A Review of Antibody Therapeutics and Antibody-Related Technologies for Oncology

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(*J Immunother* 2006;29:351–364)

INTRODUCTION

The antibodies currently approved for the treatment of diseases, including cancer, have been developed predominantly based on the understanding and identification of key targets involved in disease pathology. Thus, for oncology, currently marketed antibodies to epidermal growth factor receptors (EGFR/HER1 and HER2) and vascular endothelial growth factor (VEGF) treat cancer by blocking the function of these targets that are crucial for tumor progression. Other targets for launched products are those that are highly up-regulated on neoplastic cells, including CD20, CD52, and CD33. Antibodies are generally highly specific for their molecular targets and can be used to affect disease-specific targets, thereby sparing normal cells and causing less toxicity than traditional cytotoxic chemotherapies. Effective antibodies act through one or more of a variety of mechanisms, including (a) blocking essential cellular growth factors or receptors, (b) directly inducing apoptosis, (c) binding to target cells and recruiting ''effector functions'' such as antibody-dependent cellular cytotoxicity (ADCC), or complement-dependent cytotoxicity (CDC), and (d) delivering cytotoxic payloads such as chemotherapies, radioisotopes and toxins.

The use of informatics will be essential as we develop new waves of products that provide additional efficacy, specificity, or safety over currently marketed products. Informatics approaches can be used to (a) identify novel targets either upstream or downstream of already validated targets to enhance or complement efficacy, (b) identify targets that are more specific for tumors, thereby enhancing safety and providing a means of directing toxic agents to the tumor, (c) identify novel pathways essential for disease progression, and (d) identify Fc mutants that have enhanced immune effector function.

Received for publication October 28, 2005; accepted November 3, 2005.
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ANTIBODY TECHNOLOGY

The first monoclonal antibodies from mice were generated in 1975.[1] In humans, mouse-derived antibodies are highly immunogenic, and therefore ''chimeric'' antibodies were created by replacing mouse constant domains (non-antigen binding domains) with human constant domains.[2] This improvement considerably reduced the immune response to therapeutic antibodies. Additional modifications of framework regions within the antigen-binding variable regions further reduce immunogenicity and result in what are termed ''humanized'' antibodies. Fully human antibodies can be derived from human cells or from genetically engineered mice transgenic for human antibody genes. Human antibodies can also be generated from antibody-expressing phage libraries as single chain Fv or Fab fragments that can subsequently be converted to full-length antibodies.[2]

ANTIBODIES THAT ARE APPROVED AND IN LATE STAGE CLINICAL DEVELOPMENT

The promise of harnessing the power of antibodies to treat cancer is now being realized in clinical practice. There are currently 8 FDA-approved monoclonal antibodies for oncology indications (Table 1).[3] Most of the approved antibodies, and those in late-stage clinical trials, were designed to directly target antigens known to be expressed on tumor cells and in some instances known to mediate essential disease-critical functions. A majority of these targets are cell-surface receptors, most notably receptor tyrosine kinases that mediate signaling processes necessary for essential cellular functions and for maintaining the malignant phenotypes of tumor cells.[4] Other antibody drugs bind to antigens over-expressed on tumor cells and mediate their effects through antibody effector function or the delivery of a toxic payload. The hope is that greater efficacy can be achieved by combining antibody therapy with chemotherapy, other biologics, or radiotherapy. Animal models using these antibodies have shown additive or synergistic benefits when combined with chemotherapy or other biologic therapies.[5]

PROMISING ANTIBODIES AND TARGETS

The success in designing and developing antibody-based cancer therapy depends largely on selecting suitable targets. In general, monoclonal antibody targets need to meet the following criteria: (a) the target antigen is expressed by tumor cells at a much higher level than by

normal cells; (b) the antigen must be presented properly and stably on the tumor cell surface for its recognition by the antibody; (c) the antigen is expressed by a large percentage of tumor cells, and is expressed in a broad spectrum of different types of tumor; (d) the antigen functionally participates in the malignant disease process, and ideally, would be essential for multiple steps during such processes.

These selection criteria were well reflected by two recently approved antibody therapeutics for treating solid tumors, bevacizumab (Avastin),[6] and cetuximab (Erbitux).[7] Avastin, the anti-VEGF antibody, neutralizes the activity of VEGF, one of the most potent angiogenic growth factors. Erbitux, the anti-EGFR antibody, binds and blocks the signaling through the receptor tyrosine kinase. These two antibodies together with trastuzumab (Herceptin) highlight antibody therapeutics against cell-surface receptors or associated signaling pathways.

A review of currently approved antibody therapeutics (Table 1) reveals another major category of tumor cell surface antigens: various CD molecules. These cell surface CD molecules—CD20, CD22, CD33, and CD52—are overexpressed on tumor cells, most notably those of hematopoietic origin. Antibodies to these targets are being developed in the form of naked antibodies, antibody-conjugated toxin, or as radiolabeled antibodies (Table 2).

In addition to targeting tumor cell surface antigens, antibodies that target the tumor vasculature represent an attractive approach. Tumor growth is dependent on angiogenesis, the formation of new blood vessels. Targeting tumor vessels provides several advantages over traditional anti-tumor approaches, including the genetic stability of antigen expression on tumor endothelial cells and the resulting low likelihood of developing drug resistance, broad application to various tumor types, and low toxicity to normal tissues.[8] The anti-angiogenesis concept has been validated in clinical studies by the success of Avastin in treating metastatic colorectal cancer.[7] Antibodies have also been developed to endothelial cell adhesion molecules, integrins ($\alpha V\beta 3/\alpha V\beta 5$, and $\alpha 5\beta 1$), vascular endothelial (VE)-cadherin, occludin, E-selectin, and platelet/endothelial-specific cell adhesion molecule (PECAM). In advanced development stage are antibodies against $\alpha V\beta 3$ integrin, an antigen expressed on certain tumor cells and the surface of endothelial cells actively involved in tumor angiogenesis, but not of those lining quiescent blood vessels. Vitaxin a humanized antibody to $\alpha v\beta 3$, and CNTO 95, a fully human monoclonal antibody to $\alpha V\beta 3/\alpha V\beta 5$, have successfully completed phase 1 clinical studies in patients with advanced cancer.[9,10]

Other targets that are suitable for monoclonal antibody-based anti-cancer therapeutics may derive from tumor stromal cells. The critical role of tumor stromal cells and tumor-host interactions is now becoming increasingly appreciated.[11] The composition, integrity, and the mechanical properties of the basement membrane have substantial influence on tumor cell behaviors, from tumor growth to metastasis. Enzymes of matrix metalloproteinase (MMP) and plasminogen activator (PA) (urokinase PA and tissue PA) systems are important players in remodeling the extracellular matrix (ECM), and regulating availability of biologically active ECM-bound growth factors. Despite the disappointing clinical trial results of small molecule MMP inhibitors, the enzymes or proteins that modulate the activity of these enzymes remain attractive antibody targets. Interactions between tumor and host are also regulated by different soluble growth factors, cytokines, and chemokines. These inflammatory factors represent another group of potential targets.[12] For example, tumor necrosis factor α (TNFα) plays a crucial role in cancer progression, and blockade of TNFα with the chimeric antibody infliximab (Remicade) has demonstrated promising therapeutic efficacy in treating metastatic renal cell cancer.[13] In addition to being therapeutics, these anti-inflammatory antibodies may also have the potential to provide supportive care benefits.[14]

SUPPORTIVE CARE

Advancements toward earlier detection and improved outcomes with new targeted therapies promise to transform cancer into a chronic and manageable condition rather than a uniformly fatal disease. In this context, ameliorating symptoms caused by the underlying cancer or side effects of toxic therapies with supportive care is increasingly important in the management and treatment of cancer. The advent of molecular biologic and bioinformatic techniques makes it possible to begin to understand the pathologic basis of cancer associated cachexia, pain, and depression, for example. In addition to acting as tumor therapeutics, antibodies may be especially well-suited to cancer supportive care given their targeted nature.

TNFα is a good example of a potential antibody target for cancer supportive care believed to play a crucial role in mediating cancer-related morbidity. The utility of blocking TNFα with the chimeric antibody Remicade is under preclinical and clinical investigation. Remicade is currently FDA-approved for the treatment of immune-mediated inflammatory disorders including Crohn's disease, ulcerative colitis, psoriasis, and rheumatoid arthritis. The range of potential indications for supportive care is broad and diverse due to the pleiotropism of TNFα action and includes cancer-associated depression, fatigue, cachexia, treatment of toxicities due to chemotherapy and radiotherapy, treatment of metastatic bone pain, and graft versus host disease (GVHD).[15–19]

A wealth of evidence implicates TNFα as a mediator of cachexia.[20] In fact, TNFα was initially called "cachectin" because it caused severe wasting in rodent models of disease. TNFα has also been shown to be important for cachexia at the cellular and molecular levels, both by increasing destructive proteolysis in mature skeletal muscle and by inhibiting the differentiation of myoblasts necessary for the repair of damaged or stressed muscle tissue.[21] The molecular details of TNFα action on skeletal muscle are starting to be elucidated.

TABLE 1. Oncology Antibodies approved by the US FDA

Name (US Tradename[1])	Company	Target	Mechanism	Antibody Form[2]	Cancer Indication	US FDA Approval Date	Ref
Rituximab (Rituxan)	Genentech and IDEC Pharmaceuticals	CD20	ADCC, CDC, Directly induces apoptosis	Chimeric IgG1	NHL	11/97	108
Trastuzumab (Herceptin)	Genentech	HER2	Inhibition of HER2-mediated tumor cell proliferation and migration	Humanized IgG1	Breast cancer with HER2 overespression	9/98	109
Gemtuzumab ozogamicin (Mylotarg)	Wyeth-Ayerst and Celltech Group	CD33	Delivery of calicheamicin into leukemic cells resulting in DNA strand breaks and apoptosis	Humanized IgG4 linked to calicheamicin	AML	5/00	110
Alemtuzumab (Campath)	Ilex Pharmaceuticals and Berlex Laboratories	CD52	ADCC, CDC	Humanized IgG1	CLL	5/01	111
Ibritumomab tiuxetan (Zevalin)	IDEC Pharmaceuticals	CD20	Delivery of cytotoxic radiation, ADCC, CDC, apoptosis	Murine IgG1 ^{90}Y conjugate (murine parent form of rituximab) (Rituximab preceding Indium-111 Zevalin followed seven to nine days later by a second infusion of Rituximab prior to Yttrium-90 Zevalin)	NHL	2/02	112
Tositumomab/^{131}I-tositumomab (Bexxar)	Corixia and GlaxoSmithKline	CD20	Delivery of cytotoxic radiation, ADCC, CDC, apoptosis	Murine IgG2a ^{131}I conjugate plus unlabeled antibody	NHL	6/03	113
Bevacizumab (Avastin)	Genentech	VEGF	Inhibition of VEGF-induced angiogenesis	Humanized IgG1	Metastatic colorectal cancer	2/04	114
Cetuximab (Erbitux)	ImClone Systems and Bristol Myers Squibb	EGFR (HER1)	Inhibits EGFR-mediated tumor cell invasion, proliferation, and metastasis and angiogenesis Enhances activity of some chemotherapeutics and radiotherapy	Chimeric IgG1	Metastatic colorectal Cancer	2/04	115

[1]The suffixes of the generic name of antibodies are assigned as follows: murine antibodies are "omab", chimeric antibodies are "ximab", humanized antibodies are "zumab", and fully human antibodies are "umab".
[2]Human IgG1 is effective in inducing CDC and ADCC, whereas the IgG4 isotype is marginally effective for both.
Abbreviations: ADCC—antibody-dependent cytotoxicity; CDC—complement-dependent cytotoxicity; NHL—non-Hodgkin's lymphoma; AML—acute myeloid leukemia; CLL—chronic lymphocytic leukemia; EGFR, HER1, HER2—epidermal growth factor receptors; US—United States; FDA—Food and Drug Administration; VEGF—vascular endothelial growth factor.

TABLE 2. Oncology Antibodies in Phase III Clinical Development for Oncology Indications

Name	Company	Target	Mechanism	Antibody Form	Cancer Indication	Status	Ref
WX-G250 (cG250)	Wilex, under license from Centocor and in collaboration with the Ludwig Institute for Cancer Research	Cervical-carcinoma-associated antigen MN/CAI	ADCC	Chimeric IgG1	RCC	Phase 3	116
Oregovomab (OvaRex; B43.13)	Unither Pharmaceuticals	CA 125 antigen	Anti-idiotypic vaccine induces immune response against tumor-expressed CA125	Murine IgG	Ovarian cancer	Phase 3	117
Cotara chimeric TNT antibody (chTNT-1/B) labeled with iodine-131	Peregrine Pharmaceuticals (formerly Techniclone	histone H1/DNA complexes exposed in the necrotic core of malignant solid tumors	Delivery of cytotoxic radiation	Human IgG	Solid tumors, including glioma	Phase 3	118
IGN-101	Igeneon AG	Ep-CAM	Vaccine induces immune response against Ep-CAM- positive tumor cells	Murine IgG (17-1A (edrecolomab)	NSCLC	Phase 3	119
Catumaxomab (Removab)	Trion Pharma GmbH licensened to Fresenius Biotech GmbH	CD3 + EpCAM	Bind to cancer cells and also to T cells and macrophages, eliminating tumor cells	Trifunctional bispecific monoclonal antibody	Maglignant ascites in ovarian and other cancers	Phase 3	120–122
Zanolimumab; HuMax-CD4; MDX-CD4	Serono, Genmab and Medarex, Inc	CD4	Immunomodulation of T-cells	Human monoclonal antibody	CTCL and NCTCL	Phase 3	123
MDX-010	Medarex and Bristol-Myers Squibb (BMS)	cytotoxic T lymphocyte antigen-4 (CTLA-4),	immunostimulatory antibody MDX-010 being studied with or without the gp100-based vaccine MDX-1379 (qv)	Human monoclonal antibody	metastatic melanoma	Phase 3	124
Panitumumab (ABX-EGF)	Abgenix and Amgen	EGFr	Binds to EGFr, blocks ligand binding, receptor signalling and inhibits cell activation and proliferation	Human Ig	Colorectal, NSCLC, RCC	Phase 3	125

This information in these tables was compiled from a variety of sources including publications, scientific meeting presentations, and company websites. All efforts were made to make the tables complete and accurate but there is no guarantee.

Abbreviations: ADCC—antibody-dependent cytotoxicity; CTCL—cutaneous T-cell lymphoma; EGFr—epidermal growth factor receptor; Ep-CAM—epithelial cell adhesions molecule; NCTCL—non-cutaneous T-cell lymphoma; NHL—non-Hodgkin's lymphoma; NSCLC—non-small cell lung cancer; RCC—renal cell cancer.

Acharyya et al[22] provide evidence that TNFα, acting in concert with interferon γ (IFNγ), specifically down-regulates the expression of myosin heavy chain. These observations help to explain the molecular pathology of cancer-related cachexia and may point the way to measurable pharmacodynamic markers of anti-TNFα activity. Clinical trials are now testing the ability of anti-TNFα agents such as Remicade to inhibit wasting in cancer patients.[20,23] Additional targets that have been associated with cancer cachexia and may be attractive antibody targets include interleukin (IL)-1, IL-6, proteolysis inducing factor (PIF), and IFNγ.[23]

Several other cancer-associated conditions could be potentially attributed to TNFα activity. Cancer-related pain remains a significant unmet medical need. TNFα appears to be important both for the pain signal itself as well as metastatic bone erosion.[24,25] TNFα also appears to mediate many of the unwanted side effects of radiation therapy. Radiation-induced production of TNFα by tumor cells enhances the intended local pro-inflammatory effects of ionizing radiation, but also damages normal tissue and can cause unwanted fibrosis. Preclinical and clinical data suggest that TNFα plays a role in mediating radiation-induced normal tissue damage and fibrosis and that anti-TNFα therapy may be effective treatment of the prevention of these deleterious side effects.[26,27]

NOVEL ANTIBODY TARGET DISCOVERY IN GENOMICS AND PROTEOMICS AGE

Recent advances in gene expression analysis have enabled large-scale gene profiling to identify ''tumor-specific'' antigens. These techniques include serial analysis of gene expression (SAGE), reverse transcriptase-polymerase chain reaction (RT-PCR)-based differential display, subtractive hybridization, expressed sequence tag (EST) sequencing, and most importantly DNA microarray, for detecting overexpressed genes, alternative splicing forms, mutations, and fusion transcripts that are specific to tumor cells. With the introduction of laser captured microdissection and in vitro linear gene amplification, it has become feasible to compare the expression profile of virtually all human gene transcripts in cancerous cells versus their adjacent normal counterparts.[28] In addition to altered gene expression levels, cancer cells also exhibit dysregulation in their protein synthesis and modification machineries. For example, changes in post-translational modification are known to be responsible for unregulated cell growth and are implicated in tumorigenesis, representing yet another class of antibody targets. Therefore, it is also critical to analyze these changes in cancer cell proteins via proteomic approaches to identify novel cancer therapeutic targets.[29]

Given the importance of cell surface proteins as target antigens for therapeutic antibodies in treating cancer, it is obviously attractive to develop antibodies to tumor-specific antigens in a high throughput fashion. With the complete human genome sequence unraveled, genes encoding cell surface antigens could be reliably predicted by bioinformatic analysis. These selected genes could be cloned, synthesized, and expressed to serve as antigens. Alternatively, phage display presents another approach to generate such antibodies. Phages harboring antibody-encoding genes could be hybridized with tumor tissue sections. Phages bound to tumor cells and preferentially recognizing tumor-specific antigens can be retrieved to express such antibodies for further characterization in various tumor models. This ''reverse immunology'' approach could also be combined with proteomics technology in which monoclonal antibodies could be generated to proteins such as those derived from tumor cell membrane protein preparations that have been separated on two dimensional gel electrophoresis.[30] Proteomics used in combination with antibody engineering also provides a means to generate such antibodies as pertuzumab (Omnitarg), the new anti-HER2 antibody with a distinct mechanism-of-action to Herceptin.[31] Because Omnitarg binds to specific epitopes of HER2 receptor involved in HER2 heterodimerization with other HER receptors and sterically blocks signaling from these receptors, its anti-cancer activity is independent of high HER2 expression and could be applicable in multiple cancer types in addition to breast cancer.[32] Such approaches may be especially useful in designing and developing novel antibodies to cell surface receptors such as tyrosine kinase receptor family members.

APPROACHES TO ENHANCING ANTIBODY EFFICACY

Enhancing Antibody Immune Effector Functions

One of the more important activities of antibodies is to help trigger cellular immune responses against various targets, such as a pathogen, a pathogen-infected host cell, or a tumor cell. One way this is accomplished is for an antibody to bind to its cellular target via its antigen-binding Fab domains while simultaneously binding to IgG Fcγ receptors (FcγR) expressed on nearby immune effector cells. Engagement of FcγRs on these effector cells, which may be macrophages, monocytes, dendritic cells, natural killer (NK) cells, or neutrophils, can lead them either to phagocytose the antibody-bound target, to kill the antibody-bound target by inducing ADCC, or to kill the target by releasing soluble lytic factors. Cellular immune responses may also be induced by antibodies via complement-dependent cell cytotoxicity (CDCC), in which components of the complement cascade serve to enhance or recruit the activity of cytotoxic effector cells. Another antibody-mediated immune effector function related to CDCC that does not directly involve cellular responses is complement-dependent cytotoxicity (CDC), in which the thorough progression through the complement cascade results in formation of a cell-lysing membrane-attack complex on the antigen-expressing cell. Engineering antibody molecules to be more effective at recruiting these functions has been of great interest, particularly for cancer immunotherapy. If successful,

such efforts will lead to multiple benefits including improved clinical efficacy, a greater proportion of treated patients responding to therapy, lower dosing, and reduced costs for treatment.

General Description of FcγRs

There are two main types of FcγRs on the surface of immune effector cells when categorized by function, activating FcγRs and inhibiting FcγRs.[33,34] Both types of FcγR bind IgG molecules in a 1:1 stoichiometric ratio in the region that spans the lower hinge and upper Fc domains. The activating FcγRs, which transmit proinflammatory types of signals upon IgG binding, are the high-affinity receptor, CD64 (FcγRI), and the low-affinity receptors, CD32A (FcγRIIA) and CD16A (FcγRIIIA). CD64 is the only FcγR that readily binds to monomeric IgG. It is expressed on monocytes, macrophages, and dendritic cells, and can be induced to express on neutrophils by interferon-γ. CD32A and CD16A show minimal binding to monomeric IgG, but show very significant binding to higher-order immune complexes of IgG due to the avidity effect associated with numerous hinge/Fc domains within a complex simultaneously binding numerous FcγRs on a cell. CD32A is primarily expressed on monocytes, macrophages, dendritic cells, neutrophils, eosinophils, and platelets, whereas CD16A is primarily expressed on macrophages and NK cells. The inhibiting FcγR is CD32B (FcγRIIB), which can interrupt intracellular signaling events triggered by activating FcγRs on the same cell. CD32B is expressed on monocytes, macrophages, and B cells. The net cellular responses that result from binding of IgG immune complexes to FcγR on cells that express both activating and inhibiting FcRs probably depend on several factors, such as relative expression level of the two types of FcγR, relative avidity of the immune complexes to the different FcγRs present,[35] and the relative "strength" of intracellular signaling pathways from the opposing FcγR types.

The molecular events that lead to FcγR binding and intracellular signaling were previously suspected to involve altered Fc conformations that distinguish antigen-bound antibodies from non-antigen-bound antibodies. However, it is now widely accepted that it is the clustering of the low-affinity FcγRs by multivalent antibody-antigen immune complexes that leads to intracellular signaling. The high-affinity FcγR, CD64, is constantly binding antibodies, whether the antibodies are bound to antigen or not. Such binding leads to receptor-mediated internalization of both receptor and antibody. However, the fate of such internalized antibodies is believed to depend on whether they are clustered by antigen. Antibodies that are not clustered, as well as the CD64 receptor itself, are thought to recycle back to the cell surface, whereas clustered antibodies (higher-order immune complexes) are retained in the cell and routed through a lysosome-mediated degradation pathway.[36,37] What is not as clear is the fate of monovalent antigens bound to antibody in a simple 1:1 or 2:1 complex (ie, where antibodies are not clustered) after binding and internalization of the complex through CD64.

PRECLINICAL AND CLINICAL EVIDENCE FOR THE IMPORTANCE OF FCΓRS IN ANTIBODY THERAPY

The importance of FcγRs to the anti-tumor activity of antibodies in a mouse model was neatly demonstrated using mice that lacked either the activating FcγRs or the inhibiting FcγR. Anti-tumor antibodies were less effective at controlling tumors in mice that lacked activating FcγRs compared with wild-type mice, but were substantially more effective in mice that lacked the inhibiting FcγR.[38] These data not only confirmed the contrasting roles of the activating and inhibiting FcγRs, but also helped inspire ongoing efforts to prepare novel anti-tumor antibodies that would favor binding to the activating FcγRs over the inhibiting FcγR.

Although it has been known for some time that different individuals have slightly different variations of particular FcγRs, the importance of such allotypic variants to clinical response to antibody therapy has only recently been appreciated. Cartron et al[39] showed that non-Hodgkin lymphoma patients that were homozygous for a CD16A receptor that has a Val at position 158 had better clinical responses to rituximab (IgG1 antibody that binds CD20) than patients that were homozygous for a CD16A receptor that has a Phe at that position. Given that the Val^{158} allotype is known to bind IgG1 with higher affinity than the Phe^{158} allotype, such data was the first to convincingly implicate FcγR binding as an important part of the mechanism of action for a therapeutic antibody. A subsequent study that compared clinical responses to rituximab in follicular lymphoma patients that varied with respect to both CD16 and CD32 FcγR allotypes implicated both CD16 and CD32 FcγRs as playing a role in responses to rituximab.[40] Although there is evidence that higher antibody dosing could help to compensate for reduced binding to antibody in those patients with a lower-binding allotype of CD16,[41] the above findings helped prompt an acceleration of efforts to enhance the FcγR-mediated effector functions of antibodies.

Choosing an IgG Isotype

Probably the earliest efforts to optimize effector function of therapeutic antibodies were based on simply choosing the desired IgG isotype, wherein recombinant DNA methods were used to fuse the DNA encoding the heavy chain variable region portion of the antibody with DNA encoding the constant region (isotype) of either IgG1, IgG2, IgG3, or IgG4. The IgG1 isotype of human antibodies has long been the choice when immune effector functions such as ADCC and CDC are desired. IgG3 antibodies also show high-affinity binding to FcγRs and are potent activators of CDC, but are problematic for commercial development owing to their propensity to self-associate and form aggregates. IgG1 may be the

choice isotype even when immune effector functions are not believed to be part of the mechanism of action for an antibody. This is because binding to either of the low-affinity FcγRs, as well as triggering of the classic complement pathway, requires a local cluster of antibodies, which may not ever form in the case of soluble antibody/antigen complexes that never expand beyond one antibody molecule binding to two antigen molecules. IgG4 antibodies are viewed by some as lacking immune effector functions when in fact there are data that IgG4s are either just as active or perhaps only 5 or 10-fold less active than their IgG1 counterparts in FcγR-dependent activities[42,43] (our unpublished observations). The variable assessments of how IgG4s compare with IgG1s in Fc-mediated activities can probably be attributed to the range of biologic assays used, because there are indications that the difference between the two IgG isotypes ranges from substantial in the case of low-affinity FcγR binding, to moderate in the case of monomeric IgG binding to high-affinity CD64, to minimal or none in the case of binding assays that involve a mix of cell types in which one expresses cell-surface antigen and the other expresses CD64 FcγR. The clear exception, however, appears to be in vitro CDC activity, in which IgG4s show very little or no activity but IgG1s are highly active.

Any decision to develop an IgG4 antibody should take into consideration the phenomenon of what could be called HL exchange between unrelated IgG4 molecules.[44] Although it has been known for many years that a significant proportion of the molecules in preparations of purified IgG4 monoclonal antibodies lacks disulfide bonds between the two heavy chains, only recently has it been shown that the two HL dimers of an $(HL)_2$ tetrameric IgG4 can dissociate from each other and then reassociate with an HL dimer derived from a different IgG4 molecule. Therefore, whereas the original IgG4 antibody was bivalent and monospecific for its antigen, the process of HL exchange results in a hybrid IgG4 that is monovalent for the original antigen and bispecific. Because most naturally-occurring IgG4 has been reported to be bispecific,[45] it has been suggested that even those IgG4 molecules that do have disulfide bonds between the heavy chains may be susceptible to HL exchange due to conversion of inter-heavy chain bonds to intra-heavy chain bonds by in vivo isomerase enzymes. This whole phenomenon can be avoided for IgG4s by introducing a single Ser to Pro amino acid substitution in the hinge (giving it the same core hinge sequence as IgG1) that enables efficient and stable disulfide bonding between heavy chains.[46]

Amino Acid Sequence Variants

As suggested above, the improved understanding of the downstream events associated with binding to the different FcγRs has led to efforts to enhance antibody effector function by identifying antibody variants that show preferential binding to activating receptors over the inhibiting receptor. By systematically changing each solvent-exposed amino acid in the Fc domain of human IgG1 and studying the effects on binding to different FcγRs, Shields et al[47] identified various mutations that either enhanced affinity for an activating FcγR, or reduced affinity for the inhibiting FcγR, or both. Importantly, enhanced FcγR binding was shown to translate into greater activity in in vitro ADCC assays. Although the increases or decreases in affinity for the different FcγRs was modest in these earliest surveys of mutant IgGs, such data showed that numerous mutations in antibodies did not affect all FcγRs in the same way, and therefore provided optimism that more extensive searches for antibody variants could yield much more potent sequences that confer whichever FcγR binding profile was sought for a particular therapeutic antibody.

Fc Glycan Engineering

A different approach to enhancing antibody effector functions entails optimizing the structure of the asparagine-linked glycan attached in the IgG Fc domain. It has been known for some time that antibodies that have been enzymatically deglycosylated, or genetically mutated so that they do not get glycosylated at that site, have a dramatically reduced affinity for FcγRs. The IgG Fc glycan structure, enveloped to a considerable extent between the two Fc domain protein backbones, apparently plays a critical role in defining the conformation of the FcγR-binding site. More recently it has been learned that the presence of particular glycan structures (glycoforms) can actually increase the affinity of the antibody for particular FcγRs dramatically. For example, compared with IgG antibodies with Fc glycans that have maximal levels of the sugar fucose, IgG antibodies with Fc glycans that lack fucose have been reported to have 50-fold greater affinity for FcγR CD16.[48] The relevance of this increased affinity has been demonstrated in in vitro ADCC assays in which afucosylated antibody has been shown to be 100-fold more effective than a fully fucosylated version of the same antibody at triggering lysis of antigen-expressing target cells by CD16-expressing immune effector cells.[48–50] Another glycan manipulation that has been shown to enhance ADCC activity of antibodies at least 100-fold involves co-expression of antibodies with the enzyme β(1,4)-*N*-acetylglucosaminyltransferase III.[51] This enzyme attaches to the Fc glycan a bisecting N-acetylglucosamine sugar residue, which itself has a beneficial effect on ADCC activity, but it also apparently competes with fucosyltransferase enzyme for Fc glycan, such that less fucose gets attached. Interestingly, the various FcγRs are not all sensitive to the same structural variations, eg, whereas CD16 binding is highly sensitive to fucose content, CD64 binding does not appear to be impacted by fucose content. Hence, glycoform engineering provides another means to optimize the effector function of antibodies, and several companies are pursuing such a strategy by developing host production cells engineered to express the desired glycoforms. It remains to be seen to what extent having optimized Fc glycan structures added to an

antibody already optimized for amino acid sequence will enhance function even further.

The FcγR-Dependent Avidity Effect

Clearly, when it comes to FcγR binding, most attention tends to be focused on defined immune effector functions, such as ADCC. But FcγR binding may also confer a potent avidity effect on the antibody for its antigen, at least in those cases where antigen is on the surface of a cell, or otherwise immobilized, or perhaps in a soluble, polyvalent complex. Those antibody molecules bound to antigen on the surface of one cell and simultaneously bound to FcγR on the surface of a neighboring cell are likely to demonstrate more prolonged binding to antigen than antibody molecules that are not bound to FcγR. This is because FcγR binding serves to keep the antibody molecules in the immediate vicinity even after dissociation from antigen, making it more likely that they will then re-associate with antigen. In addition to enhancing the effective affinity for antigen, depending on the antigen target, such simultaneous FcγR binding may have biologic implications, as has been described for anti-CD3 antibodies that bind T cells.[52,53]

Enhanced Complement Activity

Another well-known immune effector function of IgG antibodies is CDC. The cascade of enzymatic events in the classic pathway of complement activation is triggered by the binding of C1q complement protein to a cluster of IgG1 or IgG3 (or IgM) Fc domains. In the case of therapeutic antibodies bound to tumor cells, the pathway would ideally culminate in the lysis of the tumor cell by the newly-formed membrane attack complex of complement. However, because mammalian cells, including tumor cells, express significant amounts of membrane complement regulatory proteins (mCRP), such as CD46, CD55, and CD59, that can inhibit the complement cascade at specific stages, the contribution of complement-mediated lysis to antibody-triggered cytotoxicity of tumors has been somewhat questionable. Efforts are ongoing to block the complement inhibition effect of these mCRPs by co-treatment with an anti-mCRP blocking antibody in addition to the anti-tumor-specific antibody.[54] Fortunately, recent data suggests that even if complement-mediated tumor cell lysis in response to antibody therapy is minimal, other beneficial effects of progressing at least part way through the complement cascade may be realized through mobilization of cellular inflammatory responses induced by intermediate-stage complement products.[55] Large numbers of complement protein iC3b have been shown to be deposited on tumor targets following rituximab (IgG1) binding,[56] and iC3b molecules on such targets may then bind CD11b/CD18 on macrophages and NK cells to activate CDCC, a killing mechanism distinct from CDC. Consequently, antibody variants that show increased affinity for C1q, to at least initiate the complement cascade regardless of whether the complement membrane-attack complex gets formed in sufficient numbers, are becoming of greater interest.

The ongoing focus to enhance immune effector functions of antibodies through engineering their amino acid sequence and/or their Fc glycan structure holds much promise. Yet it seems likely that more novel structures will eventually be engineered that offer even more advantages, eg, by engaging immune effector cells such as neutrophils that are not recruited by current antibody constructs.

IMMUNOCONJUGATES AS CANCER THERAPEUTICS

Immunoconjugates are a distinct class of therapeutics in oncology. Immunoconjugates are bi-functional molecules that combine the specificity of monoclonal antibodies to tumor antigens with the extraordinary potency of cytotoxic agents. Generally an immunoconjugate consists of three moieties: a specific tumor-targeting antibody or a functional fragment of antibodies such as a nanobody[57]; a cytotoxic agent, which can be a small molecular drug, a protein toxin, or a radioisotope molecule; and a linker, which covalently or non-covalently links the targeting agent and cytotoxic agent together. Immunoconjugates can be classified into three sub-groups: (a) drug-antibody immunoconjugates, if the cytotoxin is a small molecule drug, (b) immunotoxins, if a protein toxin is used as the cytotoxic agent, and (c) radioimmunoconjugates, if the targeting molecule is labeled with a radioisotope. Under certain circumstances drug-antibody immunoconjugates are also called tumor-activated prodrugs (TAP).[58] There are a number of comprehensive reviews of immunoconjugates.[59–61] In this section the concept of immunoconjugates and their current progress as cancer therapeutics will be discussed.

Targeting Molecule

The selection of the ''ideal'' targeting molecule is crucial for delivering a selective cytotoxic agent to cancer cells. Complete sequencing of the human genome and application of proteomic tools in discovery of new cancer biomarkers provide valuable approaches to identify new targets for cancer therapy. The basic concept for identification of tumor-associated antigens and selection of potential therapeutic targets has been discussed in detail.[62–65] A tumor antigen targeted by immunoconjugates should be a cell surface protein with selective expression in tumor tissues (tumor-specific antigen) or at least with high expression levels in tumors relative to normal tissues (selective tumor antigen). The tumor antigen chosen as target would also be one that internalizes after being bound by antibody. Internalization of the antigen-immunoconjugate complex would be followed by intracellular cleavage of the linker, leading to release of active, cytotoxic drug.

A large number of tumor-associated antigens have been selected as targets for immunoconjugates. These include receptor tyrosine kinases such as EGFR and HER2,[66] mucins such as CanAg,[67] integrin $\alpha v\beta 3$,[68] and selectins such as E-selectin.[69] In most cases, because the tumor antigen is likely to be expressed by normal tissues, it

is important to balance the relative selectivity of the targeting molecule with the potency of the agent delivered.

CYTOTOXINS

Many chemotherapeutic agents, bacterial and plant toxins or their derivatives have been conjugated to targeting antibodies. The selection of potent cytotoxic agents is another key factor to successfully develop a potent immunoconjugate. To choose a cytotoxic drug for immunoconjugation, several factors must be kept in mind. First, with current technologies, only 3–10 drug molecules can be linked to an antibody, and thus the ratio of drug to antibody is low. Second, tumor antigen densities on the cell surface are usually between 10^5 and 10^7 molecules per cell. Therefore, the number of binding sites for a particular immunoconjugate may be limited. Third, the efficiency of processes such as endocytosis of antigen-imunoconjugate complexes and drug release from the lysosome is usually not 100%. Thus, it has been hypothesized that only cytotoxins with potencies (as defined by in vitro assays) in the picomolar to nanomolar range are useful in generating immunoconjugates.[70,71] Some potent cytotoxic agents used in immunoconjugates are listed in Table 3, and they include small molecule drugs, protein toxins and radioisotopes.

LINKER

The nature of the linker between the cytotoxic agent and the targeting antibody (or functional fragment of antibodies) dictates the degree of successful delivery and release of cytotoxic agents. Ideal linkers should meet the essential criterion that they are stable in systemic circulation, but specifically cleaved once internalized into cells. Immunoconjugates are macromolecular drugs. Like any other macromolecule, the immunoconjugate will be up taken by cells via an endocytotic pathway such as pinocytosis[72] or clathrin-mediated pathways.[73] There exist alternative routes for the uptake of macromolecules, such as cavaeolae-mediated process,[74] but their roles are still unclear and might be minor. Once taken up by cells, immunoconjugates are first in the endosomal compartment. Then the endocytosed immunoconjugates will be either re-circulated back to the cell surface or further transferred into the acidic (pH 4.5–5.0) lysosomal compartment, which contains enzymes that are able to degrade immunoconjugates and release drugs such as hydrolases, peptidases and thioredoxin enzymes. A variety of linkers have been developed and used in conjugation of antibodies with cytotoxic agents and several are described here.

ACID-LABILE LINKAGES

There is a pH gradient from the extracellular environment to intracellular compartments. The pH is 7.2–7.4 outside of cells, whereas the pH value is 6–6.8 in endosomes and 4.5–5.5 in lysosomes. It has also been reported that the tumor tissues are 0.5–1.0 units more acidic than normal tissues. Acid-labile linkers have been developed based on this information. For example, the anti-tumor drug doxorubicin was coupled to the low-molecular weight protein lysozyme via the acid-sensitive *cis*-aconityl linker.[75] Results have demonstrated that the release of cytotoxic doxorubicin in the bladder can be achieved by acidification of the urine. Another type of acid-sensitive linker is the hydrazone linker, which has been broadly used in a number of drug conjugates, such as doxorubicin, 5-fluorouridine, and vinblastine. Mylotarg, an anti-CD33-calicheamicin conjugate targeting acute myeloid leukemia (AML), is the first drug immunoconjugate approved by FDA, the linker of which contains both a cleavable acylhydrazone bond and a disulphide bond.[76]

SULFHYDRYL LINKAGES

Many bacterial and plant toxins are composed of a toxic enzymatic subunit A covalently linked by a disulfide bond to the binding subunit B, such as *Pseudomonas* exotoxin A, cholera toxin, as well as the plant toxin ricin. A key event in the intracellular activation of these A-B toxins is the reduction of the disulfide bond between subunit A and subunit B.[77] This evidence demonstrates the importance of the disulfide bond in the activation of A-B toxins and also suggests that disulfide linkage is a possible way of releasing drugs from antibodies in intracellular compartments. It has been proposed that cysteine is the physiological reducing agent within endosomal compartment. However, mechanisms of reduction of a disulfide bond within cells remain unclear. A recent review by Saito, et al[78] discussed in detail where and how the disulfide bond in immunoconjugates is reduced upon entering cells. In eukaryotic cells, the ubiquitous thioredoxin system (thioredoxin + thioredoxin reductase)[79] and the glutaredoxin system (glutaredoxin + glutaredoxin reductase)[80] catalyze fast and reversible

TABLE 3. Typical cytotoxic agents used in immunoconjugates

Name	Target	Example	Reference
Auristatin	Microtubule	anti-CD30-Auristatin E	83
Calicheamicin	DNA	anti-CD33-Calicheamicin	110
Doxorubicin	DNA/Telomerase	anti-Lewis^y-Doxorubicin	126
DM1	Microtubule	anti-CanAg-DM1	66
Pseudomonas exdotoxin A	Elongation Factor 2	antiCD22-PE38	127
Iodine-131 (I^{131})	DNA	^{131}I-anti-CD20	128
Yttrium-90 (Y^{90})	DNA	^{90}Y-anti-CD20	129

thio-disulfide exchanges between cysteines in their active site and cysteines of their disulfide substrates using nicotinamide adenine dinucleotide phosphate (NADPH) and glutathione peroxidase (GSH) as a source of reducing equivalents, respectively. The newly discovered gamma-interferon-inducible lysosomal thio reductase (GILT) is the first reducing enzyme identified mainly in the endocytic pathway.[81,82] Unlike other thioreductases GILT has unique characteristics. It does not have the common motif of Cys-Gly—His/Pro-Cys-shared by members of the thioredoxin family in its enzyme catalytic site. The optimal pH for GILT's activity is 4.0–5.5 instead of neutral pH for other thioreductases.[83] The discovery of GILT may provide, at least in part, an explanation how a disulfide bond is broken up in a very acidic environment. Several immunoconjugates in which the antibody and drug are linked by a disulfide linkage have been developed. As mentioned above, the linker for Mylotarg (anti-CD33-calicheamicin) contains a disulphide bond as well as an acid-labile hydrazone bond.[76] Other antibody-drug immunoconjugates at late development stages include Cantuzumab mertansine (human anti-CanAg-DM1), BB10901 (human anti-CD56-DM1), and MLN-2704 (anti-PSMA-DM1).

ENZYME-DEGRADABLE LINKAGES

Enzyme-degradable linkers have also been designed. These linkers often have a peptide sequence that is sensitive to cleavage by lysosomal enzymes[84,85] or tumor-associated enzymes.[86] For example, doxorubicin has been conjugated to anti-Lewis Y monoclonal antibody through linkers consisting of cleavable dipeptides Phe-Lys or Val-Cit. Both conjugates demonstrated rapid and near quantitative doxorubicin release when incubated with either the cysteine protease cathepsin B or in a rat liver lysosomal preparation.[85] These immunoconjugates also demonstrated significant anti-tumor activity against a lung carcinoma expressing Lewis Y-antigen. An anti-CD30 antibody, cAC-10, has been linked with monomethyl auristatin E, a synthetic analog of the natural product dolastatin 10, by a linker containing Val-Cit peptide. This immunoconjugate has been demonstrated to be highly potent and selective against CD30+ tumor cell lines. In SCID mouse xenograft models of anaplastic large cell lymphoma or Hodgkin diseases, cAC-10-auristatin E conjugate was efficacious at doses as low as 1 mg/kg.[84] Considering its maximal tolerated dose is more than 30 mg/kg, it is apparent that cAC-10- auristatin E conjugate possesses a wide therapeutic window.

Efforts have been made to increase drug loading onto antibodies. For example, branched linkers have been developed allowing the loading of multiple drug molecules on a single targeting antibody. This technology leads to enhanced conjugate's potency.[87] To date three immunoconjugates have been approved by the FDA and are marketed, including Mylotarg, Zevalin and Bexxar. All of them are used to treat hematological cancers. This is a landmark for immunoconjugate development.

Enhancing Drug Delivery Through Ligand-Targeted Liposomes

Many cancer therapeutics are potent cytotoxics, but systemic delivery of these drugs results in cytotoxic activity against both the tumor cells and healthy cells throughout the body. Exposure of healthy cells to cytotoxics causes undesirable side effects that limit drug dose and/or dosing frequency, and this ultimately constrains drug efficacy and tumor control. Strategies that increase the therapeutic index of such drugs and reduce their toxicity to the patient are highly desirable. Ligand targeted liposomes (LTL) are one way in which the biodistribution and uptake of a cancer drug can be altered favorably to achieve these goals. LTLs also represent another strategy by which the targeting power of antibodies can help create more effective anti-cancer therapeutics.

Tumors differ in many ways from normal tissues, but one key difference that can be exploited by liposome technologies is that tumor vasculature is defective and leaky. Normal blood vessels are comprised of endothelial cells with tight junctions that do not permit liposomes to extravasate to neighboring tissue. However, the vessels in tumors are permeable, with pores that range from 100–800 nm in diameter. This raised the possibility that small liposomes, on the order of 60–150 nm, could traverse the tumor vasculature and deliver toxic payloads directly to the tumor. In addition, tumors lack lymphatic drainage, so liposomes are not readily cleared from the tumor and can continue to accumulate.

Liposomes are comprised of phospholipid bilayers that enclose an aqueous inner compartment. Usually the inner compartment is formulated to contain a hydrophilic payload, but hydrophobic drugs can be associated with the lipid bilayer. Initial experiments with "naked" liposomes injected intravenously (i.v.) showed that liposomes were cleared within minutes from the bloodstream.[88,89] It was found that serum proteins adsorbed to the liposome surface, which caused leakage and loss of integrity of the liposome.[90–92] In addition, adhesion of serum proteins led to recognition and uptake by the mononuclear phagocyte system (MPS), primarily macrophages in the liver and spleen, which led to rapid liposome clearance. Finally, antibodies may recognize directly the lipid bilayer and facilitate the removal of liposomes from the bloodstream.[93,94]

To address the limitations of naked liposomes, many attempts were made to shield the liposome from the MPS. It was found that the half-life of liposomes could be greatly increased by coating the liposome surface with a hydrophilic carbohydrate or polymer, such as polyethylene glycol (PEG).[95,96] The PEG coating is thought to sterically stabilize the liposomal membrane and resist interaction with serum proteins. PEG-coated liposomes evade recognition by the MPS, and thus their half-life is extended from minutes to many hours, up to 20 hours in mice[97] and 45 hours in humans.[98] These PEG-coated liposomes, known as Stealth liposomes, have shown greater efficacy than free drug in tumor models.

Approved Stealth liposomal drugs include Doxil/Caelyx, Myocet, and Daunosome, and there are many other Stealth liposome drug candidates in clinical trials.[99]

Doxil is an interesting example of the positive impact of formulating a cytotoxic (doxorubicin) in a Stealth liposome. Doxorubicin's half-life in vivo is on the order of minutes, whereas that of Doxil is many hours. More importantly, up to 10% of an injected dose of Doxil accumulates in patients' tumors, which is about 10-fold higher than the accumulation of free drug.[100] The side effect profiles of each drug are also quite different. Patients receiving doxorubicin experience myelosuppression, alopecia and nausea, and there is significant risk of cardiotoxicity with cumulative dosing. The latter side effect ultimately limits administration of the drug, though doxorubicin continues to have anti-tumor activity. In contrast, Doxil's primary dose-limiting side effect is skin swelling and redness (palmar/plantar erythrodyesthesia (PPE)), with stomatitis and nausea also observed, but no cardiotoxicity. The dermatologic side effect occurs because liposomes traverse the lymphatics and accumulate in the skin, in addition to extravasating in the tumor vasculature. The key message is that efficacy of doxorubicin and its side effect profile are improved through the Stealth formulation.

LTLs further refine Stealth liposomes by insertion of a tumor targeting ligand into the lipid bilayer. Stealth liposomes allow drug accumulation and release near tumor cells, but a targeting moiety on the liposome could facilitate internalization and drug release within tumor cells, which could lead to greater efficacy. The most popular ligands include antibodies and antibody fragments (Stealth immunoliposomes), though other ligands are used as well. One concern about LTLs is the potential for development of an immune response to the LTL. Early experiments with whole IgG inserted into Stealth liposomes demonstrated rapid clearance from the bloodstream, which was likely due to recognition of the Fc portion of the antibody by the MPS.[101] When single chain Fv (scFv) or Fab' fragments that lack the Fc were used instead as targeting moieties, the half-life of the Stealth immunoliposome was restored to that of Stealth liposomes. Thus it is possible to design appropriate ligands that allow retention of the desirable half-life shown by Stealth liposomes.

A key question to address is whether the presence of a targeting ligand impacts the biodistribution of the liposome, particularly because most, if not all, ligands will recognize receptors present abundantly on tumor tissue, and at lower levels on normal tissue. Studies have shown that LTLs and Stealth liposomes accumulate to similar levels in tumors,[102,103] indicating that the biodistribution of the drug is determined by the liposome carrier, with minimal influence of the targeting agent. Once the LTL reaches the tumor, however, internalization of the drug can be demonstrated, in contrast to Stealth liposomes that remain on the tumor periphery.[102,103] Thus, this novel drug class exhibits both passive targeting based on the liposome carrier, and active targeting based on the targeting ligand. An additional advantage is that the toxicity of LTLs should be predictable based on the biodistribution of Stealth liposomes.

The way the targeting ligand is inserted into the liposome has an impact on the efficacy of the molecule. Targeting ligands can be attached in two ways to the liposome, either inserted directly into the liposome bilayer, or attached to the distal end of the PEG chain (pendant type). The latter approach makes the targeting ligand more available for interaction with its receptor, and this appears to result in greater binding to target in vivo.[104] Other characteristics of the targeting ligand also impact efficacy. For example, higher valency (30–50 targeting molecules per liposome) appears to enhance binding to tumor in vivo.[104] However, higher avidity may not be an advantage, as high-avidity LTL may bind preferentially to the first tumor cells encountered at the periphery of the tumor (''binding site barrier'' hypothesis[105]). Lower-avidity targeting molecules may enable better penetration of the tumor interior. Finally, studies suggest that the tumor cell itself should express at least 10^4–10^5 copies of the receptor to aid binding and internalization of the LTL.[106,107]

Most importantly, LTLs have shown increased efficacy in preclinical tumor models, as compared with non-targeted Stealth liposomes. For example, LTLs designed with antibody fragments specific for CD19,[107] Her2,[103,106] and B1 integrins,[108] among others, all show significantly enhanced anti-tumor activity compared with non-targeted liposomes. The increased efficacy observed with LTLs in multiple model systems strongly suggests that this advance in liposome technology may have clinical benefit, and it is expected that such novel drugs will soon move into clinical trials.

CONCLUSIONS

Therapeutic antibodies are currently providing clinical benefit to many patients suffering with cancer. Many other antibodies are currently in late stage clinical development, providing optimism that more patients will benefit from antibody therapy in the future. Antibodies marketed and in development have been selected on the basis of their tumor specificity or their mechanism of action, and many have been termed ''targeted therapies'' because they are selective and have marginal effects on non-cancerous cells. Ongoing research to define novel targets and technological approaches should provide us with more effective and tolerable therapies that will help to convert cancer into a manageable and chronic, rather than fatal, disease.

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