT, J., GREENE, M.I. and WEINER, D.B., 1991, Design of bioactive peptides based on antibody hypervariable region structures, *J. Biol. Chem.*, **266**, 5182–5190.

WILLINS, J.D. and SGOUROS, G., 1995, Modelling analysis of platinum-195m for targeting individual blood-borne cells in adjuvant radioimmunotherapy, *J. Nucl. Med.*, **36**, 315–319.

WINTER, G., GRIFFITHS, A.D., HAWKINS, R.E. and HOOGENBOOM, H.R., 1994, Making antibodies by phage display technology, *Annu. Rev. Immunol.*, **12**, 433–455.

WOOD, C.R., BOSS, M.A., KENTEN, J.H., CALVERT, J.E., ROBERTS, N.A. and EMTAGE, J.S., 1985, The synthesis and *in vivo* assembly of functional antibodies in yeast, *Nature*, **314**, 446–449.

WOODHEAD, J.S., ADDISON, G.M. and HALES, C.N., 1974, *Br. Med. Bull.*, **30**, 44.

WRIGHT, A. and MORRISON, S.L., 1994, Effect of CH2-associated carbohydrate structure on the functional properties and *in vivo* fate of chimeric mouse–human immunoglobulin G, *J. Exp. Med.*, **180**, 1087–1096.

WU, X.C., NG, S.C., NEAR, R.I. and WONG, S.L., 1993, Efficient production of a functional single-chain antidigoxin antibody via an engineered *Bacillus subtilis* expression–secretion system, *Bio/Technol.*, **11**, 71–76.

YALLOW, R.S. and BERSON, S.A., 1960, Immunoassay of endogenous plasma insulin in man, *J. Clin. Invest.*, **39**, 1157–1175.

YAMAGUCHI, Y., KIM, H., KATO, K., MASUDA, K., SHIMADA, I. and ARATA, Y., 1995, Proteolytic fragmentation with high specificity of mouse immunoglobulin G, *J. Immunol. Methods*, **181**, 259–267.

YAMAIZUMI, M., MEKADA, E., UCHIDA, T. and OKADA, Y., 1978, One molecule of diptheria toxin fragment A introduced into a cell can kill the cell, *Cell*, **15**, 245–250.

YANG, W.P., GREEN, K., PINZ-SWEENEY, S., BRIONES, A.T., BURTON, D.R. and BARBAS, C.F., III, 1995, CDR walking mutagenesis for the affinity maturation of a potent human anti-HIV-1 antibody into the picomolar range, *J. Mol. Biol.*, **254**, 392–403.

YARNOLD, S. and FELL, H.P., 1994, Chimerization of antitumour antibodies via homologous recombination conversion vectors, *Cancer Res.*, 54, 506–512.

YARRANTON, G.T. and MOUNTAIN, A., 1992, Expression of proteins in prokaryotic systems – principles and case studies, *Protein Engineering, a Practical Approach* (Rees, A.R., ed.), pp. 303–325, Oxford University Press, Oxford.

YEDNOCK, T.A., CANNON, C., FRITZ, L.C., SANCHEZ-MADRID, F., STEINMAN, L. and KARIN, N., 1992, Prevention of experimental autoimmune encephalomyelitis by antibodies against $\alpha 4\beta 1$ integrin, *Nature*, **356**, 63–66.

YEUNG, D., GILL A., MAULLE, C. and DAVIES, R.J., 1995, Detection and quantitation of biomolecular interactions with optical biosensors, *Trends Anal. Chem.*, 14, 49–56.

YOKOTA, T., MILENIC, D.E., WHITLOW, M. and SCHLOM, J., 1992, Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms, *Cancer Res.*, **52**, 3402–3408.

YOKOYAMA, K., IKEBUKURO, K., TAMIYA, E., KARUBE, I., ICHIKI, N. and ARIKAWA, Y., 1995, Highly sensitive quartz crystal immunosensors for multisample detection of herbicides, *Anal. Chim. Acta*, **304**, 139–145.

YORKE, E.D., BEAUMIER, P.L., WESSELS, B.W., FRITZBERG, A.R. and MORGAN, A.C., JR, 1991, Optimal antibody–radionuclide combinations for clinical radioimmunotherapy: a predictive model based on mouse pharmacokinetics, *Nucl. Med. Biol.*, **18**, 827–835.

YOUNG, W.W., TAMURA, Y., WOLOCK, D.M. and FOX, J.W., 1984, Staphylococcal protein A binding to the Fab fragments of mouse monoclonal antibodies, *J. Immunol.*, **133**, 3163–3166.

YUAN, Q., STRAUCH, K.L., LOBB, R.R. and HEMLER, M.E., 1996, Intracellular single-chain antibody inhibits integrin VLA-4 maturation and function, *Biochem. J.*, **318**, 591–596.

ZALIPSKY, S. and LEE, C., 1992, Use of functionalized poly(ethylene glycol)s for modification of polypeptides, *Poly(Ethylene Glycol) Chemistry* (Milton Harris, J., ed.), pp. 347–371, Plenum, New York.

ZALUTSKY, M.R., MCLENDON, R.E., GARG, P.K., ARCHER, G.E., SCHUSTER, J.M. and BIGNER, D.D., 1994, Radioimmunotherapy of neoplastic meningitis in rats using an α-particle-emitting immunoconjugate, *Cancer Res.*, **54**, 4719–4725.

ZHU, Z., LEWIS, G.D. and CARTER, P., 1995, Engineering high affinity humanized Anti-p185HER2/anti-CD3 bispecific $F(ab')_2$ for efficient lysis of p185HER2 overexpressing tumor cells, *Int. J. Cancer*, **62**, 319–324.

ZHU, Z., PRESTA, L.G., ZAPATA, G. and CARTER, P., 1997, Remodeling domain interfaces to enhance heterodimer formation, *Protein Sci.*, **6**, 781–788.

ZHU, Z., ZAPATA, G., SHALABY, R., SNEDECOR, B., CHEN, H. and CARTER, P., 1996, High level secretion of a humanized diabody from *Escherichia coli*, *Bio/Technol.*, **14**, 192–196.

ZOU, Y.R., MULLER, W., GU, H. and RAJEWSKY, K., 1994, Cre-loxP-mediated gene replacement: a mouse strain producing humanized antibodies, *Current Biol.*, **4**, 1099–1103.

ZU PULITZ, J., KUBASEK, W.L., DUCHENE, M., MARGET, M., VON SPECHT, B. and DOMDEY, H., 1990, Antibody production in baculovirus-infected insect cells, *Bio/Technol.*, **8**, 651–654.

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Antibodies are increasingly being used for research and development, as diagnostic agents and are now a new class of pharmaceuticals to combat human disease. *Applications and Engineering of Monoclonal Antibodies* discusses how such antibodies are designed and produced, and the features which can be incorporated to improve their usefulness.

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David King is head of protein biochemistry and project leader for antibody engineering at Celltech Therapeutics. His research on antibody engineering began with some of the first recombinant antibodies produced and has developed since then into design and development of recombinant antibody therapeutics for cancer and inflammatory diseases.

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