Journal of Cellular Biochemistry 96:305–313 (2005)

Recombinant Polyclonal Antibodies for Cancer Therapy

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Abstract Although monoclonal antibodies are increasingly used for cancer therapy, remissions are only temporary due to emergence of tumor cell escape variants that are no longer affected by the antibody. The emergence of escape variants could be minimized by multi-targeting of tumor cells with polyclonal antibodies, which would also be more efficient than monoclonal antibodies at mediating effector functions for target destruction. A technology for generating recombinant polyclonal antibodies for cancer therapy has been developed based on the construction and selection of tumor-reactive Fab phage display libraries. The selected Fabs are mass-converted to full-length polyclonal antibody libraries (PCALs) of any isotype and any species. Prototypic PCALs generated against human colorectal cancer cell lines showed that libraries of diverse recombinant antibodies, enriched for reactivity to the cancer cells compared to normal human cells, can be obtained. The success of recombinant polyclonal antibodies as cancer therapeutics will depend on the ability to generate, characterize, and mass-produce PCALs with high ratios of cancer-to-normal reactivities that cross-react with many cancers of the same type. J. Cell. Biochem. 96: 305–313, 2005.

Key words: phage display; polyclonal antibody libraries; colorectal cancer

The antibody response of vertebrates to most antigens is polyclonal and targets multiple epitopes on the antigen surface [Sharon, 1998]. This characteristic has co-evolved with the effector mechanisms mediated by the Fc regions of antibodies—complement binding with production of C3b, opsonization/phagocytosis, and antibody-dependent cellular cytotoxicity (ADCC)—that serve to eliminate the antigen [Janeway et al., 2005]. Thus, triggering of effector functions requires a high density of antibodies on the antigen surface for sufficient cross-linking of Fc receptors on effector cells and for efficient C1q binding [Herlyn et al., 1985; Welt et al., 1987; Bindon et al., 1988]. When the immunogen is a living cell, such as a microbe or a tumor cell, the polyclonality of the antibody response helps guard against the development of cell "escape variants" that are no longer recognized by the antibodies, because the probability that a given cell will simultaneously lose all the target epitopes is exceedingly small.

Attempts to treat cancer with polyclonal antibodies, in the form of anti-tumor sera, date back to the 1880s [Currie, 1972; Rosenberg and Terry, 1977]. Treatment of mice with allogeneic anti-tumor serum was shown to prevent development of a transplantable murine tumor [Gorer and Amos, 1956]. Vaccination with polyclonal rabbit IgG against purified tumor associated antigens significantly decreased the frequency of chemically induced colon cancer in rats [Zusman et al., 1996]. And treatment of mice with rabbit polyclonal IgG against murine endothelial cells induced apoptosis and inhibited the growth of tumors including murine melanoma and angiosarcoma and human colorectal carcinoma [Scappaticci et al., 2003]. Furthermore, occasional successful treatment of human malignancies, such as melanoma and renal cell carcinoma, with anti-tumor sera derived from experimental animals or from humans, has been reported [Currie, 1972; Rosenberg and Terry, 1977]. Finally, intravenous administration of human IgG, pooled from normal donors, was shown to inhibit growth and limit the spread of tumor cells in hematologic

Grant sponsor: National Institutes of Health (to JS); Grant number: R01 AI23909; Grant sponsor: Symphogen A/S.

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Received 26 April 2005; Accepted 28 April 2005

DOI 10.1002/jcb.20536

malignancies, presumably due to natural anti-tumor antibodies [Jonsson et al., 2000].

Despite their potential efficacy, the use of conventional polyclonal antibodies for cancer therapy is limited by short supply of serum antibody from immunized animals or from humans; inability to modify the antibodies because the genes are not available; and the likely effective loss of the antibody population if negative selection were performed to eliminate major cross-reactivities with normal human cells, because many cell surface antigens are expressed on many different cell types.

In contrast, hybridoma-derived monoclonal antibodies (MAbs) are available in unlimited supply and can be modified as desired because their genes can be cloned from the hybridoma cell lines. To date, eight MAbs have been shown to have clinical efficacy and have been approved for cancer treatment by the US Food and Drug Administration (FDA) [ACS, 2005b]. Most of these antibodies had been modified from the original mouse hybridoma antibodies by replacement of the murine constant (C) regions with human C regions to obtain "chimeric" antibodies or by further replacement of the murine framework regions of the variable (V) domains to obtain "humanized" antibodies. The FDA-approved MAbs include: Rituxan®, a chimeric anti-CD20 antibody approved for the treatment of non-Hodgkin's lymphoma; Campath®, a humanized anti-CD52 antibody approved for treatment of chronic lymphocytic leukemia; Herceptin®, a humanized anti-HER2/neu antibody approved for treatment of breast cancer; Erbitux®, a chimeric anti-endothelial growth factor receptor (EGFR) antibody and Avastin®, a humanized anti-vascular endothelial growth factor (VEGF) antibody, both approved for the treatment of metastatic colorectal cancer; radiolabeled murine anti-CD20 antibodies Zevalin® and Bexxar®, approved for the treatment of non-Hodgkin's lymphoma; and Mylotarg®, a humanized anti-CD33 antibody linked to a cytotoxic antibiotic, approved for the treatment of acute myeloid leukemia. In addition, Panorex (CO17-1A [Herlyn et al., 1979, 1980]), a murine MAb reactive with the tumor associated antigen Ep-CAM—was approved in Germany for the treatment of colorectal cancer [Riethmuller et al., 1994, 1998]. Although remissions have been noted using MAbs for cancer treatment, most were only temporary [Divgi and Larson, 1995; Riethmuller et al., 1998; Ben-Efraim, 1999; Macdonald, 1999; Normanno et al., 2003]. The emergence of tumor cell "escape variants," which are no longer affected by the antibody, remains the major problem. Therefore, a multi-pronged attack on cancer cells should be advantageous.

Indeed, the development and use of combination cancer therapies is increasingly becoming a strategy of choice. The therapeutic efficacy of antibodies has been enhanced by combining them with conventional chemotherapeutics or radiation therapies. For example, the anti-epidermal growth factor receptor (EGFR) antibody Erbitux® combined with irinotecan, 5-fluorouracil and leucovorin in the third-line treatment of refractory colorectal cancer showed a 23% objective response rate compared to 11% in patients treated with antibody alone [Cunningham et al., 2003]. However, the toxicity of conventional anti-cancer therapeutics remains an issue. Combining anti-tumor antibodies represents a promising approach that may yield enhanced therapeutic efficacy without the added toxicity of chemo/radiotherapeutics. In pre-clinical studies presented by ImClone Systems at the 2005 American Association of Cancer Research (AACR) conference in Anaheim, California, treatment with Erbitux® in combination with an anti-insulin-like growth factor 1 receptor (IGF1R) antibody or anti-vascular endothelial growth factor receptor (VEGFR) antibody resulted in a greater inhibition of growth of human pancreatic carcinoma xenografts in immunodeficient mice when compared to monotherapy [Tonra et al., 2005]. Analysis of the anti-tumor effects resulting from these combinations indicated that the inhibition of the EGFR pathway combined with either the IGF1R or VEGFR2 pathways results in a greater tumor growth inhibition than predicted from an additive effect—the combinations were synergistic. At the same conference, Centocor Inc. presented studies in an orthotopic human breast cancer model, showing that combination therapy with antibodies targeting both the breast cancer xenograft and mouse derived tissue factor resulted in synergistic inhibition of tumor growth and a decrease in the number of palpable tumors to two out of eight mice compared to seven out of eight mice in the monotherapy group [Ngo et al., 2005]. Finally, in the past several years, bispecific antibodies—antibodies capable of binding two different antigens—were shown to have potential appli-

cations as cancer therapeutics [Kufer et al., 2004]. The polyclonal antibody approach to cancer treatment, which will target many more than two antigens on a single tumor cell, is expected to have even higher potential.

POLYCLONAL ANTIBODY LIBRARIES (PCALS)

Our laboratory has developed a technology for generating recombinant polyclonal antibodies. This technology enables the perpetuation of standardized mixtures of polyclonal antibodies specific for microbes or tumor cells. Therefore, it combines the advantages of hybridoma-derived monoclonal antibodies and serum-derived polyclonal antibodies (see Table I).

The generation of recombinant polyclonal antibodies with desired specificities is made possible by the technology for displaying Fab antibody fragments on the surface of phage particles to generate so called "Fab phage display libraries" [Barbas et al., 1991; Hoogenboom et al., 1991; Kang et al., 1991]. In this system, the genetic material encoding Fab fragments is obtained from the B lymphocytes of immunized animals, or from humans, and cloned into a phage vector such that the genetic material encoding the Fab is attached to the genetic material encoding a phage coat protein (cp). Upon transformation of bacteria with these phage vectors, phage particles that display Fab on their surface are generated because the Fab is embedded in the phage coat via the coat protein to which it is attached. As each phage particle contains a different vector molecule for its genome, each phage particle displays a different Fab on its surface. The beauty and power of the phage display system is the coupling of a selectable function (binding to an antigen) to the genetic material that encodes that function. This is because the selected phage have a replication function and therefore a few phage particles can be amplified indefinitely if they are used to infect the bacterial host. This system has been used to select and clone-out phage encoding monoclonal antibody fragments to haptens, proteins, viruses, and cancer cells [O'Brien and Aitken, 2002], which can be converted to full length MAbs as an alternative to the hybridoma technology.

In addition to production of MAbs, our laboratory has adapted the Fab phage display system to generate polyclonal antibody libraries (PCALs) [Sharon et al., 2002; Williams and Sharon, 2002a; Williams et al., 2002b; Chen et al., 2003a,b; Liebman et al., 2004]. Repertoires of expressed light and heavy chain variable (VL and VH) region genes are obtained by RT-PCR from B cell and plasma cell RNA derived from immune humans or experimental animals, and cloned as VL-VH region gene pairs into a phage display vector to generate a combinatorial Fab phage display library. But instead of isolating monoclonal Fab phage particles, the Fab phage display libraries are maintained as polyclonal mixtures and subjected to positive and negative selection. For an anti-cancer library, positive selection is done to recover phage particles with anti-cancer specificities, and negative selection is done, on normal cells, to deplete phage particles that cross-react with normal tissues. After amplification of these selected sublibraries, we transfer the selected VL-VH region gene pairs, in mass, from the phage vector population to a mammalian vector that provides complete constant region genes and appropriate transcription regulatory elements for expression of full length, glycosylated antibodies in mammalian cells. Thus, the sublibrary that was selected at the Fab phage display level can be expressed—after transfection of the mammalian vector population into mammalian cells—as a library of polyclonal full length antibodies that can

TABLE I. Comparison of PCALs With MAbs and Serum-Derived Polyclonal Antibodies

Characteristic	Serum-derived polyclonal antibodies	MAbs	PCALs
Density on target	**High**[a]	Low	**High**
Efficiency at mediating effector functions	**High**	Low	**High**
Target cell escape variants	**Unlikely**	Likely	**Unlikely**
Supply	Limited	**Unlimited**	**Unlimited**
Genes	Unavailable	**Available**	**Available**
Amplification of desired specificities	Not possible	Not applicable	**Possible**

[a]Advantages are bold.

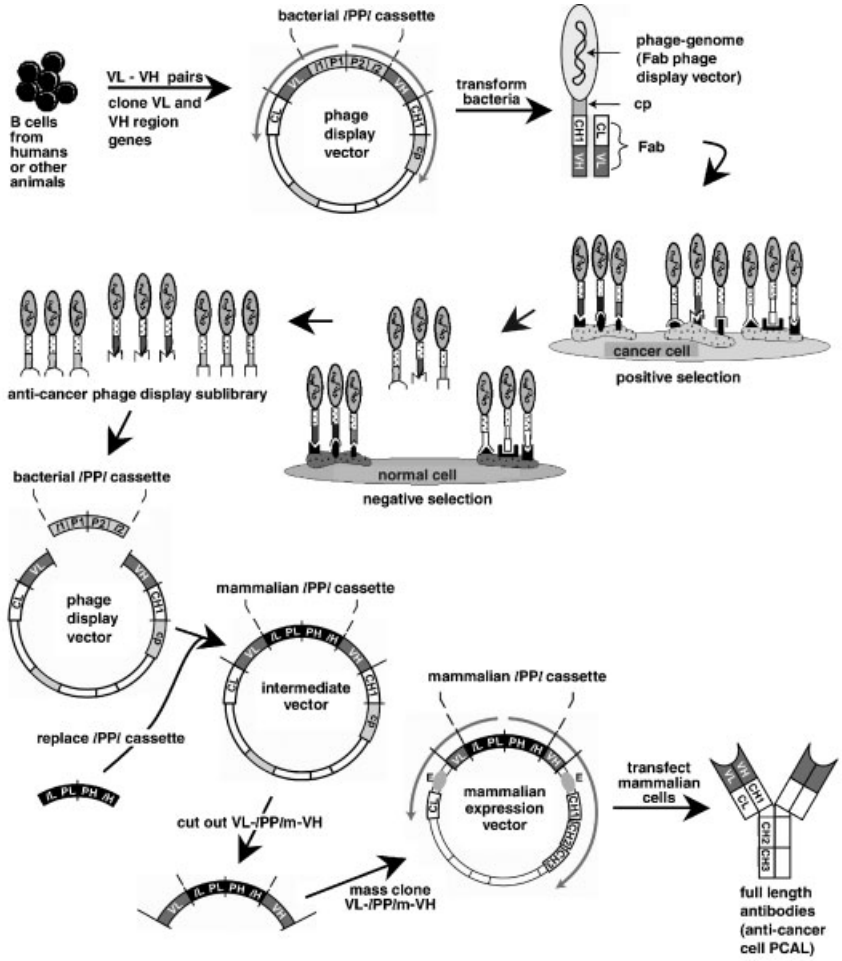

Fig. 1. Scheme for construction of an anti-cancer polyclonal antibody library (PCAL). CL, light chain constant region/domain; CH1, CH2, CH3, antibody heavy chain constant domains 1, 2 and 3, respectively; cp, coat protein (of phage); E, enhancer; l, leader; P, promoter; VH, variable region of heavy chain; VL, variable region of light chain; Arrows surrounding vectors denote transcriptional orientation. The bidirectional orientation of the H and L chain transcriptional units facilitates the mass transfer of the selected V region gene pairs from the phage display vector to the mammalian expression vector because each VL-VH region gene pair remains together on the same DNA molecule.

mediate effector functions. Mammalian vectors that contain C region genes of any isotype and any species can be used. Because the transfected cells are immortal, the cell population producing the PCAL can be perpetuated indefinitely, just like hybridoma cells (see Fig. 1).

PCALs are intended to contain individual antibodies which may or may not be specific for the target cancer cells. However, the collection of the individual antibodies will recognize the antigenic profile of the target cancer cell with a high signal-to-noise ratio above the background of any cross-reacting normal cell (see Fig. 2). Because effector functions are inefficient at low antibody density, low-level cross-reactivity with normal cells will likely be tolerated in therapeutic applications.

The premise—that the antigenic profile of cancer cells differs from the antigenic profiles of normal cells—has been substantiated by the finding of: "tumor-associated antigens (TAAs)" that are not expressed in significant or measurable amounts on the surface of normal cells, such as oncofetal proteins; "tumor-specific antigens (TSA)" in the form of peptides that are presented on the cell surface in association with major histocompatibility complex (MHC) class I molecules, and are not detected on most normal cells; and different antigenic profiles on tumor versus normal cells, as assessed by serologic analyses [Sharon, 1998; Janeway et al., 2005]. The notion of different antigenic profiles is further supported by catalogs of expressed genes compiled by the Cancer Genome Anatomy

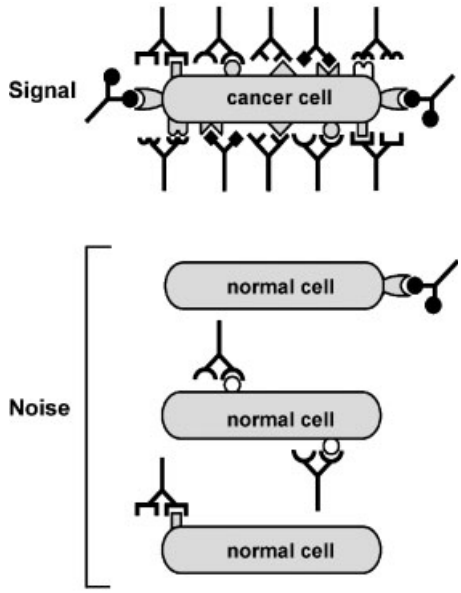

Fig. 2. Schematic representation of cancer cell recognition, by PCAL antibodies, with high signal-to-noise ratio.

Project (CGAP), which indicate that several thousand genes are expressed in cancer tissues but not in normal tissues [Strausberg, 2001].

Molecular analysis of changes that occur during transformation from a normal cell to a cancer cell, and potentially contribute to the transformation process, have revealed mutations in three major gene categories [Aziz, 1998; Sharon, 1998]: (1) genes whose products regulate apoptosis; (2) genes whose products cause cell division; (3) genes whose products inhibit cell division including tumor suppressor genes (e.g., p53). Such mutations plus other alterations that occur during the unchecked cell division of cancer cells have been categorized into TSAs and TAAs. In the case of colorectal cancer, four categories of sporadic cancer have been defined based on common initiating mutations and associated characteristics. The most common category is characterized by mutations in the adenomatous polyposis coli (*APC*) gene, chromosomal instability with loss of heterozygosity (LOH) and mutations in several tumor suppressor genes, most notably p53 LOH and 18q (DCC) LOH [Kennedy and Hamilton, 1998]. Because a tumor suppressor gene is expected to suppress a defined set of other genes, inactivation of the tumor suppressor gene should result in activation of the entire set of genes, which would provide shared antigens for all tumors with p53 LOH and 18q LOH. This premise is supported by cDNA microarray data showing correlations between up-regulated genes in colorectal cancers and LOH molecular markers [Birkenkamp-Demtroder et al., 2002].

Anti-cancer PCALs are intended to include antibodies to intracellular and secreted tumor antigens that are displayed as peptides complexed with MHC molecules on the tumor cell surface. Indeed, antibodies, as expected from their ability to recognize a variety of both native and linear epitopes [Janeway et al., 2005], have been shown to recognize peptides in peptide-MHC complexes [Messaoudi et al., 1999; Denkberg et al., 2002].

PROTOTYPIC PCALS TO HUMAN COLORECTAL CANCER

We have chosen colorectal cancer, the second leading cause of cancer death in the United States [ACS, 2005a], as a test application for the PCAL technology. This choice was supported by the availability of human colorectal cancer cell lines that can form tumor xenografts in immunodeficient mice, allowing evaluation of anti-cancer PCALs in preclinical studies. Furthermore, the clinically used [Riethmuller et al., 1998] anti-colorectal cancer murine MAb, CO17-1A [Herlyn et al., 1979, 1980], is available for comparative studies.

A combinatorial Fab phage display library was generated from the antibody variable region genes of each of two BALB/c mice immunized with the human colorectal cancer cell lines SW480, SW948, and SW837 [Williams and Sharon, 2002a; Williams et al., 2002b]. These libraries were shown to be diverse by nucleotide sequencing and diagnostic restriction enzyme digestion (fingerprinting) of individual members. The two libraries were combined and selected on the colorectal cancer cells by two different methods to preserve as many anti-colorectal cancer reactivities as possible. In selection method 1 (conventional wash method), the Fab phage display library was selected by incubation with a suspension of formaldehyde-fixed human colorectal cancer cells in two successive rounds of selection and phage amplification by infection of bacteria [Williams et al., 2002b]. In selection method 2 (density gradient method), the library was selected by incubation with a suspension of native human colorectal cancer cells, in one round, using density gradient centrifugation, instead of washes, to

separate cell-bound and free phage; the density gradient consists of a layer of fetal bovine serum (FBS) on a "cushion" of Percoll density medium, where cells with bound phage sediment at the FBS-Percoll interface and free phage remain on top of the FBS layer [Williams and Sharon, 2002a].

All selected libraries were analyzed for binding to the three colorectal cancer cell lines by enzyme linked immunosorbent assay (ELISA) of each library as well as individual clones, and polyclonality was assessed at the DNA level by fingerprint analysis of individual binding clones. The first round of selection, by both methods, yielded a high percentage of antigen-reactive clones (54% and 91% for the conventional wash and density gradient selection methods, respectively) and a high degree of polyclonality. In the conventional wash selection method, the second round-selected library showed 94% antigen reactive clones but more limited diversity as compared to the first round. These results demonstrated that high quality polyclonal anti-colorectal cancer Fab phage display libraries can be selected.

To test the hypothesis that a PCAL—that binds to colorectal cancer cells with a high signal-to-noise ratio compared to normal human cells—could be produced, we started with a 1:1 mixture of two Fab phage display libraries that had been obtained by one round of positive selection on formaldehyde-fixed [Williams et al., 2002b] or native [Williams and Sharon, 2002a] human colorectal cancer cell lines SW480, SW948, and SW837. The combined library phage were subjected to two sequential absorptions on 1:1 mixtures of erythrocytes and leukocytes. The absorbed phage as well as a sample of the unabsorbed phage were then separately subjected to positive selection on the SW480 colon cancer cell line, in native form, by the density gradient centrifugation method [Williams and Sharon, 2002a]. This yielded a negatively selected (absorbed) library of 9.2×10^4 members and a paired, non-negatively selected (unabsorbed) library of 3.8×10^5 members [Liebman et al., 2004], demonstrating a fourfold reduction in library size due to negative selection.

ELISA analysis of the absorbed Fab phage display library showed that 70% of tested clones reacted to colorectal cancer cells. Reactivity of the library was reduced 10-fold to erythrocytes and 4-fold to lymphocytes or granulocytes, with many clones unreactive to one or more of the blood cell types. However, the absorbed library remained polyclonal, as shown by DNA fingerprint analysis of colorectal cancer-binding clones.

The H and L chain V region gene pairs of the absorbed library were then transferred in mass from the Fab phage display vector to a mammalian vector and expressed as murine IgG2b following transfection, by spheroplast fusion, into Sp2/0 myeloma cells [Liebman et al., 2004]. Transfected cells were plated in 96-well plates, and ELISA analysis of cell supernatants showed that 79% of transfectants expressed IgG and of those 74% were positive for binding to the SW480 human colorectal cancer cell line. A template of 95 SW480-reactive wells was assembled, designated Lib-Col2.1, and IgG purified from a consolidated mass culture. The purified Lib-Col2.1 IgG was compared to the clinically used anti-colorectal cancer MAb CO17-1A, by ELISA and flow cytometry, for binding density on SW480 colorectal cancer cells and on normal human blood cells. The results showed that Lib-Col2.1 bound to SW480 cells at higher density than MAb CO17-1A and that its binding to normal human blood cells was reduced 2.4–24 fold relative to an unabsorbed anti-SW480 serum. Furthermore, Lib-Col2.1 was more effective than MAb CO17-1A at inhibiting the growth of SW480 cells in culture [Liebman et al., 2004].

These results suggested that a polyclonal antibody library with preferential reactivity to cancer cells as compared to normal cells could be generated. Evaluation of Lib-Col2.1 in nude mice bearing human colorectal cancer xenografts—is underway.

FUTURE PERSPECTIVES

Although recombinant polyclonal antibodies have the potential to be much more efficacious than MAbs for cancer therapy, their clinical success will depend on the ability to generate, characterize, and mass-produce PCALs with high ratios of (titer-based) cancer-to-normal reactivity (CNR). The optimal CNR—which will maximize the therapeutic effect while minimizing toxicity—will depend on the histologic origin of the cancer, whether the tissue of origin is essential such as lung or dispensable such as prostate. Thus, a lower CNR for the tissue of origin (CNRO) might be acceptable compared to

the general CNR (CNRG) for other tissues or cell types. In that regard, it should be noted that FDA approved antibodies such as Rituxan and Erbitux, specific for CD20 and epithelial growth factor receptor respectively, target molecules that are expressed at high levels on some normal cells. For a PCAL, CNRs will have to be determined both for the library as a whole and for the individual library clones. As a starting point, a CNR cutoff of "10" might be chosen, for the library as a whole, based on reports in the literature on ratios of tumor- to-normal reactivities for "good" anti-tumor MAbs [Stein et al., 1990]. Lower CNRs could be tolerated for some of the individual library clones because each clone will contribute only a small fraction of the total antibody dose.

How many cancer-reactive antibody clones should a PCAL contain? The number might fall between 50 and 100. This would still provide the advantages of multi-targeting while allowing the characterization of each library member for antigen specificity and separate storage and cryopreservation of PCAL clones in individual wells of a single 96-well plate. Knowledge of the antigen specificity of each PCAL member and its effect on tumor cell growth, in vitro and in mouse models of human tumor xenografts, would permit the exclusion of any "enhancing antibodies" that may act as agonists of tumor growth. Keeping the number of PCAL members below 100 would also facilitate the mass-production of PCAL protein for clinical applications. To ensure minimal variability between lots, mass-production will probably begin with separate expansion of the individual PCAL clones, followed by step-wise consolidation into increasingly larger vessels.

Recombinant polyclonal antibodies for cancer therapy could be chimeric, with murine V regions and human C regions, derived from mice immunized with primary human tumors. Thus, the same V region gene pairs could be expressed either in all-mouse antibodies for preclinical studies in mouse models of human tumor xenografts or in mouse-human chimeric antibodies for clinical applications. Mouse-human chimeric antibodies are efficient in mediating effector functions in humans and have longer half-lives in vivo compared to murine antibodies [White et al., 2001]. Thus, of the first 10 therapeutic monoclonal antibodies approved by the US FDA, four are chimeric [ACS, 2005b]. These chimeric monoclonal antibodies do not elicit human anti-mouse antibody (HAMA) responses and anti-idiotypic antibody responses (to the mouse V regions) have been found in less than 1% of treated patients [White et al., 2001]. Use of polyclonal antibodies with xenogeneic V regions further reduces the induction of anti-idiotypic antibodies because, in a polyclonal antibody preparation, no one V region pair should be present in sufficient concentration to be immunogenic; and even if a particular V region pair induces an anti-idiotypic response which eliminates it, it should be inconsequential because the activity of a polyclonal antibody preparation does not depend on a single member. This was shown by the anti-idiotypic response of patients to the OKT3 anti-human thymocyte monoclonal antibody but the lack of an anti-idiotypic response to polyclonal rabbit or horse anti-human thymocyte globulins [Regan et al., 1997]. Similarly, human MAbs elicit an anti-idiotypic antibody response in humans [Koda et al., 2001], just as mouse MAbs elicit an anti-idiotypic response in mice [Paul, 2003].

As an alternative to chimeric antibodies, recombinant polyclonal antibodies could be all-human, derived from xeno-mice—transgenic mice containing human antibody genes [Green, 1999; Gallo et al., 2000], or from human Fab phage display libraries [Hoet et al., 2005]. Humanized versions of recombinant polyclonal antibodies would also be possible, although the "humanization" would have to be done individually for each library clone, which might not be worth the effort because the advantage of humanized PCALs over chimeric PCALs is expected to be minimal if any.

An additional consideration regarding recombinant polyclonal antibodies for cancer therapy is whether the same PCAL could be used to treat different patients with the same cancer type. At one extreme, all patients with a particular cancer type, e.g., breast cancer, could be treated with the same PCAL whereas at the other extreme, a new PCAL would have to be generated for each breast cancer patient. It is likely that, as with MAbs, a PCAL would cross-react with many cancers in the same category and, therefore, could be used to treat multiple patients. Thus, breast cancer patients whose cancer cells over-express HER2/neu are eligible for treatment with Herceptin [Cobleigh et al., 1999].

In conclusion, recombinant polyclonal antibodies would provide replenishable multi-targeted cancer therapeutics which will minimize the chance of emergence of tumor cell escape variants and will be efficient at mediating effector functions. It is expected that the same PCAL could be used to treat many patients with the same cancer type.

ACKNOWLEDGMENTS

We thank Jae Kim for help with the preparation of the manuscript and the figures. J. Sharon has a significant financial interest in Symphogen A/S. B.R. Williams is employed by ImClone Systems, Inc.

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