Monoclonal antibodies in the detection and therapy of micrometastatic epithelial cancers

Gert Riethmüller and Judith P. Johnson

Institute of Immunology, University of Munich, Munich, Germany

The initial promise of monoclonal antibodies as major therapeutic agents in human epithelial cancer has not been realized. Inaccessibility of cells in solid tumors due to factors such as the nature of the vascular endothelia and high pressure in the tumor are primarily responsible for the failure of antibody therapy. Although new strategies employing recombinant antibodies and immunoglobulins designed to actively engage the immune system may prove beneficial, micrometastatic tumor cells (at the stage of minimal residual disease) are likely to be the only suitable targets for antibody therapy. The diagnostic approaches to identify and characterize these cells and their use for prognosis and monitoring adjuvant immunotherapy is discussed.

Current Opinion in Immunology 1992, **4**:647–655

Introduction

The hope that monoclonal antibodies (mAbs), with their exquisite specificity and multiple effector functions, would revolutionize the diagnosis and treatment of cancer, has failed to materialize. While mAbs have successfully replaced polyclonal antisera for the detection of tumor markers in the serum, their therapeutic use in patients with solid tumors can only be viewed as a disappointment, even with improvements in the ability of antibodies to target and destroy tumor cells in model systems. Thus, fundamental changes in the strategies of using mAbs to treat tumors are required. In this review we focus on several such strategies, on the one hand, involving new developments in antibody production and use, and on the other, focusing on the target of the therapy itself. As more has been learned about the biology of the interaction of tumors with the vascular system and the extracellular matrix, it has become clear that much of the difficulty with passive antibody therapy is related to the accessibility of the tumor cells to these reagents. In light of this, micrometastasis, or the stage of minimal residual disease, may not be only an important field for diagnostic studies but also the best target for antibody mediated therapy.

Monoclonal antibodies in diagnosis: determination of minimal residual disease

The metastatic spread of small localized primary tumors in the absence of clinical signs of the disease in distant parts of the body can, at present, be diagnosed only in retrospect when the true extent of the disease manifests itself by an overt clinical relapse. For example, nearly 25% of breast cancer patients who do not show signs of disease in the regional lymph nodes, do in fact suffer from disseminated disease, borne out by the occurrence, often many years later, of distant metastases [1]. The concentration of tumor antigens shed from a few tumor cells is too low for detection by the most sensitive immunoassays and even the most sophisticated physical diagnostic procedures available, including nuclear magnetic resonance and photon emission tomography, are not specific and sensitive enough to detect individual tumor cells disseminated to distant organs. Thus, in the absence of the diagnosis of disseminated disease, the development of effective antibody-mediated (adjuvant) therapies proceeds at a scandalously slow pace. As with all adjuvant therapies they must first show some efficacy in 5 year trials on terminal patients with a heavy tumor burden before they can be tested on earlier stages. It is evident that in this dilemma, a firm diagnosis of minimal residual disease would be of invaluable help to identify patients actually in need of adjuvant therapy. Furthermore, the monitoring of therapeutic effects would enormously benefit the development of adjuvant therapies.

Since the early years of the last decade there have been several attempts to use mAbs to distinguish infiltrating tumor cells from hematopoietic bone marrow cells [2], an approach which was greatly expanded by Neville's group at the Ludwig Cancer Institute in London [3]. The general feasibility of this method was

Abbreviations

ADCC—antibody-dependent cell-mediated cytotoxicity; **APAAP**—alkaline phosphatase–anti-alkaline phosphatase; **CTL**—cytotoxic T lymphocyte; **Fv**—single chain monoclonal antibody; **H**—heavy; **HLA**—human leukocyte antigen; **L**—light; **mAb**—monoclonal antibody; **MHC**—major histocompatibility complex; **PCR**—polymerase chain reaction; **SEA**—staphylococcal enterotoxin A; **TCR**—T-cell receptor; **V**—variable.

soon demonstrated using different mAbs in a variety of epithelial tumors (Table 1). Because of the generally low concentration and considerable heterogeneity of expression of tumor-associated membrane antigens, we chose to use the abundant intracellular cytokeratin proteins [4,5] as markers for detecting epithelial cancer cells in the bone marrow [6••]. Using an immunocytochemical alkaline phosphatase–anti-alkaline phosphatase (APAAP) detection system it is possible to detect epithelial cells at a fequency of 10^{-5} to 10^{-6} bone marrow cells [7,8••,9•,10]. Patients without epithelial malignancies essentially lack cytokeratin-positive cells [7,11], while between 10 and 40% of these samples exhibit staining with antibodies directed against the membrane antigens epithelial membrane antigen and human milk fat globule mucin [6••,12,13]. Further evidence for the specificity of this method was obtained from double marker studies using a combination of immunoautoradiography and APAAP staining. In the bone marrow from carcinoma patients, the expression of the leukocyte common antigen, CD45 and cytokeratin 18 were never found on the same cell [7].

Although this method appears to have a high level of specificity, the low frequency of tumor cells in the bone marrow makes it likely that they are not uniformly distributed and suggests that a considerable sampling error occurs when only one site is sampled [14]. In fact, triple site aspiration (left and right iliac crest plus sternum) increased the percentage of positive stage M_0 breast cancer patients from 10.7% to 28.2% [15] (M denotes distant metastases and M_0 refers to tumors where there is no evidence of distant metastases). For routine purposes, a two-sided aspiration of the pelvic crest is currently performed on the operating table, immediately prior to the operation.

The judicious use of double staining techniques allows one to define additional markers on the individual disseminated cells. Thus cytokeratin-positive cells in the bone marrow of tumor patients were shown to express proliferation-associated molecules such as the the Ki 67 nuclear antigen and receptors for transferrin and epidermal growth factor [15]. Similar to cells in the metastatic lesions themselves, cytokeratin-positive bone marrow cells also often lacked HLA class I expression. In one study, 50% of the breast cancer patients with positive tumor cells in the bone marrow had only class I negative epithelial cells in the bone marrow [8••]. These cells not only express proliferation-associated markers but are able to grow *in vitro*. Epithelial cells could be expanded from bone marrow aspirates by the use of various combinations of growth factors [16] and the rate of successful expansions was substantially increased when the culture vessels were precoated with extracellular matrix proteins. Using a serum free culture system, Hay *et al.* [17] recently reported the growth of small-cell lung cancer cells in 100% of positive bone marrow samples.

What is the clinical significance of the presence of epithelial cells in the bone marrow? In a number of studies on breast carcinoma patients [14,18••] as well as in colorectal cancer [19] and neuroblastoma [20], the presence of micrometastases in the bone marrow has now been shown to be associated with a shorter disease-free interval. Surprisingly, while bone marrow micrometastatic cells were found in 33% of the colorectal tumor patients (Dukes C stage, i.e. a trans-serosal tumor with regional lymph node involvement), most of the manifest metastases involved the liver, indicating that the presence of tumor cells in the bone marrow is associated with the probability of metastases development in general, and not necessarily with the manifestation of

Table 1. Detection of bone marrow micrometastasis in various types of epithelial cancer.

Origin of tumor	Marker antigens	Correlation with established risk factors	Prognostic value	References
Breast	Epithelial membrane antigen (EMA)	+	+	[14]
	Cytokeratin	+	n.t.	[7]
	MBr1	–	n.t.	[57]
	Cytokeratin	+	n.t.	[18••]
Colorectum	17.1A			
	Cytokeratin	+	+	[19]
Stomach	Cytokeratin	+	+	[6••]
Prostate	PSA + Cytokeratin + EMA	+	n.t.	[72]
	Cytokeratin	+	+	Oberneder*
Small-cell lung	SM1	+	n.t.	[58]
cancer	LCA_1, LCA_2, and LCA_3	+	n.t.	[59]
Non-small-cell	Cytokeratin	+	+	Pantel*
lung cancer				
Bladder	Cytokeratin	+	n.t.	Oberneder*
Renal cell	Cytokeratin	+	n.t.	Oberneder*
carcinoma				

*(Unpublished data); n.t., not tested.

skeletal metastasis. Taking all of the published studies together, a good correlation exists between the presence of epithelial cells in bone marrow and the conventional risk factors based mainly on the extent of tumor dissemination. Furthermore, the total tumor burden may be estimated from the number of epithelial cells in the bone marrow [18••,20]. The evaluation of tumor cells in the bone marrow is, therefore, a potentially powerful diagnostic tool since it allows one to look at a part of the tumor burden which the surgeon has left behind. Not only could this provide a way to monitor the effectiveness of various therapies, but with a further characterization of these cells (oncogene, tumor suppressor gene expression, etc) it may eventually be possible to more accurately predict the metastatic potential of these cells.

Monoclonal antibodies in therapy: new strategies for production and use

Although passive antibody therapies have been effective against established tumors in many experimental systems, the therapy of solid tumors with mAbs in the clinic has had a history of failure. A brief look at the literature covering the years of 1984–1991 provides no hint of a consistent therapeutic efficacy of mAbs. In a review of 12 studies comprising 196 patients with a variety of solid tumors, Gisler lists only two complete remissions (Table 2) and the number of partial remissions remains within the realm of the anecdotal (R Grisler, in *Cancer and the Immune System*, Proceedings of European School of Oncology, Venice 1990). The general conclusion that has been drawn from this conspicuous failure is that antibodies as naked mouse immunoglobulins are inefficient, and a number of new strategies are being undertaken to create new antibodies and to use mAbs to actively engage the host's own immune response against the autologous tumor.

The development of new reagents that react with tumors through recombinant DNA technology

While emphasis in recent years has been on using genetic engineering to 'humanize' and 'Fc customize' rodent antibodies, the future almost certainly lies in the production of human antigen-binding molecules from combinatorial libraries in *Escherichia coli* [21]. In this method, independent cDNA libraries are created from the mRNAs encoding variable (V) light (L) and heavy (H) chain antibody regions, using the polymerase chain reaction (PCR). These two libraries are then randomly combined and cloned into a phage vector which directs the expression of one VL and one VH polypeptide chain. The use of vectors which allow the Fab fragments to be expressed as intact antigen-binding sites on the phage surface, either in monomeric or multimeric form, have greatly facilitated screening and selection of desired antibodies [22•]. In addition, the most recent developments indicate that high affinity antibodies of virtually any specificity can be obtained by applying rounds of random mutagenesis and selection to the Fabs produced from the cDNA of naive IgM^+ B lymphocytes, a situation which should obviate the need for immune donor cells [23••].

Once the desired antigen-binding moieties have been selected, the VL and VH regions can be produced as a single polypeptide (with the regions separated by a flexible linker) to generate single chain mAbs or Fv molecules. These can be further engineered to contain toxins or

Table 2. Mouse monoclonal antibodies in clinical trials on solid tumors.

Tumor	Mouse antibody	Isotype	Number of patients	Effect	References
Gastrointenstinal tumors	CO17-1A	IgG2a	20	20 ±	[60]
			22	22 ±	[61]
			8	8 −	[62]
			20	20 ±	[63]
Breast, colon, ovarian and lung cancer	L6	IgG2a	19	1 + +, 18?	[64]
Pancreatic adenocarcinoma	BW494	IgG1	18	18 −	[65]
	CO17-1A	IgG2a	19	4 +, 15 −	[66]
Neuroblastoma, melanoma	3F8 anti-GD2	IgG3	17	1 + +, 6 +, 10 −	[67]
Melanoma	9.2.27	IgG2a	20	20 −	[68,69]
Melanoma	R24 (anti-GD3)	IgG3	21	4 +, 17	[70]
			12	3 +, 2 ±, 7 −	[71]

+ +, Complete response; +, partial response; ±, minor response; −, no response.

other effector domains [24••]. Fvs frequently refold into a conformation that retains the binding characteristics of the divalent mAb and may penetrate tissues much more efficiently than intact immunoglobulins. Such characteristics can be used to predict whether they will be important anti-tumor reagents and their preliminary use in mouse models confirms this [25].

Use of monoclonal antibodies to engage the host's immune system

The passive administration of tumor-reactive mAbs is increasingly being replaced by immunotherapeutic schemes designed to engage the host's own immune system in the destruction of the autologous tumor. Among the most widely used strategies at present is the attempt to exploit the idiotypic network [26••,27• ,28•]. This approach is based on the theory that certain anti-idiotypic antibodies (the Ab2βs) will express the internal image of the original immunizing epitope [29]. Treatment with such antibodies is, therefore, equivalent to immunizing patients with the tumor antigen, or rather with a single epitope of this antigen. In point of fact, patients treated with Ab2s frequently produce Ab3s which react with the original tumor antigen [30,31,32•] and in some cases, even appear to develop a cellular reactivity against this antigen although the nature of this reactivity remains poorly characterized [30,33,34].The clinical consequences of active immunization in those instances where the tumor associated antigen, e.g. the 17.1 A colorectal carcinoma epithelial antigen, is also widely expressed on normal epithelia are unclear.

Despite the common production of Ab3s that react with tumor cells in the anti-idiotype treated patients, these clinical trials have generally not been any more successful than those using Ab1s, antibodies to the original epitope, [27• ,28•]. However, Mittelman *et al.* [32•], recently reported a significant increase in survival time in melanoma patients producing Ab3s against the chondroitin sulphate proteoglycan, a surface antigen of melanoma cells. This result could not be accounted for by higher general immune reactivity (i.e. performance status) of the patients. While such discrepant results may reflect, in part, the fact that immunization with these reagents induces an immune response against a single epitope that may have varying functional and structural characteristics (e.g. density, sensitivity to modulation, functional significance), studies in a mouse anti-tumor model (reviewed in [26••]) indicate that selection of the Ab2, used in such trials is of critical importance. In this system, four different Ab2s, which were very similar in their interaction with the Ab1 and in their ability to induce anti-tumor antigen humoral and cellular responses, were examined for their ability to vaccinate against tumor growth. Only one Ab2 was able to induce protective immunity; more disturbing was the observation that one actually appeared to potentiate tumor growth [35]. The finding of a linear sequence homology between the second hypervariable region of the protective Ab2 L chain and the tumor antigen [26••], not only suggests a molecular basis for the similarity between the internal image and the tumor antigen, but means that selection of appropriate Ab2s may eventually be possible through sequence comparison of the antibody variable regions with the sequence of the antigen. However, this is not possible for carbohydrate antigens that are among the best candidates for tumor surrogate antigen vaccines given their effectiveness as tumor target structures [36•]. This would be cumbersome enough for protein antigens, but it would be even more difficult for carbohydrate antigens because of the difficulty of producing them in large quantities; despite this, the effectiveness of carbohydrate antigens as tumor target structures means that they are among the best candidates for surrogate antigen vaccines [36•].

A conceptually simpler approach to engage the host immune system is the use of bi-specific antibodies to target host effector cells to the tumor. These reagents combine, in a single molecule, specificity for a tumor associated antigen with specificity for an effector cell molecule, most commonly the T-cell receptor (TCR)-associated CD3 complex [37,38]. Addition of such conjugates to interleukin-2- or anti-CD3-activated leukocytes prior to their injection reduces the growth of established tumors in nude mice more effectively than the activated cells alone [39], a result which was also obtained in a small clinical trial [40]. Most cells that are targeted to the tumor by this approach are not directed against tumor antigens. But bi-specific heteroconjugates can also increase the killing of autologous tumors by clones of tumor-specific tumor-infiltrating lymphocytes and cytotoxic T lymphocytes (CTLs) provided the tumor cells express the target antigens [41•]. However, if the tumors are negative or heterogeneous for target-antigen expression, the presence of heteroconjugates can inhibit tumor-specific cytotoxicity, presumably by blocking the TCR. Given the universality of tumor cell heterogeneity, this could be a serious drawback to the successful use of such reagents.

An approach which might overcome this problem is one which takes advantage of the characteristics of bacterial toxin superantigens [42]. These molecules bind with high affinity to MHC class II antigens and once bound, demonstrate a specificity for particular families of TCR Vβ chains. Reactivity with the TCR leads to T-cell activation, cytokine release and killing of the cell expressing MHC class II antigen. By coupling a colon carcinoma reactive mAb to staphylococcal enterotoxin A (SEA), Dohlstein *et al.*, [43••] have been able to replace class II dependent T-cell activation with a tumor antigen specific T-cell activation and have achieved killing of carcinoma cell lines by CTLs bearing the appropriate Vβ chains. Since SEA will not engage the T cell until it is 'presented' by mAb binding, activation of the T cells and cytokine release should occur only locally, in the immediate environment of the tumor cells. Thus, like the use of cytokine transfected tumor cell vaccines [44], which also lead to spatially and temporally concordant expression of tumor antigens and cytokines, SEA–mAb conjugates may also trigger development of the host's own anti-tumor response.

Micrometastatic tumor cells as targets for immunotherapy: the way to secondary prevention

Even in the face of major improvements in the ability of antibodies to bind to and kill tumor cells, the problem of the accessibility of the tumor cells to these reagents remains unresolved. In fact, recent data on the microcirculation and extracellular matrix of tumors indicate that this may in fact be the most important impediment to successful antibody treatment of solid tumors [45,46].

The organization of the tumor's vascular system is largely determined by the histogenic type of tumor (i.e. tissue of origin), its differentiation state, proliferation rate and location. The non-fenestrated capillaries often found in epithelial tumors prevent antibodies from entering the tumor tissue. Another major factor obviating the transcapillary transport of macromolecules is the tumor interstitial pressure which is generally much higher than the surrounding normal tissue and which increases with tumor size [47]. The pressure gradient from the center to the periphery of a tumor leads to an outward convection of the interstitial fluid so that extravasated mAb will be transported to the periphery of the tumor. In the interstitium itself, the dense network of glycosaminoglycans presents a formidable obstacle for free diffusion of antibody. In addition, the antigens shed from the tumor, when immobilized in this interstitium, may neutralize antibodies and lead to precipitation of antigen–antibody complexes. A final barrier that must be overcome in differentiated metastases of adenocarcinomas is the shielding of target antigens from diffusing antibodies by the intercellular tight junctions.

While the clinical trials have only resulted in a very few well documented antibody-induced regressions (which may more easily be explained by derangements in the tumor vasculature or extracellular matrix rather than by the inherent characteristics of the antibody), they have clearly shown that antibody therapy is remarkably free of toxicity and serious side effects. Thus, the stage would now seem to be set for applying passive antibody therapy to its most logical target — minimal residual disease (or micrometastasis), a state which applies to 1/3 to 1/2 of patients with epithelial cancer (the most common type of cancer), following (curative) radical surgery of the primary lesion.

One of the first hints that micrometastatic cells might be particularly suitable targets for passively administered antibody was provided by Schlimok *et al.* [7] who demonstrated that tumor cells in bone marrow of patients with colorectal cancer can be labelled with mouse immunoglobulin *in vivo*. In a later report [8••], the therapeutic effects of antibody infusions over a period of several months up to 2 years were monitored by immunocytochemical analysis of bone marrow. In 12 of the 23 Dukes C patients with clinically manifest metastases, micrometastatic cells were repeatedly identified and clinical relapse occurred in nine of these patients.

In order to monitor directly the therapeutic elimination of micrometastatic cells, patients with high numbers of micrometastatic cells in the bone marrow were treated with an IgG3 anti-Lewis Y antibody displaying high complement-dependent cytotoxicity [48•]. (This antibody was selected because IgG3 mouse antibodies are particularly effective in the activation of human complement and ADCC). While no regressions were observed in these patients with extensive metastatic disease, a dramatic decrease in the number of tumor cells in bone marrow was observed, an effect not seen in placebo treated patients (G Schlimok, G Riethmüller, K Pantel *et al.*, abstract 709, *Proc Am Soc Clin Onc* 1991, **10**:212). To analyze the efficacy of antibodies against micrometastatic cells in colorectal cancer a prospective randomized trial was initiated into which only patients with regional disease (Dukes C1 and C2; metastases in 1–3 regional lymph nodes and 4 or more, respectively) were recruited. The trial comprising a total of 176 patients in a treatment and control arm will be closed at the end of 1992 with a median observation time of 5 years. A preliminary analysis at a median observation time 4.5 years indicates differences between treated and control patients in terms of overall survival and recurrence rate of distant metastasis (G Riethmüller, G Schlimok *et al.*, unpublished data). Although another prospective randomized trial on pancreatic cancer patients treated with an anti-carbohydrate antibody failed to show therapeutic effects [49••], the state of minimal residual disease in these patients can be disputed as the partial pancreato-duodenectomy (removal of the pancreas and duodenum) employed for tumor resection may not have been complete in many of them.

Conclusions and perspectives

While it is clear that a major consideration for the successful application of antibody therapy in solid tumors is the accessibility of the tumor cells, other aspects also need to be taken into account. Heterogeneity of tumor cells is the major obstacle of any antigen-targeting therapy. To cope with antigen heterogeneity, which increases with tumor progression, a carefully selected combination of antibodies directed to independently controlled membrane antigens should be applied as early as possible, i.e. immediately after the removal of the primary tumor. Published data demonstrate that selected pairs of antibodies directed against different epitopes of a particular antigen exhibit up to a 100-fold synergistic increase in cell killing *in vitro* [50] or *in vivo* [51] or result in augmented binding [52–54]. As passive antibody therapy imitates a natural effector mechanism, a well defined oligoclonal combination of antibodies, in which each has been shown to eliminate micrometastatic cells, should be as acceptable for clinical use as current registered polyclonal anti-thymocyte globulin preparations.

The optimal combination of such antibodies would consist of human or 'humanized' immunoglobulins. Since normal epithelia are less accessible to cytotoxic antibodies (they are located behind a dense basal membrane which is quasi-impermeable to non-secretory immunoglobulin), an absolute specificity for therapeutic antibodies appears less critical. Indeed, toxicity to normal epithelia has been negligible in the trials reported thus far. Although the inactivation of complement by homol-

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.