
How Does the Immune System Attack Cancer?

An understanding of how immunotherapy may impact tumors begins with a description of the important effector cells and cytokines. Classically, the immune system is considered to have 2 arms: cell-mediated immunity and humoral, or antibody-mediated, immunity. The principal effector cells in the cellular immune response to cancer are cytotoxic T cells and NK cells, whereas the humoral immune response is mediated by antibodies produced by B cells.

Cytotoxic T Lymphocytes

Cytotoxic T lymphocytes (CTL) are CD8⁺ T cells that recognize, via their T cell receptor (TCR), tumor-derived peptides presented on the surface of the tumor cells by major histocompatibility molecules. This can lead to direct destruction of the tumor cells (see below).¹ However, the context in which antigens are recognized is important. The “two-signal” model of T cell recognition of antigen indicates that, in addition to TCR engagement by a cognate antigen-major histocompatibility complex (MHC), the additional interaction of the T cell surface receptor CD28 with CD80 (B7.1) or CD86 (B7.2) on the antigen-presenting cell (APC) is necessary for generation of antigen-specific immune responsiveness. Without this co-stimulation, antigen-specific T cells enter a state of tolerance and possibly apoptosis. Since tumors will often, through a natural selection process, evolve to lose expression of MHC molecules and key co-stimulatory molecules to avoid activating CTL, other so-called “professional” APC, namely dendritic cells (DCs), are a key component of CTL responses to tumors (see below).

NK Cells

Natural killer (NK) cells mediate innate immunity against pathogens and tumors.² They comprise up to 15% of peripheral blood lymphocytes and are found in peripheral tissues, including the liver, peritoneal cavity, and placenta. NK cells use the same killing mechanisms as CTL

(described in detail below) and can secrete similar cytokines to those secreted by CTL. Unlike B and T cells, NK cells do not express a single, invariant receptor molecule. Instead, they express multiple activating and inhibitory receptors, and seemingly it is the synthesis of these signals that determines the functional response of the NK cell. Not all NK cells carry the same group of receptors; distinct groups of receptors are expressed by different NK cells. Unlike T cells, antigen recognition is not via MHC molecules presenting antigen. In fact, MHC class I molecules can inhibit NK cells by interacting with killer-inhibitory receptors (KIR) on the surface of the NK cells. In the absence of MHC class I molecules, the target cell is lysed. Consequently, NK cells are involved in the immune response to tumors, particularly when tumors down-regulate MHC class I expression. In some instances, it seems that NK cells can reject tumor cells expressing NKG2D ligands, despite MHC class I expression.

Cellular immune responses to cancer are mediated by cytotoxic T cells and NK cells. These cells can kill tumors cells through 2 different mechanisms: the Fas-Fas-L pathway, and the perforin-granzyme pathway. Fas-mediated apoptosis starts with the binding of a FasL trimer on the effector cell surface to a Fas trimer on the target cell membrane, which results in the formation of the death-inducing signaling complex (DISC) around the cytoplasmic chain of Fas. DISC formation leads to the sequential interaction of Fas with the adaptor protein, Fas-associated death-domain protein (FADD), through homologous association of their death domains. Subsequently, a chain of events leads to the eventual apoptosis of the target.

Regarding the perforin-granzyme pathway, the pore-forming molecule perforin is stored in cytotoxic granules, together with granzymes. On recognition of a target cell, the granules are released, and perforin monomers insert themselves into the target cell membrane and interact to create pores. These perforin pores can cause osmotic lysis of the target cell and allow granzymes to enter the target cell and induce apoptosis through various downstream effector pathways.

B Cells

B cells recognize tumor antigen via the B cell receptor, which is a monospecific immunoglobulin or antibody expressed on the surface of B cells. Although some large molecules can directly stimulate them, B cells normally receive 2 stimulating events, antigen binding by the antibody receptors and cytokine stimulation from stimulated T helper (Th) cells. Typically, most antigens are first processed by APC and then presented to the B cells. These same APC are also capable of presenting antigen to Th

cells. The stimulated Th cells then release lymphokines that act on “primed” B cells, inducing B cell proliferation and differentiation. Once stimulated, the B cells themselves can function as APC to recruit even more B and T cells to the inflammatory site. Recognition of antigen results in differentiation of the B cell into a plasma cell, which secretes large quantities of antibody.

Antibodies patrol the body and tag invaders for destruction by other cells of the immune system. There are 2 mechanisms of antibody-mediated killing that can potentially impact tumors. Human antibodies, especially IgG1 and IgG3, can direct the killing of tumor cells by antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). ADCC is triggered by an interaction between the Fc region of an antibody that has bound, through its antigen-binding region, to a tumor cell and the Fc receptors (FcRs), particularly FcRI and FcRIII, on immune effector cells such as neutrophils, NK cells, and macrophages. Depending on the type of effector cell involved, the tumor cell is eliminated either by phagocytosis or lysis. In CDC, recruitment of the complement component C1q by IgG bound to the tumor cell surface triggers a proteolytic cascade to activate complement. This can potentially lead to the formation of a membrane attack complex that kills the target cell by rupturing its cell membrane. Alternatively, tumor cell-bound C1q can bind to complement receptors, such as C1qR, CR1 (CD35), and CR3 (CD11b/CD18), on effector cells, such as neutrophils, macrophages, and NK cells. This can also trigger cell-mediated tumor cell lysis or phagocytosis, depending on the type of effector cell.

Dendritic Cells

Although NK cells and CTL are the direct effector cells of the cellular immune response, several other cells facilitate their function. DC play a fundamental role in immunity, and also have a role in tumor immunity.³ DC are found throughout the body, particularly in areas that can be portals of entry for infectious organisms. DC are generally thought to derive from bone marrow precursors and can be found circulating in the bloodstream most likely on the way to peripheral sites (such as the epidermis of the skin, where they are called Langerhans cells). DC function to capture, process, and present antigens from dying tumor cells and traffic to draining lymph nodes where they prime CTL and Th and B cells. DC are therefore sometimes referred to as the “sentinels” of the immune system. DC take up protein antigen by receptor-mediated events and through macropinocytosis. Although exogenous antigens taken up by DCs are typically thought to enter the class II MHC pathway for presentation to

CD4⁺ T cells, some exogenously supplied antigen can be presented by class I MHC molecules to D8⁺ T cells. Therefore, protein antigens taken up by DCs can be used to induce CTL responses and Th responses. DCs are particularly potent at stimulating immune responses because of the high level of expression of both MHC class I (HLA-A,B,C in humans) and MHC class II (HLA-DR,DP,DQ). In addition, DC have a high level of expression of co-stimulatory molecules that provide the second necessary signal for T cell activation in addition to the appropriate peptide within MHC molecules. Several different states of DC maturation exist, based on surface marker expression and function. Immature DC are very efficient at endocytosing antigen and processing the antigenic proteins into short peptides. However, they lack high levels of MHC molecules and co-stimulatory molecules that are required for optimal T cell priming. DC can be “matured” by a variety of agents, including cytokines and other stimuli (eg, interferon- γ [IFN- γ] with CD40L trimer). These “matured” DC are not so efficient at capturing and processing antigen, but have high levels of surface MHC molecules and co-stimulatory molecules (eg, CD80 [B7.1] or CD86 [B7.2]), and secrete cytokines that stimulate T cell function (eg, IL-12). Thus, these matured DC are very potent stimulators of T cell activation. DC also act as APC for B cells and prime B cells. How DC are being used as immunotherapeutic agents has been reviewed extensively.⁴

Th Cells

Th cells are CD4⁺ T lymphocytes that, as the name suggests, provide “help” for other immune cells. Specifically, they secrete cytokines that promote the proliferation and activation of CTL, B cells, DC, and other immune cells. They, too, recognize antigen in the context of peptide presented by MHC class II molecules via their TCR. Again, although tumors can be recognized directly by this mechanism, generally Th cells are primed by DC or B cells acting as APC. Different subsets of Th cells have pronounced differences in their effect on the immune response and will be discussed in greater detail below. An important second signal for B cells has been identified as being provided by CD40L, which is expressed on the helper T cell surface after activation. At this point, the T cell surface alone is strongly stimulatory and will activate even naive B cells.

NKT Cells

NKT cells⁵ are a subset of lymphocytes that express both T cell and NK cell markers. They recognize glycolipid antigen in the context of the

MHC-like molecule CD1d via their semi-invariant surface receptor. NKT cells are an area of intense study and are thought to play a role in biasing the immune response toward Th1 or Th2 imbalance, are implicated in immune surveillance and protection against carcinogenesis, and conversely, a regulatory or immunosuppressive subset of NKT cells has also recently been reported. NKT cells recognize α -GalCer in the context of the MHC-class-I-like molecule CD1. Although the number of NKT cells is small, they can produce large amounts of interleukin-4 (IL-4) or IFN- γ , cytokines that promote humoral or cellular immunity, respectively.

Cytokines

Cytokines are molecules that deliver signals from 1 immune effector to another, link innate and adaptive immunity,⁶ and can be both immunostimulatory or immunosuppressive. In particular, cytokines released at the site of tissue damage from infectious agents and tumors serve as danger signals to the immune system. These cytokines such as tumor necrosis factor- α (TNF- α), CD40 ligand (CD40L), and IFN- γ act, in part, by stimulating the maturation of DC, particularly in appropriate combinations and temporal sequences. When fully matured, DC produce cytokines such as IL-12, IL-15, and IL-18, which drive antigen-specific Th1-type immunity. In turn, Th1 cytokines (IFN- γ , IL-2, TNF- α) appear to be important for the generation of antitumor CTL activity.

IL-12 has pleiotropic effects on a variety of different cell types.⁷ These effects include: 1) stimulation of T and NK cell proliferation, IFN- γ production, and cytolytic activity; 2) stimulation of Th1 development; 3) augmentation of B cell IgG production and proliferation; 4) promotion of myelopoiesis in conjunction with other growth factors; 5) activation of neutrophils; and 6) activation of DC.

Overexpression of IL-15 in transgenic mice has shown that this cytokine can enhance both CTL and NK cell function and augments their antitumor effects.⁸

IFN- γ modulates the host immune response and has direct effects on tumor cells.⁹ Regarding the stimulation of host immunity, IFN- γ has been shown to augment NK cell cytolytic activity, enhance survival and proliferation of CD8⁺ T cells indirectly through the stimulation of IL-15 production, promote DC maturation, stimulate T cell IFN- γ production, and induce the expression of TNF-related apoptosis-inducing ligand (TRAIL) on CD4⁺ and CD8⁺ T cells. IFN- α has also been shown to enhance humoral immunity and promote isotype switching, in part through its effects on DC. IFN- γ can also augment the immunogenicity of tumor cells through the up-regulation of MHC class I expression. In

addition to its immune-mediated and direct antiproliferative effects, IFN- γ has also been shown to promote tumor regression by inhibiting bFGF-dependent angiogenesis.

IL-2, predominantly derived from activated T cells, increases the cytolytic activity of NK and activated T cells. Naïve T cells do not express functional IL-2 receptors, but high affinity receptors are up-regulated on activated T cells, and memory T cells express intermediate affinity receptors. IL-2 induces T cell proliferation and cytokine production, and augments TCR-mediated and TCR-independent cytotoxicity.

TNF- α is a cytokine produced primarily by monocytes and macrophages, but which can also be produced by activated NK and T cells.¹⁰ The main effects of TNF include: 1) the release of prostaglandins, leukotrienes, nitric oxide, and reactive oxygen intermediates; 2) protein induction, including IL-1, TNF, IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN- γ , MHC class I and class II, tissue factor, and adhesion molecules; and 3) cytotoxicity. TNF activates endothelial cells, leading to expression of adhesion molecules, chemokines, and procoagulants. TNF can also induce morphologic changes in endothelial cells that contribute to increased microvascular permeability and to extravasation of neutrophils, lymphocytes, and monocytes. Monocytes/Macrophages and neutrophils are activated by TNF; the effect on neutrophils includes the up-regulation of adhesion molecules, which enhances the ability of neutrophils to stick to activated endothelial cells. Superoxide production by neutrophils and nitric oxide production by monocytes/macrophages contribute to TNF-induced endothelial injury. TNF also stimulates T cell proliferation, enhances the cytotoxic activity of NK cells, and promotes proinflammatory cytokine production and adhesion to endothelial cells by T and NK cells. In addition to these effects on the immune system and endothelial cells, TNF also has direct cytotoxic activity against select transformed cell lines, with cells dying by either necrosis or apoptosis, and this direct cytotoxic effect of TNF can be significantly augmented by combining it with alkylating agents or topoisomerase-II inhibitors.

GM-CSF is produced by a variety of different cell types, including T cells, B cells, NK cells, macrophages, fibroblasts, mast cells, and endothelial cells. Factors capable of inducing GM-CSF production include IL-2 or IL-12 for T and NK cells, LPS for macrophages, and IL-1 or TNF- α for fibroblasts and endothelial cells. In addition to its well-known effects on differentiation of neutrophils and monocytes, GM-CSF also has immunomodulatory effects that include the activation and chemotaxis of monocytes, macrophages, and DC.

Overview of Immunotherapy Strategies

Cancer Vaccines. Cancer vaccines are intended to activate immune effectors in vivo against specific tumor targets. Unlike infectious disease vaccines, cancer vaccines presently are aimed at the eradication of existent tumor. In addition, infectious disease vaccines target highly immunogenic, foreign antigens, whereas cancer vaccines typically target self antigens. Finally, although most infectious disease vaccines approved for clinical use are believed to act through the generation of protective humoral immunity, most experts believe that this will not be the case for cancer vaccines. Instead, the induction of tumor-specific cellular immunity will likely be necessary to induce a clinically effective immune response. A successful cancer vaccine requires an antigen expressed by the tumor and immune effectors that can recognize it as their signal to initiate the cascade of events that lead to tumor destruction. Vaccines must present the antigen in a manner that leads to immune activation instead of anergy, and a vaccine must provide or recruit other sources of cytokines to promote a more vigorous immune response.

Tumor Antigens for Cancer Vaccines. It has been recognized that tumors express antigens that make them appear “foreign” to the immune system, and therefore immunogenic. Although rejection of the tumor is not guaranteed merely because it expresses antigens recognized by the immune system, many tumor antigens have indeed been found to be the target for immune effectors that lyse tumor cells. These antigens may be epitopes on native, surface-expressed proteins or carbohydrates that are recognized by spontaneously present or intentionally activated antibodies, or they may be processed antigens that can be bound within the groove of MHC molecules and presented at the cell surface for recognition by T cells as small fragments of the antigen. What makes a tumor antigen a T cell-targeted, rejection antigen has been amply discussed,¹¹ but in general, the antigen should be presented by MHC molecules, should be recognized by T cells, and should trigger T cells to lyse or cause apoptosis of the tumor. Although it is not known whether all proteins have immunogenic epitopes, it has been possible to induce immune responses in vitro against even unmutated self antigens under the appropriate conditions.

Tumor antigens may be relatively tumor restricted or widely expressed, they may be mutated or overexpressed or aberrantly expressed, and they may be derived from proteins usually expressed on the cell surface or those that are purely intracellular or secreted. Finally, they may be identified or previously unidentified and may be unique to a particular

patient. They may be classified by the type of immune response deployed against them, cellular ($CD4^+$ or $CD8^+$) or humoral. The list of identified tumor antigens grows yearly and includes cancer testis antigens that are expressed in a variety of tumors, but in few normal tissues (eg, MAGE 1,3,4,6,10, GAGE 1,2, BAGE, RAGE, NY-ESO-1, SSX2, SCP1), over-expressed antigens (eg, HER2/neu, CEA, AFP), mutated antigens (eg, p53, ras, BCR-abl), unique antigens for certain tumors such as the melanocyte differentiation antigens (eg, Mart1/Melan A, gp100, tyrosinase) or prostate-expressed antigens (eg, PSA, PSMA), and antigens that are either nonprotein based (eg, gangliosides, carbohydrates) or recognized in a non-MHC restricted fashion (MUC1). The case for and against the use of identified antigens in tumor vaccines is often discussed. In brief, identified antigens permit the development of vaccines that can be produced en masse and bottled for off the shelf use. This reduces costs and from a regulatory standpoint is advantageous because reproducibility can be more readily assured. Furthermore, it is easier to measure the immune response induced. Unfortunately, the actual tumor rejection antigens may vary from person to person and the use of defined antigens does not permit the customization of vaccines appropriate to each individual.

There is also frequent discussion as to whether a single antigen or multiple antigens should be used in a tumor vaccine. The heterogeneity of tumors suggests that targeting a single antigen may not be sufficient to trigger an immune response capable of destroying all tumor cells. Therefore, a vaccine including multiple antigens may be preferred. Nonetheless, it could also be the case that attempting to stimulate immune responses against several antigens at the same time may result in weak responses against the less immunogenic antigens. Fortunately, this seems not to be the case because polyepitope immunizations have generally not been found to weaken the immune response in murine models. In addition, epitope spreading may result in a broader immune response.¹² This occurs when immunization against a small number of antigens ultimately results in the development of immune responses against a wider variety of antigens. This may occur when tumor cell destruction in response to an immune-mediated attack results in the release of more antigens, and induction of new antigen-specific T cells.

Presentation of Antigens in Cancer Vaccines. T cells (both $CD8^+$ cytolytic T cells and $CD4^+$ T cells that provide help to B cells and $CD8^+$ T cells) require multiple signals to ensure activation and not anergy. The first signal is the peptide epitope that can be recognized by the TCR within the groove of the appropriate MHC molecule. The second signal

comes from co-stimulatory molecules such as CD80 and CD86, which bind to CD28 on T cells. The third signal, provided through cytokine secretion, involves skewing of the T cells toward Th1 or Th2 pathways. If the first signal (antigen) is delivered, but the second (co-stimulatory) is not, then anergy can occur. Therefore, the antigen must be presented in such a way that these other signals are also delivered. This can be accomplished by delivering the antigen with an inflammation-inducing adjuvant, or by supplying the co-stimulatory signals in close proximity to the tumor antigen. Operationally, this means mixing the antigen with an adjuvant such as DETOX or QS21 or an inflammatory bacteria such as bacille Calmette Guérin (BCG), conjugating it to an immunogenic protein such as KLH, administering a cytokine such as GM-CSF into the site of the antigen injection, creating plasmids or viral vectors containing genes for the antigen and co-stimulatory molecules, injecting genes for co-stimulatory molecules into the tumor, or delivering antigens loaded into APC such as DC. The response to the antigen may be improved by delivering it by several different methodologies (prime boost). Finally, once an immune response is stimulated, it may be possible to augment it by providing cytokines such as IL-2 or IL-12. Vaccines can supply these cytokines either by encoding them within plasmids or viral vectors, or these cytokines may be supplied exogenously.

Therapeutic Vaccines in Clinical Development

Whole Cell Vaccines. Vaccines derived from tumor cells¹³ were among the first “specific” vaccines tested. The most obvious advantage is that it is neither necessary to have identified the tumor rejection antigens nor to have synthesized them even if they are known. They also provide multiple epitopes that would reduce the likelihood of antigenic escape and provide antigen generally in the form of protein or lengthy peptides that might eliminate the need for HLA restriction. Tumor-based vaccines may be autologous or allogeneic. Autologous tumor has an undeniable appeal since it should contain most of the antigens relevant to the elimination of a particular individual’s tumor and has shown promise in some clinical studies. Complexities involved in obtaining sterile, viable, autologous tumor, though, make this approach cumbersome for widespread use. In addition, resected tumor specimens are also typically heterogeneous in many ways that may be undesirable. They may be composed of a substantial amount of stroma that creates difficulties for disaggregation of the tumor. The primary tumor and metastases may have different antigenic content due to mutations that occur once the tumor disseminates. Allogeneic tumor derived from well-characterized cell lines has

appeal because it can be produced in large quantities from a master cell bank and kept in storage until needed. Allogeneic tumor vaccines, such as CanVaxin, are the furthest along in development. An advantage is that alloimmune responses may contribute to the potency of the vaccine. Complexities affecting allogeneic vaccines involve the extensive need to characterize the cell lines, particularly for the presence of adventitious organisms.

Vaccines based on tumor cells come in several different forms. Initially, whole cancer cells, irradiated to prevent replication, were used. Subsequently, lysates were created by infection with viruses such as vaccinia or Newcastle Disease Virus. Tumors may also be used to produce lysates or extracts by other means. For example, Melacine consists of lysates from melanoma cell lines mixed with DETOX as an adjuvant. Lysates from tumors may also be used to load DC or may be further processed to obtain derivatives such as heat shock proteins, shed antigens, or exosomes. As acellular products, these derivatives are easier to store and administer.

The recognition that tumor cells themselves may have limited immunogenicity prompted the development of strategies for transferring genes that could enhance the immune response. Cytokines studied have included GM-CSF, IL-2, IL-4, IL-12, and others, with IL-2 or GM-CSF being the most effective. It appears that the secretion of the cytokine leads to an inflammatory response, destruction of the immunizing tumor cell with release of tumor antigens, and cross-presentation by DC. There have also been attempts to deliver genes to tumors that provide co-stimulatory molecules such as CD80 or other immunostimulatory molecules such as allogeneic MHC molecules. These strategies also likely involve cross-presentation of tumor antigens. These gene transfer studies initially used autologous tumor and have been tested in clinical trials in prostate cancer, melanoma, and pancreatic cancer and are being commercially pursued as the GVAX vaccines. Because of the unreliability of gene transfer to autologous tumor specimens, this approach is now being extended to allogeneic tumor cell lines. Alternatively, tumor cell may be co-injected with bystander cells such as fibroblasts that secrete the cytokine of interest and cause the same inflammatory response. The advantage of this approach also is that controlled and prolonged local production of cytokine will occur. Other ways to increase tumor cell immunogenicity include treatment with DNP to create haptenated, and therefore more immunogenic antigens. There are ongoing studies with this approach in melanoma and ovarian cancer. The greatest challenge to their use may ultimately be regulatory issues since it is difficult to fully characterize

them for purity and comparability. It is also challenging to develop assays for the potency of these agents or to detect immune responses induced against them.

Peptide Vaccines. Peptide vaccines, as well-defined molecules, are a solution to the regulatory issues described above. They can be easily evaluated for purity and identity, and tested for their potency and comparability. Their development required the discovery of the epitopes against which tumor-infiltrating lymphocyte (TIL) clones reacted. These clones were used to screen cDNA libraries of human tumors to identify which genes encoded the proteins containing epitopes against which the clones reacted. It is also possible to elute peptides from tumor cells or from MHC complexes derived from tumor lysates and separate them by high performance liquid chromatography. Once the amino acid sequences are identified, they can be readily synthesized in large quantities. Assays testing the ability of candidate peptides to bind and stabilize MHC molecules have aided the selection of peptides for further testing. Studies of the sequences of the highest affinity peptides and crystallographic studies of the MHC molecules have also contributed to a greater understanding of which peptides are likely to bind the best. For example, certain “anchor” positions on peptides may be fairly constrained. Knowledge of the immunodominant peptides also allows further manipulations to them to improve their binding to MHC molecules or by improving interactions with and triggering of the TCR.

Peptides are typically MHC class I or II restricted, but may be promiscuous (Pan DR epitope [PADRE]) or non-MHC restricted in some cases (MUC-1). Most of the peptides that have been tested are 9 amino acids long and MHC class I restricted, and in particular, are HLA A1, A2, or A3 restricted, although HLA A24, B1, B35 peptides have been developed. Therefore, there is a need to HLA type patients, and this reduces the number of potential candidates who can receive a particular peptide. This must be considered in clinical trials to which accrual may be severely limited if patients with tumors that express the particular antigen and are of the correct HLA type are few in number.

Peptides are generally administered in 1 of several ways (reviewed by Parmiani and colleagues¹⁴): alone; mixed in an adjuvant such as incomplete Freund's adjuvant; administered with either systemic or injection site cytokines such as IL-2, IL-12, or GM-CSF; or loaded onto DC or other carriers such as dexosomes. An important consideration has been whether class I-restricted, class II-restricted peptides, or both, should be used. If class I peptides alone are used, it is possible that the lack of T cell help will limit any immune response. Despite this concern, immune

responses have been identified when only class I peptides were used.¹⁵ There is a considerable amount still unknown about peptides. First, the best dose remains to be established. Most of the injected peptide probably does not bind directly to in situ APC, but is removed by lymphatics or is degraded before it can be presented. Additions to peptides to direct them to APC or to improve their uptake or presentation such as addition of a lipid tail, as described above, may increase their potency.

Protein Vaccines. To supply multiple epitopes without the need to restrict HLA type, one could administer the full-length tumor antigen protein. For proteins that have oncogenic potential such as HER2/neu, truncated portions of the protein such as the intracellular domain can be used. This approach has had the most success when the tumor antigen is idiotype protein derived from lymphomas or myeloma. These B cell malignancies produce immunoglobulins that are unique to each patient and are not found on any normal tissues in the body since they are produced by somatic mutations. The immunoglobulin idiotype may be purified from the peripheral blood if a monoclonal spike is present or may be generated by recombinant means using genetic material derived from the malignant clone. The protein may then be administered with an adjuvant or cytokine or conjugated to an immunogenic protein such as KLH. Other recombinant proteins have been tested, including MUC1, CEA, HER2/neu ICD, PSA, and KSA. In general, protein vaccines induce antibody responses and lymphoproliferative responses, but CD4 and CD8⁺ antigen-specific immune responses have been observed. Another type of protein vaccine is the idiotype vaccine in which immunoglobulins engineered to contain idiotypic determinants that mimic the structure of antigens are delivered. Again, antibody responses predominate, but T cell responses have been identified as well. Mimics for CEA (CEA-Vac) and GD3 (BEC2) are in advanced clinical trials. In order for T cell responses to be induced, the idiotype is presumably taken up and processed into immunogenic peptides within APC. The major drawback of protein vaccines is that producing proteins is more complicated than producing peptides. In addition, proteins from the production system (for example, *Escherichia coli* bacterial proteins) could contaminate the vaccine and lead to allergic reactions or complicate assessment of the induction of immunity.

Carbohydrate Vaccines. Although typically thought of as vaccines to induce antibodies against infectious organisms, carbohydrate vaccines can induce T cell responses in some cases. Purified gangliosides such as GM2, GD2, or Globo H conjugated to KLH have been administered in clinical trials of melanoma or prostate cancer (reviewed by Livingston¹⁶).

IgM and IgG antibody responses seem to correlate with the clinical outcome. The Theratope vaccine (STn conjugated to KLH) also is a carbohydrate vaccine, but interestingly T cell responses have been demonstrated. This likely occurs when antibodies mediate destruction of tumor cells and the released antigens are taken up, processed, and presented by in situ DC.

Genetic Vaccination. Immunization with genetic material encoding tumor antigens represents a simpler alternative to protein immunizations and offers the possibility of greater T cell responses. Genes may be delivered in plasmids, encoded within viral vector genomes, or as RNA. A typical plasmid used for genetic vaccination includes a promoter such as the Rous sarcoma virus long terminal repeat or the CMV promoter, the gene or genes of interest, a polyadenylation signal to produce stable mRNA, and a selection marker such as the Kanamycin resistance gene. If more than a single gene is encoded, then they are either separated by an internal ribosomal entry site or another promoter. Because the plasmids are derived from bacterial DNA, there are unmethylated CpG repeats throughout the plasmid that contribute to immunogenicity because of the inflammatory response induced by cells of the innate immune system on binding unmethylated CpG containing DNA. It should also be recognized that the selection markers may also contribute to the immunogenicity of the transfected cell if the gene encodes an enzyme foreign to the host. Plasmid DNA is the simplest form of genetic immunization, is easy to manipulate and propagate, and stable enough to be stored lyophilized at room temperature. Unfortunately, they are taken up poorly by mammalian cells, and various manipulations must be performed to use them. For in vivo immunization, particle bombardment or intramuscular injection may be required. Naked plasmid DNA injected intramuscularly results in uptake of the gene by muscle cells and production of the antigen of interest, which is then released during apoptosis or an inflammatory response to the muscle cell. In situ DC take up the antigen, process it further, and present it to T cells (cross priming). Alternatively, following intradermal injection, in situ DC take up the plasmid and transcribe and translate the encoded protein, which is then processed and presented on MHC molecules.

Genetic material delivered by viral vectors has been tested extensively in a variety of tumors. Adenovirus has been among the most extensively tested. There are more than 100 members of the adenovirus family, but serotype 5 is perhaps the best characterized for gene transfer. They are relatively easy to propagate, although not as easy as plasmids. To render them replication defective and increase the amount of available space for

gene insertion, the E1 and E3 regions are generally deleted, although this then requires a complementing cell line for in vitro propagation. These viruses are fairly versatile because of their ability to infect a wide range of cells. An inflammatory response may increase immunogenicity, but antibody responses could render repeat immunizations ineffective. The first orthopox vectors to be developed for immunotherapy were vaccinia, which had been used to eradicate smallpox. Although generally well tolerated with side effects mainly due to the development of a pustule at the injection site, occasional cases of disseminated infection or encephalitis occurred. To reduce the virulence, an attenuated vaccinia (modified vaccinia Ankara MVA) was developed. It can infect mammalian cells and undergo replication, but a packaging defect prevents the generation of infectious virus. Another method for reducing virulence is to use a limited host range virus such as the avian poxviruses. These can infect mammalian cells, but are replication defective in humans. Because the genes are expressed in the cytoplasm, they must be under the control of a poxvirus promoter. Large genes may be inserted into pox vectors by replacing nonessential genes such as the thymidine kinase.

A new viral vector approach involves the alphaviruses, which are + strand RNA viruses with several features that may be relevant to cancer immunotherapy. The replicon consists of 4 genes that encode a replicase with the ability to replicate large amounts of RNA rapidly. By replacing structural protein genes with genes of interest, large amounts of the RNA and ultimately protein for the antigen of interest will be produced. It is possible to take advantage of the alphaviral replicon in 3 ways: as a replication-deficient recombinant viral particle, as naked alphaviral RNA, or as a plasmid that encodes the replicon and also contains a eukaryotic RNA polymerase to allow for expression of the replicon. VEE has tropism for DC, allowing direct targeting to the APC necessary for presenting the antigen of interest. Immunization with these vectors may induce antibody and CTL responses. Lower amounts of alphavirus-based plasmids are required to induce immune responses compared with conventional plasmid vaccines.

A particularly exciting observation made with the use of genetic vaccination has been that greater immune responses may be induced with prime boost strategies with heterologous vectors. Among the most successful have been plasmid DNA, followed by pox vectors and vaccinia, followed by canarypox. The immune response induced against the encoded antigen was much diminished with the reverse strategies. Explanations have included: 1) the low level of expression of plasmid-encoded gene induces small numbers of high affinity T cells that are

boosted in number by the immunogenic viral vector, and 2) the presence of certain viral antigens may deviate the immune response away from the antigen of interest and that certain prime–boost strategies avoid this.

Advantages of genetic vaccinations include: 1) production of large amounts of plasmids or of viral vectors encoding the antigen of interest may be easier than attempting to synthesize large amounts of peptide or protein antigen; 2) some viral vectors more selectively target APC, unlike proteins or peptides that diffuse from the site of injection to be taken up randomly by APC; and 3) other molecules important for stimulating the immune response such as Th epitopes or co-stimulatory molecules may be the antigen of interest.

DC Vaccines. Therapeutic vaccines based on DC loaded with tumor antigens have been of special interest because of the central role DC play in immunity. Although many different types of DC have been described, most clinical trials use DC generated from monocytoïd precursors *ex vivo* whereas a few use DC isolated from peripheral blood.¹⁷ Antigen-loading strategies for DC include those that require uptake by the DC followed by further processing (eg, loading with DNA, viral vectors, mRNA, lysates, proteins, and tumor cells) and those that do not (eg, peptide loading).

Other Ways to Use Vaccines as Antitumor Therapy. It is possible to activate immune responses against other components of the tumor progression cascade without directly targeting the tumor. For example, antibodies may be induced against growth factors required by tumors. For example, the G17-DT vaccine is a fragment of gastrin conjugated to diphtheria toxin. Some colorectal and pancreatic tumors produce gastrin, and are induced to proliferate by a gastrin autocrine loop. Neutralizing the gastrin may slow tumor growth. The tumor vasculature represents another potential target for antigen-specific immune responses. Immunizations may be used to induce immune responses against neovasculature, which could theoretically cause a vasculitis in tumor vasculature with eventual tumor destruction. In summary, there are numerous ways to immunize against malignancies. Future development in this area will ultimately require choosing the candidates with the greatest potential activity so that they may be tested in large randomized studies.

Overview of Cytokine-Based Immunotherapies

IFN- α . IFN- α is used today to treat a variety of hematologic malignancies and solid tumors, including CML, multiple myeloma, hairy cell leukemia, B and T cell lymphomas, melanoma, renal cell cancer, and Kaposi's sarcoma.¹⁸ Direct growth-inhibitory effects of IFN- α have been demonstrated more consistently in CML than in melanoma. In addition to

its direct use, IFN- α enhances the development of antigen-specific immune responses against tumor cells in certain animal models, and this provides the rationale for using IFN- α as a vaccine adjuvant in combination with peptide or DC vaccines. Common toxicities associated with IFN- α therapy include both acute and chronic side effects. Acute toxicities include flu-like symptoms, hair thinning, skin rash, neutropenia, and liver function test abnormalities. Chronic toxicities include fatigue, anorexia, depressed mood, and impaired cognitive functioning. Many of these side effects have been attributed to the release of pro-inflammatory cytokines, including TNF- α , IL-1, IL-2, IL-6, IFN- α , and IP-10.¹⁹

Interleukin-2

Because of its effects on enhancing proliferation and cytolytic activity of T and NK cells, IL-2 has been used as a nonspecific immune stimulant and in conjunction with tumor vaccines. Identification of the lymphocyte subsets involved in the antitumor effect of IL-2 has come from studies involving murine models of sarcoma and melanoma. In mice treated with high-dose IL-2, NK cells appeared to mediate the eradication of micrometastases independent of tumor cell MHC class I expression, whereas T cells mediated the regression of class I-expressing macrometastases.^{20,21} IL-2 appeared to be ineffective against macrometastases devoid of class I antigens. In mice treated with low-dose IL-2, only micrometastases from weakly immunogenic/class I-expressing tumors were eradicated, and this was mediated by T cells. Other experiments showing that the antitumor effect of IL-2 against macrometastases is unaffected in beige mice lacking NK cells but diminished in athymic mice,²² as well as reports demonstrating the ability of IL-2 to potentiate the antitumor effect of adoptively transferred tumor-specific CTL,²³ further implicate T cells as the primary effector cell in IL-2-mediated tumor regression.

The therapeutic efficacy of IL-2 is restricted by dose-limiting toxicities involving multiple organs. Organ damage induced by IL-2 is initiated at the level of the postcapillary endothelium and occurs as a result of 2 pathological processes. These include the development of a vascular leak syndrome, and lymphocytic transendothelial migration and parenchymal infiltration.^{24,25} Neutrophil and lymphocyte activation by IL-2, coupled with endothelial cell activation by TNF- α and IL-1, leads to enhanced neutrophil and lymphocyte adhesion in the microcirculation. Activated neutrophils and lymphocytes injure endothelial cells through the release of proteases and perforins, and this injury is further compounded by nitric oxide, platelet aggregation and microvascular thrombosis, and Fas-mediated apoptosis. Chemokines released by activated lymphocytes and

endothelial cells lead to the further recruitment and infiltration of leukocytes, and organ damage results from hypoperfusion, direct toxic effects of TNF- α and nitric oxide, and immune-mediated cytotoxicity. Although immune activation by IL-2 is likely the primary cause of endothelial cell activation/injury and vascular leak, a recent report demonstrated that a 3 amino acid sequence motif, (x)D(y), present in toxins such as ricin and in IL-2, can bind to and damage endothelial cells.²⁶ This raises the possibility that IL-2 may have a direct effect on endothelial cells that can initiate vascular leak syndrome.

Tumor Necrosis Factor- α

Animal models using tumors such as Meth A-induced sarcoma, which is insensitive in vitro to direct effects of TNF- α , have shown that TNF can induce hemorrhagic necrosis of tumors that have grown large enough to have a microvasculature. These experiments have provided evidence that the host response to TNF plays a role in its antitumor activity. The hemorrhagic necrosis induced by TNF is reduced in nude mice, suggesting that T cells play an important role in the induction of a local inflammatory state and endothelial injury. However, TNF can still induce the regression of certain tumors in nude mice, suggesting that this may be mediated through a direct effect of TNF on tumor cell viability. TNF has not been particularly successful in preventing experimental metastases, underscoring the fact that tumor cells are often insensitive to the direct effect of TNF alone, and micrometastases that have not yet acquired a microvasculature will therefore be able to escape the effects of TNF. Nonetheless, TNF has found a role in isolated perfusion of the liver or limbs to treat malignancies such as melanoma.²⁷ Common toxicities of TNF include fever/chills, anorexia, myalgia, and hypotension. Higher doses can cause a toxicity syndrome that resembles septic shock and includes capillary leak, oliguria, acute renal tubular necrosis, hypoglycemia, hemorrhagic infarction of the gastrointestinal tract, and disseminated intravascular coagulation (DIC). Excessive prostaglandin and thromboxane synthesis, nitric oxide production, and the action of other TNF-induced proinflammatory cytokines, along with the interaction of activated neutrophils, lymphocytes, and platelets with activated endothelial cells, all contribute to the vascular injury that underlies the hypotension and organ failure.²⁸

Interleukin-12

The role of IL-12 in neoplastic diseases has been established in animals through the demonstration that IL-12 can eliminate tumors and prevent

the development of metastases in murine models of melanoma, renal cell cancer, sarcoma, mammary cancer, and bladder cancer.²⁹ In mice, combinations of IL-12 + IL-2 or IL-12 + IL-18 have been more effective than IL-12 alone, suggesting that the synergistic activation of the immune system by these cytokine combinations can have profound effects on the generation of antitumor immunity. Direct cell-mediated tumor killing and antiangiogenic effects are both operative in IL-12-mediated antitumor responses. These activities suggest that IL-12 may have activity alone or along with vaccines. IL-12 has completed phase I studies and will begin phase II studies along with IFN shortly. The toxicity of IL-12 includes flu-like symptoms, stomatitis, nausea, anorexia, fatigue, liver function test abnormalities, transient neutropenia/thrombocytopenia, and anemia.²⁹ Toxicity can be profound when multiple daily doses are administered without a break or without a prior test dose, or when large doses of IL-12 and IL-2 are given concurrently on a daily basis. This can result in a septic shock syndrome, with hypotension, renal failure, hepatic dysfunction, coagulopathy, and gastrointestinal hemorrhage. NK cell activation appears to play an important role in the toxicity from IL-12 or IL-12 + IL-2,³⁰ and although toxicity often correlates with the magnitude of IFN- α production, other factors aside from IFN- α appear to mediate the more severe toxicity observed with the combination of IL-12 + IL-2.

GM-CSF

When used alone at pharmacologic doses, in addition to its well-known effects on neutrophil function and efficacy in preventing febrile neutropenia in patients receiving chemotherapy, GM-CSF has been used for its immunomodulatory effect alone or as an adjuvant with peptide, protein, and DNA vaccines due to its ability to recruit DC. Another frequent use of GM-CSF in immunotherapy has been as part of a cytokine “cocktail” for generating DC ex vivo for vaccines. Tumors genetically engineered to secrete GM-CSF have been used for immunizations. When used in these vaccines, immunization sites were shown to be densely infiltrated with DC, macrophages, T cells, and eosinophils.

Overview of Adoptive Immunotherapy

Adoptive immunotherapy involves removal of an individual’s lymphocytes, generation of tumoricidal immune effector cells, and re-infusion of these effectors into the tumor-bearing host. Although the early attempts at adoptive transfers used lymphokine-activated killer (LAK) cells or T lymphocytes from the resected tumors of melanoma patients, these early approaches yielded low response rates, short response durations, and

severe toxicities associated with the concurrent administration of high doses of IL-2. Similarly, infusions of polyclonal donor T lymphocytes into patients with relapsed leukemia after allogeneic bone marrow transplants induced remissions in the majority of patients, but were associated with severe toxicity from graft-versus-host disease (GVHD). More recently, the identification of target antigens has facilitated the development of adoptive immunotherapy using tumor antigen-specific T cells expanded *ex vivo*. This strategy has yielded encouraging results in patients with post-transplant lymphoma. The infusion of EBV-specific T cells isolated from the bone marrow donor and expanded *in vitro* into patients who underwent allogeneic bone marrow transplant³¹ has been effective in restoring T cell responses to EBV and in inducing the regression of life-threatening EBV-induced post-transplant lymphoma. Several preclinical studies have demonstrated the ability to generate T cell products with potent *in vitro* cytolytic activity. To date, the results with adoptive immunotherapy in other settings have been modest,³²⁻³⁴ but recent experience in melanoma patients³⁵ has rekindled interest in this approach.

Challenges to Immunotherapy

One challenge to adoptive immunotherapy is delivery of insufficient numbers of specific, antitumor CD8⁺ T cells. In mice, eradication of established tumors by adoptively transferred CD8⁺ T cells is clearly dose dependent.³⁶ One way to increase antigen-receptor T cells involves cloning receptors for tumor antigens into T cells so that all the T cells recognize the antigen of interest.³⁷

Tumor-specific T cells may be deleted in the periphery of tumor-bearing hosts,³⁸ become anergic,³⁹ or become unable to “see” antigen presented on tumor cells.⁴⁰ It is also possible that the T cells generated from tumor-bearing hosts do not have sufficient avidity for tumor cells⁴¹ or do not produce the appropriate cytokines or lytic activity.⁴² Studies have also suggested that secretion of local immunosuppressive factors, such as TGF- β 1, IL-10, nitric oxide, and prostaglandin E2, by the tumors contributes to the immune escape.⁴³ Furthermore, they may fail to efficiently infiltrate tumors after administration.⁴⁴ A detailed understanding of the chemokines governing lymphocyte homing⁴⁵ will facilitate the design of effective T cell populations that can infiltrate tumor tissues effectively.

A potential hurdle faced by all forms of antigen-specific immunotherapy is loss of antigen expression by tumors. For example, tumors may down-regulate MHC expression.⁴⁶ In some instances, this can be reversed

by IFN- γ . For tumors that continue to express tumor antigens as native molecules at the cell surface, it may be possible to harness developments in chimeric TCR technology based on grafting T cells with an antigen-specific, recombinant receptor that recognizes antigen as 3-dimensional structures in a non-MHC-restricted fashion.⁴⁷ The antigen-binding domain of the receptor consists of a single-chain antibody fragment (scFv) that is derived from a monoclonal antibody and binds to the tumor antigen expressed on cell surface. The intracellular signaling domain is derived from the cytoplasmic part of the TCR- ζ chain or the Fc receptor for IgE γ chain to induce cellular activation. The recent addition of co-stimulatory molecule CD28 to the chimeric TCR further enhances its utility.⁴⁸

Clinical Application of Immunotherapy for Surgical Malignancies

Melanoma. Melanoma is considered the model for immunotherapy of malignancies because of the well-known ability of modulators of the immune system to induce clinical responses. Although much of the research on the immunotherapy of melanoma has been devoted to nonspecific immune stimulators such as cytokines (see recent reviews^{49,50}) or bacterial derivatives, specific immunotherapy including vaccines and the adoptive transfer of immune effectors aimed at melanoma-associated antigens have garnered increasing attention. This is in part due to the large number of tumor antigens that have been identified in melanomas. These include the differentiation antigens (tyrosinase, gp100/pmel-17, MART-1/Melan-A, TRP-1/gp75, TRP-2, MSH receptor, high molecular weight melanoma antigen, P polypeptide, KU-MEL-1, GM2, GD2, GD3), cancer-testis antigens (MAGE, GAGE, XAGE, MMA families, and NY-ESO-1), and mutated self antigens (CDK4, β -catenin, MUM-1, p15, Caspase-8, CDC27, LDLR/FUT). Although most of the antigens identified in melanomas to date are recognized as short peptides within the MHC class I complex by CD8⁺ T cells,⁵¹ several class II-restricted epitopes have been discovered and some of the epitopes such as the gangliosides GM2, GD2, and GD3 are recognized by antibodies. Furthermore, with the exception of small studies with a chimeric monoclonal antibody (KM871) against GD3⁵² and the immunocytokine (hu14.18 anti-GD2 antibody conjugated with IL-2), most of the specific immunotherapy approaches under development involve vaccines or adoptive transfer of T cells.

Adoptive Cellular Immunotherapy

Adoptive cellular immunotherapy for melanoma began 2 decades ago with nonspecific effectors, such as LAK cells, NK-like cells activated in vitro with IL-2. The most recent incarnation of this in vitro-activation approach is being tested in a phase I trial being conducted at St. Luke's Medical Center, in which peripheral blood mononuclear cells (PBMC) are activated with an anti-CD3 monoclonal antibody, IL-2, and IL-12 for 2 weeks before reinfusion. TILs with autologous tumor-specific cytotoxic activity have also been utilized, but they are still relatively nonspecific. Clones derived from TILs or PBMC of vaccinated patients increases their specificity, but when administered with IL-2 in a phase I study at the National Cancer Institute (NCI) had limited activity.³⁴ Subsequently, this group modulated the host environment by using "immunoablative" chemotherapy to create "space" within the T cell repertoire and demonstrated markedly improved clinical activity.³⁵ Major emphasis is now centering on ways to increase T cell longevity after transfer, including the administration of low-dose IL-2⁵³ and transduction of T cells with IL-2⁵⁴ or antiapoptosis genes such as Bcl-X(L).⁵⁵

Despite these exciting results, there has been concern that the autologous T cells may be anergized against the "self" melanoma antigens, and that the most effective approach would be to adoptively transfer allogeneic T cells to create a graft-versus-tumor response.⁵⁶ This may be done with less toxicity using nonmyeloablative doses of chemotherapy and subsequent allogeneic hematopoietic stem cell transplantation, with or without delivery of allogeneic T cells. Clinical trials of this approach are ongoing at several centers (Fred Hutchinson Cancer Research Center; University of Chicago Cancer Research Center; National Heart, Lung and Blood Institute; Duke University Medical Center).

Specific Active Immunotherapy

Vaccines Containing Tumor Cells or Cell Lysates. Vaccine strategies utilizing whole tumor cells or their lysates have been the most extensively studied in melanoma. Some groups have focused on autologous tumor administered with adjuvant or modified to increase immunogenicity, whereas others have used allogeneic tumor cell lines. The results from these studies are highlighted in Table 1. The study by Dillman and colleagues⁵⁷ demonstrates the complexity of using autologous tumor since they were able to generate short-term autologous tumor cell lines for only 108 of 170 (64%) patients with metastatic melanoma. In a recurring theme, they observed a tumor-specific delayed-type hypersensitivity

TABLE 1. Tumor cell-based vaccine clinical trials

Tumor cell preparation	Adjuvant therapy	n	Study design¹
Allogeneic tumor cell lines (polyvalent melanoma vaccine)	Cimetidine, indomethacin, or low-dose cyclophosphamide	136	RUT
Autologous tumor cell lines	BCG, GM-CSF, IFN- α or IFN- γ (various combinations)	122 (69 vaccinated)	NUT
Autologous tumor cells	BCG, $\pm$ GM-CSF	35 (15 with BCG, 20 with BCG + GM-CSF)	NUT
DNP-modified autologous tumor cells	Cyclophosphamide, BCG	97 (83 evaluable)	NUT
Allogeneic melanoma lysate (Melacine)	Detoxified Freund's adjuvant (DETOX, Corixa Corp, Seattle, WA)	600	MRCT
Allogeneic melanoma lysate (Melacine)	Cyclophosphamide, IFN- α	47 (39 evaluable)	NUI

Abbreviations: DC, dendritic cell(s); DFS, disease-free survival; DNP, dinitro-fluorobenzene (a dinitrophenolic hapten); DTH, delayed-type hypersensitivity; GM-CSF, granulocyte-macrophage colony-stimulating factor; n, number of patients; NED, no evidence of disease; OS, overall survival; OR, overall response; SD, stable disease.

¹Trials: N, nonrandomized; R, randomized; C, controlled; U, uncontrolled; M, multi-center; T, trial.

²Stage is denoted "metastatic" (MET) in cases of metastatic disease not otherwise specified.

³Clinical responses include CR, PR, and minor responses (not mixed responses or disease stabilization).

(DTH) conversion rate of 18 of 44 (41%) and a low objective tumor response, but the overall survival rate was increased for those with DTH reactivity, in particular those who had no evidence of disease at trial entry (median survival 61.4 versus 9.7 months). Similarly, the study by Berd and colleagues⁵⁸ of a haptenated autologous tumor cell vaccine demonstrated that the DTH response to unmodified cells was significantly associated with prolonged survival, as was a clinical response to the vaccine. In addition, this study pointed out that tumor regression required prolonged periods of time to become evident. The study by Leong and colleagues⁵⁹ of an autologous melanoma vaccine plus bacillus BCG is notable for the fact that there was a 20% response rate (10% complete) for those who also received GM-CSF into the injection site, but there were no responders in a second study when GM-CSF was not administered.

TABLE 1. (Continued).

Stage(s) ²	Immunologic responders/total # of subjects evaluated	Clinical responders/total # of subjects ³	Reference
IIIA, IV	13/26 (humoral-autologous tumor) 97/136 (DTH) 33/40 (proliferation)	9/40 evaluable	Morton et al., 1992 ⁶⁰
MET	18/44 (DTH) (Note: + DTH correlated with better survival)	3/26 evaluable	Dillman et al., 1998 ⁵⁷
IV	Not evaluated	4/35 (all in BCG + GM-CSF group)	Leong et al., 1999 ⁵⁹
III or IV unresectable	47% (DTH vs tumor only; 87% vs tumor-DNP)	6/83	Berd et al., 2001 ⁵⁸
II (Node Neg., 1.5–4.0 mm thick)	Not evaluated	No significant difference in relapse-free survival	Sondak et al., 2002 ⁶³
MET	Not evaluated	OR 10%, SD 64%	Vaishampayan et al., 2002 ⁶⁵

Because autologous tumor is difficult to manipulate and standardize, allogeneic tumor cell lines may ultimately be more useful for general use. Morton and colleagues have performed a series of studies with an allogeneic polyvalent tumor vaccine now known as Canvaxin™ (Cancer-Vax, Carlsbad, CA), which consists of a combination of 3 melanoma cell lines that together express most known melanoma antigens. The vaccine has been documented to induce humoral, cellular, and complement-dependent immunity, and improved disease-free survival (DFS) was associated with these responses.^{60–62} Two large phase III multi-center, prospective, randomized controlled trials currently underway are described in Table 2. These trials will test the hypothesis that the polyvalent melanoma vaccine increases DFS or overall survival (OS) in patients no evidence of disease (NED) following surgical resection for stage III, IV, or recurrent melanoma.

Allogeneic tumor cell lysates, particularly the combination referred to as Melacine, have also been tested extensively. A randomized phase III trial recently reported by the Southwest Oncology Group (SWOG) demonstrated no disease-free survival benefit for this approach as an adjuvant therapy in patients with intermediate-thickness, node-negative melanoma,⁶³ although HLA type was subsequently found to impact the outcome.

TABLE 2. Ongoing tumor cell vaccine clinical trials¹

Vaccine	Adjuvant therapy	Trial phase	Stage(s)
Autologous DC/autologous irradiated melanoma cell line	Vaccine administered with GM-CSF	I/II	IV or recurrent
Polyvalent melanoma vaccine (Canvaxin™)	BCG	III	III or recurrent (NED after surgical resection)
Polyvalent melanoma vaccine (Canvaxin™)	BCG	III	IV (NED after surgical resection)
Intra-Lymphatic IFN- α - or IFN- γ -treated autologous or allogeneic, irradiated tumor cells	Cyclophosphamide; GM-CSF	II	High risk of recurrence/metastases

¹See Table 1 for abbreviations.

Specifically, among those who were HLA-A2 positive and/or HLA-C3 positive, the 5-year relapse-free survival for vaccinated patients did exceed that for the control group.⁶⁴ Furthermore, despite a low response rate (10%), there was a high rate of prolonged disease stabilization (64%) when Melacine was administered to patients with metastatic melanoma and followed by IFN- α .⁶⁵ These studies suggest that careful patient selection may be the key to observing activity of these vaccines.

Other methods of using tumor cells as part of the vaccine preparation include DC-melanoma fusions to create hybrids that express class II MHC, CD40, CD54, CD83, CD86, and IL-12,⁶⁶ and pools of shed melanoma antigens from several cultured melanoma lines. The polyvalent melanoma shed antigen vaccine showed a statistically significant increase in the median DFS (1.6 versus 0.6 years, $P < 0.05$) and nonsignificant trend toward increased OS in a small, randomized, placebo-controlled clinical trial of stage III patients.⁶⁷

Several investigators have been interested in enhancing the effectiveness of tumor cell-based vaccines by selecting a more efficacious route of delivery. One method of achieving this goal might be to administer the vaccine directly into the lymphatic system, which could enhance antigen presentation and effector cell activation. Wiseman and colleagues have employed dorsal pedal intralymphatic vaccination of irradiated tumor cells for a variety of malignancies including melanoma, and found that adjuvant administration of IL-2 augmented the humoral immune response.⁶⁸ This group has initiated a phase II trial to evaluate the intralymphatic administration of irradiated tumor cells. In this trial,

TABLE 2. (Continued)

Study design	Projected accrual	Primary outcome variable(s)	Study chair, institution
NUT	30–80	Safety	R. O. Dillman, Hoag Memorial Hospital Presbyterian
RCMT	1118	OS, DFS	D. L. Morton, John Wayne Cancer Institute/NCI
RCMT	670	OS, DFS	D. L. Morton, John Wayne Cancer Institute/NCI
NUT	20–40 (variety of tumor types)	Feasibility, toxicity, clinical and immunologic response	C. L. Wiseman, St. Vincent Medical Center, Los Angeles

autologous or allogeneic tumor cells are also treated with IFN- α or IFN- γ , and adjuvant systemic GM-CSF and cyclophosphamide treatment is administered (Table 2).

Melanoma Protein/Peptide Antigen-Based Methods

Defined-antigen melanoma vaccines containing polypeptide antigens have received intense interest over the past decade and have been evaluated in several completed or ongoing clinical trials (Tables 3 and 4).⁷⁰⁻⁷⁹ Although MHC class I-restricted peptide epitopes have been thought of as the primary target of the melanoma-specific CD8⁺ immune response, the role of MHC class II-restricted epitopes that present antigen to CD4⁺ cells is becoming increasingly clear. Consequently, efforts have broadened to include the search for MHC class II-restricted melanoma peptides or to use proteins that can be processed into a variety of peptides, some of which would be MHC class II restricted and some class I restricted. Most published clinical trials of peptide/protein-based melanoma vaccines have employed 1 to 3 HLA-A2-restricted antigenic peptides previously shown to represent immunodominant peptides of MAA. To improve the effectiveness of these vaccines, the majority of recent or ongoing trials employ some type of vaccine adjuvant, such as incomplete Freund's adjuvant (IFA), lipid agents such as montanide ISA-51 or QS21, or systemic agent(s). Alternatively, peptides have been loaded onto DC and other APC and administered as a cellular vaccine.⁸⁰⁻⁸⁴ Many of these studies have been small, uncontrolled (with respect to vaccine effect), phase I or II trials, increasing the difficulty of

TABLE 3. Peptide and protein-based vaccine studies¹

Antigen(s)	Adjuvant, APC	n	Study design
MART-1 + gp100 + tyrosinase peptides (6 total)	None	6	NUT
MART-1	IFA	23	NUT
gp100 or gp100 (210M) mutant peptide	IFA, $\pm$ IL-2	51 (31 received IL-2)	NUT
MAGE-3.A1 (HLA-A1)	None	39 (25 completed)	NUT
gp100 (210M)	IFA, $\pm$ IL-2, IL-12 or GM-CSF	54	NUT
MART-1	IFA, CRL 1005 (nonionic copolymer)	25	NUT
MAGE-3 + PAN-DR epitope (PADRE) (HLA-A1)	IFA	18	NUT
3 NY-ESO-1 peptides	GM-CSF (if no disease progression by day 50)	9 Melanoma (12 total)	NUT
MAGE-1 + MAGE-3 (HLA-A1); or MART-1, gp100 + tyrosinase (A2)	Autologous DC	14	NUT
MART-1 + gp100 (210M)	Autologous DC, $\pm$ IL-2 (2 pts)	10	NUT
Tyrosinase	+ GM-CSF	18	NUT
MART-1 + MAGE-3 + tyrosinase + gp100	Autologous CD34 ⁺ derived DC	18	NUT
MAGE-3 or MART-1	IL-12, Autologous PBMC	15	NUT
4 codon-161 mutated N-RAS peptides (HLA not an entry criterion)	+ GM-CSF	10	NUT
gp100, or gp100 + helper tetanus peptide, or gp100-helper tetanus peptide fusion	ISA-51 or QS21	22	RUT
Tyrosinase (370D) + gp100 (210M) peptides	Autologous DC	16	NUT
gp100 (210M) + tyrosinase (370D)	IFA, $\pm$ IL-12	48	RUT

¹See Table 1 for abbreviations.Adopted from Weber JS, Aparicio A: Novel immunologic approaches to the management of malignant melanoma. *Curr Opin Oncol* 2001; 13:124–8.

TABLE 3. (Continued)

Stage(s)	Immunologic response no. pos/total (test)	Clinical response, no. pos/total	Reference
II-IV	5/6 (microcytotoxicity)	1/6 (a minor response)	Jaeger et al., 1996 ⁶⁹
IV	5/6 (DTH - Tyrosinase)		
	10/18 (microcytotoxicity)	0/23	Cormier et al., 1997 ⁷⁰
	15/18 (IFN- γ release)		
MET	15/38 (IFN- γ release)	17/51 (13/31 in IL-2 group)	Rosenberg et al., 1998 ⁷¹
III and IV	0/4 tested (microcytotoxicity)	7/25	Marchand et al., 1999 ⁷²
IV	25/54 (IFN- γ release)	6/54 total (all 6 in + IL-2 group)	Rosenberg et al., 1999 ⁷³
High-risk resected IIB, III and IV	10/22 (IFN- γ release)	16/25	Wang et al., 1999 ⁷⁴
	13/25 (DTH) 12/20 (ELISPOT)		
	0/24 (proliferation)		
High-risk resected III or IV	5/14 (microcytotoxicity)	N/A (9/18 disease free at 14 months)	Weber et al., 1999 ⁷⁵
	6/16 (DTH, but 7/16 anergic)		
	2/16 (IFN- γ release)		
	0/16 (proliferation)		
IV	8/9 (DTH)	2/9	Jager et al., 2000 ⁷⁶
	7/9 (microcytotoxicity to any peptide)		
	8/9 (ELISPOT)		
Advanced	4/14 (DTH)	1/13	Mackensen et al., 2000 ⁸⁰
	1/14 (in vitro CTL)		
MET	1/5 tested (IFN- γ release)	1/7 (an immune assay not responder)	Panelli et al., 2000 ⁸¹
MET or high-risk resected	4/5 (ELISPOT)	1/18	Scheibenbogen et al., 2000 ⁷⁷
IV	10/14 (DTH to any of 4 melanoma peptides)	7/17	Banchereau et al., 2001 ⁸²
IV	8/15 (IFN- γ release)	6/15 (all immune assay responders)	Gajewski et al., 2001 ⁸³
II or III	8/10 (DHT)	N/A	Hunger et al., 2001 ⁷⁸
	2/10 (proliferation)		
IIB-IV (high-risk resected)	3/21 tested (ELISPOT, IFN- γ release)	N/A (OS 75% at 4.7 years)	Slingluff et al., 2001 ⁷⁹
	4/21 (DTH)		
IV	3/16 (DTH) 5/16 (IFN- γ release) to either peptide	1/16	Lau et al., 2001 ⁸⁴
III-IV (high-risk resected)	34/40 gp100 (DTH),	N/A	Lee et al., 2001 ⁸⁵
	0/42 tyrosinase (DTH),		
	33/38 (IFN- γ release)		
	37/42 (MHC:peptide tetramer)		

TABLE 4. Ongoing clinical trials with peptide vaccines¹

Antigen(s)	Adjuvant therapy	Trial phase	Stage(s)
Melan-A, MART-1, gp100 antigen, tyrosinase, MAGE-3, NY-ESO-B	DC vs QS21 (plus KLH, flu matrix)	I/II	II, III
gp100, MART-1, tyrosinase peptides	NA17.A2 antigen	III	Nonmetastatic primary, medium/large ocular melanoma
MART-1 peptide loaded autologous DC	None	I/II	IV or recurrent disease
MART-1, gp100, MAGE-3 peptides loaded autologous DC	GM-CSF prior to DC prep; DC also loaded with KLH and treated with IFN- α -2b	I	IV (measurable disease)
gp-100 peptide	ISA-51 (VA), high-dose IL-2	II	IV or recurrent disease
Tyrosinase, gp100, and MART 1 peptides	ISA-51; $\pm$ GM-CSF; $\pm$ IFN- α -2b	II	IV (measurable disease)
Tyrosinase, gp100, MART-1 peptides	GM-CSF	III	III, IV, recurrent disease (NED after excision)
Tyrosinase, gp100, and MART 1 peptides	ISA-51; $\pm$ GM-CSF	I	IV (measurable disease)
Tyrosinase, Melan-A, NY ESO-1a and -1b MAGE-10.A2 peptides	GM-CSF	I	III, IV, recurrent disease
gp100 peptide	ISA-51, low-dose IL-2	II	IV, recurrent disease
Mutated gp100 peptide (plus HPV-16 E7 peptide)	ISA-51	II	I-III with primary tumor at least 1 mm thick
Tyrosinase, gp100, MART-1 peptides	IFA, $\pm$ anti-CTLA-4; IL-12; + alum or GM-CSF	II	IIB, IIC, III, IV (NED after surgical resection)
Tyrosinase, gp100, MART-1 peptides	ISA-51; anti-CTLA-4	I	III, IV (NED after surgical resection)
Tyrosinase, gp100, MART-1 peptides	IFA; $\pm$ anti-CTLA-4; $\pm$ IL-12	I/II	III, IV (NED)
Tyrosinase, gp100, MART-1 peptides	DC treated ex vivo with	II	III, IV
loaded autologous DC	GM-CSF, CAST; systemic IL-2		disease)
TRP-2 peptide	ISA-51; systemic IL-2	II	IV (measurable disease)

TABLE 4. (Continued)

Study design	Projected accrual	Primary outcome variable(s)	Study chair, institution
RUT	50	Immunologic response, toxicity	N. Bhardwaj, Rockefeller University
RCMT	600	Liver metastasis, OS	V. Brichard, EORTC, J. Prause
NUT	18–24	Safety; clinical and immunologic response	J. E. Economou, Jonsson Comprehensive Cancer Center/NCI
NUT	12	Safety/Toxicity immunologic response	J. W. Fay, Baylor University/NCI
RCT	90	Clinical response, OS, toxicity, Ag-specific immune response	D. M. Gustin, Univ. of Illinois/NCI
RCMT	92	Immune response; clinical response, OS, DFS; toxicity	J. M. Kirkwood, ECOG/NCI
RCMT	600	OS, DFS; T cell response; circulating DC frequency	D. H. Lawson, ECOG/NCI
RCT	30	Safety/Toxicity, preliminary efficacy data	S. Markovic, Mayo Clinic Cancer Center (Rochester, MN)/NCI
RCT	20	Safety; clinical and immunological responses	K. P. Papadopoulos, Herbert Irving Comprehensive Cancer Center/NCI
NUT	25–50	Clinical response, DFS	J. D. Roberts, CALGB/NCI
RCT	36	Toxicity, Ag-specific immune response	J. W. Smith, Earle A. Chiles Research Institute/NCI
RCT	60	Immune reactivity	J. S. Weber, Univ. Southern California/Norris Cancer Center/NCI
NUT	18	Safety/Toxicity, Ag-specific T cell/immune response	J. S. Weber, Univ. Southern California/NCI
NUT	72	Safety/Toxicity, preliminary efficacy data	J. S. Weber, Univ. Southern California/Norris Cancer Center/FDA
NUT	21–41	Clinical and immunologic responses, toxicities	J. S. Weber, Univ. Southern California/Norris Cancer Center/FDA California Norris Cancer Center/NCI
RCT	83	Clinical and immunologic response	NS/NCI

TABLE 4. (Continued)

Antigen(s)	Adjuvant therapy	Trial phase	Stage(s)
2 gp100 peptides	ISA-51; Anti-CTLA-4	I	IV (measurable disease)
MHC class I restricted MART-1 and gp100 peptides plus class II gp100 peptide	± Systemic IL-2	II	IV
gp100, tyrosinase peptides	ISA-51; ± systemic IL-2	II	II or III (NED)
MAGE-12 peptide (note: 4 times/week)	ISA-51; ± systemic IL-2	II	IV (measurable/evaluable disease)
tyrosinase or TRP-1 or gp100 peptide	IFA	II	IV (measurable disease)
gp100 peptide	IFA; systemic high-dose IL-2	III	III or IV (measurable disease)
peptide loaded autologous DC	IL-2	I	IV (measurable/evaluable disease)
Modified (high HLA-binding) MART-1 and gp100 peptides	± IL-2	II	IV (measurable disease)
gp100 and MART-1 peptides	ISA-51; ± IL-2	NS (I/II)	IV (ocular melanoma)
Telomerase peptide (up to 4 times/week)	ISA-51; ± IL-2	II	IV (evaluable disease)

Abbreviations: Anti-CTLA-4, MDX-CTLA-4 monoclonal antibody; CAST, *Candida* skin-test antigen; CD40L, CD40 ligand; DC, dendritic cell(s); DFS, disease-free survival; DTH, delayed-type hypersensitivity; ELISPOT, enzyme-linked immunospot assay; EORTC, EORTC Melanoma Cooperative Group; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFA, incomplete Freund's adjuvant; ISA-51, montanide ISA-51 (vaccine adjuvant); N/A, not available; n, number of patients; NED, no evidence of disease; OS, overall survival.

¹Enrolled patients were HLA-A2⁺, except where noted.

²Trials: N, nonrandomized; R, randomized; C, controlled; U, uncontrolled; M, multi-center; T, trial.

³Stage is denoted "metastatic" (MET) in cases of metastatic disease not otherwise specified.

⁴Clinical responses include CR, PR, and minor responses (not mixed responses or disease stabilization).

interpretation of clinical and immunologic responses. Nonetheless, class I-restricted peptide-based vaccine preparations, in conjunction with lipid adjuvants or DC, have met with highly inconsistent and mostly disappointing results. Larger-scale trials are currently underway in hopes of establishing with greater confidence whether there is a benefit of these vaccines. For example, there is an active phase II trial at the NCI in which patients with measurable metastatic melanoma are vaccinated with

TABLE 4. (Continued)

Study design²	Projected accrual	Primary outcome variable(s)	Study chair or P.I., group/sponsor
NUT	41	Safety/Toxicity, clinical and immunologic response	NS/NCI
NUT	75	Safety/Toxicity, clinical and immunologic response	NS/NCI
NUT	291	Safety/Toxicity, clinical and immunologic response	NS/NCI
RCT	112 (variety of tumor types)	Safety/Toxicity, clinical and immunologic response	NS/NCI
NUT	457	Safety/Toxicity, clinical and immunologic response	NS/NCI
RCMT	185	Safety/Toxicity, clinical and immunologic response	NS/NCI
NUDT	42	immunologic response, toxicities	NS/NCI
NUT	103	Clinical and immunologic response	NS/NCI
NUT	300	Safety/Toxicity, clinical and immunologic response	NS/NCI
RUT	162 (variety of tumor types)	Safety/Toxicity, clinical and immunologic response	NS/NCI

tyrosinase, TRP-1, or gp100 peptide in IFA and are being monitored for evidence of a clinical and/or immunologic response. This study is expected to accrue over 400 patients. A phase II trial at the Earle A. Chiles Research Institute is examining vaccination of stage I to III patients (with melanomas greater than 1 mm in thickness) using a vaccine containing mutated gp100 plus human papillomavirus-16 E7 peptide helper epitope in ISA-51 adjuvant. In an effort to further augment antitumor immunity and to decrease the probability of tumor escape through the antigen-loss mechanism, there has been an increasing interest in vaccines employing a larger number of MAA epitopes. Bhardwaj and colleagues at Rockefeller University have initiated a randomized trial in which 6 MAA are either loaded onto DC or administered with QS21 adjuvant in patients with stage II or III melanoma.

Several actively enrolling studies are examining the hypothesis that adjuvant cytokine treatment will enhance the antitumor efficacy of peptide-based vaccines. A phase II multi-center trial of patients with

metastatic or recurrent melanoma is being directed by the University of Illinois. In this study, patients are randomized to receive a gp100-based vaccine in ISA-51, followed by high-dose IL-2 according to 1 of 3 schedules. As another example of this strategy, a phase II gp100-based vaccine emulsified in ISA-51 with low-dose IL-2 in patients with metastatic or recurrent melanoma is also being performed through the Cancer and Leukemia Group-B (CALG-B). Finally, a randomized, controlled multi-center phase III trial sponsored by the NCI is evaluating the clinical and immunologic response to gp100 peptide with ISA-51 in conjunction with high-dose IL-2 versus IL-2 alone for patients with stage III or IV melanoma.

Lee and colleagues have examined the role of adjuvant systemic IL-12 therapy in conjunction with mutated gp100 and tyrosinase peptides in IFA for patients with high-risk resected stage III and IV melanoma.⁸⁵ Thirty-four of 40 patients had DTH responses to gp100 (but none to tyrosinase), and most patients had immunologic evidence of antigen-specific immunity, as evidenced by IFN- γ ELISA and MHC-peptide tetramer analysis. IL-12 was well tolerated and appeared to warrant further study in larger, randomized trials.

A prospective, randomized, controlled, multi-center phase III trial is being directed by Eastern Cooperative Oncology Group (ECOG) to examine the benefit of vaccination with multiple peptide epitopes in conjunction with GM-CSF as an immune adjuvant. This trial will involve immunization of patients with stage III or IV or recurrent melanoma with tyrosinase, gp100, and MART-1 peptides combined with systemic GM-CSF treatment and is expected to accrue 600 patients. Those patients who are HLA-A2 negative are also enrolled in the trial, but are only randomized to the GM-CSF or placebo group. This study is well designed with proper controls to establish the benefit of both the multiepitope vaccine preparation and adjuvant GM-CSF. Another study being overseen by ECOG is a phase II trial of tyrosinase, gp100, and MART-1 peptides in conjunction with GM-CSF and/or IFN- α -2b. In this multi-center trial, patients with metastatic melanoma are randomized to receive 1 or both adjuvant cytokines.

Increased affinity of a peptide to a particular MHC complex is often associated with increased ability to stimulate peptide-specific T cell immunity on binding to the cognate TCR. Immunodominant peptide epitopes of MAA may be further modified so as to increase affinity to its appropriate MHC complex, and several of these have been shown to induce potent antigen-specific T cell responses in preclinical studies. Whether these changes will be translated into increased efficacy in

melanoma vaccines is unknown. To address this hypothesis, investigators at the NCI are conducting a phase II trial of vaccines containing MART-1 and gp100 peptides modified so that they exhibit increased binding affinity to HLA-A*0201 complexes. Patients with metastatic melanoma receive the peptides alone or peptides in conjunction with high-dose IL-2.

An intense area of investigation has focused on providing appropriate T cell help and co-stimulatory signals in melanoma vaccines. One solution recently developed is to construct a single molecule that includes the class I antigenic peptide epitope, as well as both a T cell helper epitope and an adjuvant moiety. To achieve this, Le Gal and colleagues created a lipopeptide construct in which MART-1(27–35) is covalently linked to a tetanus toxoid helper epitope TT830–843 and a lipid tail.⁸⁶ Using a transgenic HLA-A2 mouse model, the authors showed that this novel lipopeptide construct could induce a strong anti-MART-1-specific CTL response without the need for a separate adjuvant. The authors anticipate testing this unique lipopeptide in a clinical trial. Another strategy reported by Weber and colleagues involves the coadministration of a pAn-DR T cell helper peptide Epitope (PADRE).⁷⁵ A phase II trial at the NCI is examining the combination of MHC class I and class II peptides in a single vaccine. This vaccine includes both a gp100 class I and class II peptide and a MART-1 class I peptide; for nonresponders, additional cycles are administered in conjunction with systemic IL-2. The use of peptide-loaded DC-based vaccines represents an alternative method of providing critical co-stimulatory signals as well as T cell help. Vaccination with autologous DC loaded with MHC class II-restricted melanoma antigens in patients with metastatic melanoma has been shown to induce antigen-specific Th1-type T cell responses.⁸⁷ Whether DC will ultimately be a necessary component of peptide-based melanoma vaccines, as opposed to cell-free preparations of peptide antigens plus adjuvant agents, is unknown at this time.

Another potential problem associated with melanoma vaccines is a suboptimal dosing schedule. Since the vast majority of protocols have examined vaccine administration on a weekly to monthly basis, yet are ineffective, 1 hypothesis is that vaccination must occur on a much more frequent basis, such as several times per week. Clinical trials being conducted at the NCI are examining dosing regimens as frequent as 4 times per week. For example, 1 study is enrolling patients with MAGE-12-expressing tumors including melanoma into a vaccine program in which patients are randomized to receive injections of MAGE-12 peptide in ISA-51 adjuvant 4 times per week either every week or every 3 weeks

for up to 24 cycles. Those patients who do not exhibit tumor regression are candidates for systemic IL-2 treatment.

Although activation of T cell co-stimulatory molecules such as CD28 and CD40 promotes antigen-specific T cell activation and cytokine production, some co-stimulatory signals are actually inhibitory. For example, cytotoxic lymphocyte-associated molecule-4 (CTLA-4) is a TCR whose ligands are B7.1 and B7.2 on APC. This protein is related to the TCR CD28, whose engagement provides a proper co-stimulation for T cell activation when its TCR is bound to a cognate peptide-MHC complex. In contrast to the function of CD28, CTLA-4 engagement provides inhibitory co-stimulatory signals and produces a state of specific T cell tolerance or anergy.⁸⁸ There are several active phase I to II trials examining the value of blocking CTLA-4 monoclonal antibodies in the context of melanoma peptide-based vaccines. All these trials employ 2 or more distinct peptides, as well as vaccine adjuvants. In addition, in an effort to establish a proper milieu for the development of effective Th1 immunity, 2 of these further incorporate IL-12 treatment. Despite these efforts to promote Th1 responses, however, the magnitude of this response could be blunted if CD4⁺CD25⁺ regulatory T cells were activated. Depletion of CD25⁺ regulatory T cells along with an antitumor vaccine produce rejection of the murine melanoma B16.⁸⁹

Vaccines Based on Antigenic Epitope Mimicry

Anti-idiotypic vaccines have been tested in a limited fashion in melanoma. A phase IB study showed evidence for generation of specific humoral immunity in 12 of 13 patients who received anti-idiotypic monoclonal antibodies (Melimmune I and Melimmune II) to human high molecular weight proteoglycan.⁹⁰ Interestingly, sera from these patients neither had direct tumoricidal activity nor blocked binding of the primary antibody. However, the T cell proliferation assays demonstrated tumor-specific immunity in all patients. In another phase I trial, 20 patients with advanced melanoma were vaccinated with an anti-idiotypic antibody generated to mimic N-glycolyl-containing gangliosides, and 16 of 17 patients who completed at least 4 doses were found to have specific antibodies.⁹¹ These early results suggest that further investigation into this strategy, possibly focusing on a different antigen or a combination of antigens, is warranted.

An alternative approach to the generation of vaccines that mimic a tumor antigen epitope is the use of phage display technology. In this method, large numbers of bacteriophage displaying random peptide sequences are screened according to binding affinity to a target of interest

until a small number of candidate phage are selected. An antibody to the human high molecular weight melanoma-associated antigen (HMW-MAA) was used to screen a phage display library and has identified candidate peptides that mimic HMW-MAA epitopes.⁹² This approach has the advantage of being able to generate novel peptide antigens mimicking a variety of melanoma-associated antigens. In addition, investigators are hopeful that it will induce both humoral and cellular antitumor immunity, with the potential for an associated increase in vaccine efficacy. This method has not yet been tested in clinical trials.

Carbohydrate Antigen-Based Vaccines

Tumor-associated carbohydrate antigens include gangliosides (eg, GM2, GD2, and GD3), glycolipids, and glycoproteins. Although congeners of this group of antigens are typically poorly immunogenic T cell-independent antigens, several of them are expressed on melanomas and have been targeted by immunologic therapies. In a phase II trial (E2695), the ganglioside antigen GM2 plus adjuvant KLH/QS21 was administered in conjunction with high-dose IFN- α -2b to 107 resected stage IIB and III melanoma patients.⁹³ The treatment was well tolerated, and IFN- α -2b treatment at least appeared to have no adverse effect on the development of humoral immune response to the vaccine. Unfortunately, in a large, randomized, controlled trial (E-1694) of 880 resected stage IIB and III melanoma patients, this preparation yielded a lower DFS and OS than high-dose IFN- α -2b treatment and was consequently closed early after an interim analysis.⁹⁴ Another randomized controlled trial evaluated treatment of stage III patients rendered NED following surgery with GM2/BCG vaccine (58 patients) versus BCG adjuvant alone (64 patients); all patients received cyclophosphamide.⁹⁵ The investigators found that most patients developed GM2-specific antibodies, and that DFS and OS were increased in these patients; however, DFS and OS were not improved in the GM2/BCG as a whole. More recently, the European Organization for Research and Treatment of Cancer (EORTC) opened a phase III randomized, controlled multi-center trial (EORTC 18,961) in which stage II melanoma patients are treated with a vaccine consisting of GM2-KLH conjugate with QS-21 (an adjuvant) versus observation.⁹⁶ This study is projected to accrue to 1300 patients.

Heat-Shock Protein-Based Vaccines

Because heat-shock proteins (HSP) act as molecular chaperones for a wide variety of polypeptides, including melanoma peptides, and HSP-peptide complexes isolated from autologous tumors have been used as

TABLE 5. Cytokine gene modified tumor cell vaccines¹

Vaccine preparation	Adjuvant therapy	n	Study design ¹
Autologous tumor cells retrovirally transduced with IFN- γ gene	None	20 (13 completed)	NUT
Autologous tumor cells infected with adenovirus-GM-CSF gene construct	None	9	NUT
Vero (monkey fibroblast) stably transfected with hIL-2 cDNA	None	28	RUMT

¹See Table 1 for abbreviations.

Ongoing trials (from www.ClinicalTrials.gov).

vehicles to generate antitumor immunity in animal models, they have been tested in melanoma patients. In a preliminary study of patients with stage IV melanoma, autologous, tumor-derived heat-shock protein gp96-peptide complexes ((HSPPC-96, Oncophage; Antigenics, Woburn, MA) were generated from 42 of 64 patients. The 4 weekly immunizations were well tolerated. Of 28 patients with measurable disease, 2 had a complete response (CR) and 3 had stable disease (SD). ELISPOT assays on peripheral blood mononuclear cells demonstrated increased numbers of melanoma-specific T cells following immunization in 11 of 23 patients.⁹⁷ An active phase III randomized, controlled, multi-center study is examining whether treatment of metastatic melanoma patients with HSPPC-96 yields a survival benefit compared with IL-2 and/or dacarbazine/Temozolomide treatment.

Exosome-Based Melanoma Vaccines

DC-derived exosomes, derivatives of late-stage endosomes that contain abundant MHC molecules, have recently entered clinical trials. A phase I study of dexosomes purified from DC supernatants and loaded with MAGE-3A.1 and MAGE-3.DP04 was performed in 12 patients with stage III and IV melanoma; all patients had progressive disease before enrollment.⁹⁸ One patient with stage III melanoma achieved SD that lasted greater than 11 months. A second patient experienced a mixed response with regression of subcutaneous (s.c.) disease and progression of pulmonary disease. There were no toxicities referable to the immunizations. Based on these data, a phase II study in patients with stage IV melanoma is being initiated.

TABLE 5. .(Continued)

Stage(s)²	Immunologic response no. pos/total (test)	Clinical response, no. pos/total³	Reference
III or IV	8/13 (humoral)	4/13	Abdel-Wahab, 1997 ⁹⁹
Locally advanced, unresectable or MET	3/9 (DTH), 5/7 (microcytotoxicity)	0/9	Kusumoto, et al., 2001 ¹⁰¹
MET	3/28 humoral, no Th1 response (plasma and tumor cytokine expression)	0/28	Rochlitz, 2002 ¹⁰³

Gene Therapy-Based Vaccines

Gene modification of tumor cells before administering them as a vaccine is a rational strategy for increasing the immunogenicity of tumor cell vaccines. Gene modification of autologous melanoma cells by retroviral transduction of the IFN- γ gene is an effective method of providing Th1 cytokines, as well as up-regulating class I and class II MHC on the tumor cells.^{99,100} This strategy has been shown to be feasible and safe in phase I clinical trials and has induced humoral immunity in the majority of patients completing the vaccination schedule (8 of 13 patients) (Table 5).^{99,100} Kusumoto¹⁰¹ attempted to immunize melanoma patients with autologous tumor engineered to secrete GM-CSF by use of an adenoviral vector encoding GM-CSF. The complexity of this approach was demonstrated by the fact that only 9 of 30 patients could actually receive therapy. These patients received intradermal and s.c. doses of 2 to 10 million cells from 2 to 10 times, every 4 to 8 weeks. In patients who received 10 vaccinations CTL activity in the peripheral blood increased in 5 of 6 patients evaluated. Another group has examined the role of IL-12 gene-modified autologous melanoma cells in preclinical investigations and a phase I clinical trial.¹⁰²

Intratumoral administration of gene-modified cells has also been attempted as a means to direct an immune response directly to the tumor. In a phase II study, patients with metastatic melanoma received Vero cells (a monkey fibroblast cell line) engineered to secrete IL-2 at 2 dose levels intratumorally on days 1, 3, and 5 every 4 weeks for 4 cycles, and every 8 weeks thereafter, until evidence of progressive disease. SD was noted in

6 (43%) and 8 patients (57%) at the 5×10^5 and the 5×10^6 dose levels, respectively. Interestingly, there was a trend toward an increase in intratumoral Th2 cytokine (IL-4 and/or IL-10) levels, which raises the question of whether this approach would be desirable given the prevailing opinion that Th1 responses are preferable.¹⁰³

Gene modification of DC has also been investigated as a means of providing optimal tumor antigen presentation. Transduction of human DC with a retroviral construct containing the gp100 gene resulted in expression of multiple class I-restricted antigenic epitopes to which antigen-specific T cells were raised.¹⁰⁴ More surprising, however, was the ability to cultivate T cells specific for a novel class II-restricted epitope, the minimal determinant of which was gp100 (174–190). These results confirm the feasibility of generating a polyvalent DC-based vaccine with both class I- and class II-restricted melanoma antigenic epitopes, properties that make this an attractive strategy for use in clinical trials.

DNA and RNA derived from tumor tissue have also been used as a source of melanoma antigens for genetically modifying DC. A randomized, phase I trial being conducted by H. F. Seigler and colleagues is currently underway at Duke University Medical Center, in which patients with metastatic melanoma will be randomized to receive a vaccine consisting of melanoma RNA-pulsed DC with or without low-dose IFN- α -2b. This study will address the question of the feasibility of this approach for the treatment of melanoma patients, establish its safety profile, and evaluate the immunologic and preliminary clinical responses to this vaccine strategy.

DC may also be modified by the use of viral vectors. A recent report described leukoderma in 3 metastatic melanoma patients treated with an adenovirus-gp100/MART-1-infected DC vaccine preparation in their early results with this strategy.¹⁰⁵ The investigators were encouraged by this finding, although all 3 patients experienced progression of disease under treatment, indicating that there was no clinically significant antitumor effect.

It has also been possible to employ genetic vaccination directly without ex vivo gene transfer. Plasmid DNA encoding immunostimulatory molecules may be injected directly intratumorally. Allovectin-7 (a plasmid DNA encoding the genes HLA-B7 and β 2-microglobulin complexed with a cationic lipid mixture, DMRIE/DOPE) has been administered intratumorally to 52 patients with metastatic melanoma in a phase II study. Regression of the injected lesion was observed in 18% of patients, including 1 complete response, 3 partial responses, and 5 minor responses. An overall response rate of 4% (2 partial responses) was

documented, and 9 patients (18%) maintained SD for at least 11 weeks.¹⁰⁶ Further phase II studies are ongoing with this agent, including testing with a formulation with a higher concentration of plasmid DNA.

A plasmid vector-based system was used to deliver a polyvalent gp100 and TRP-2 DNA vaccine by IM or intranasal injection in a murine model of melanoma with evidence for induction of antigen-specific and antitumor immunity.¹⁰⁷ A similar strategy has been translated into a clinical trial that is currently open for enrollment. A phase I study being conducted by ImClone Systems (Somerville, NJ) and the Memorial Sloan Kettering Cancer Center is examining the safety and efficacy of direct IM administration of gp75 DNA in patients with metastatic melanoma. CTL ImmunoTherapies Corporation (Chatsworth, CA) is currently sponsoring a multi-center, phase I/II dose-escalation study of a vaccine containing a plasmid vector with a MART-1 gene construct. In this trial, the DNA vaccine will be administered intranodally by continuous infusion, potentially increasing the immunogenicity of the vaccine.

Viral vectors are increasingly being used for the expression of MAA or co-stimulatory molecules. A randomized, controlled phase III trial directed by the NCI is currently evaluating the effectiveness of a fowlpox-gp100(209 to 217; 210 mol/L) construct in IFA plus high-dose IL-2 compared with high-dose IL-2 treatment alone in patients with metastatic melanoma. A potential problem with vaccine strategies that do not provide necessary co-stimulatory signals, whether in the form of cytokines or APC, is that antigen presentation may be inadequate. In another study sponsored by the NCI, investigators are evaluating the IM or IV vaccination of metastatic melanoma patients with a fowlpox-gp100(209 to 218; 210 mol/L) construct in which the mutant gp100 peptide sequence is preceded by an endoplasmic reticulum insertion sequence. It is hoped that the addition of the signal sequence will enhance peptide presentation by APC. Another promising strategy employs a recombinant vaccinia viral vector-TRICOM construct, which encodes 3 co-stimulatory molecule transgenes (B7.1, LFA-1, and ICAM-1) in an effort to promote antigen presentation. This has been delivered in an intratumoral fashion in a phase I trial,¹⁰⁸ and continues to be evaluated in an ongoing trial for recurrent or metastatic melanoma. Several actively enrolling gene therapy-based melanoma vaccine trials are listed in Table 6.

Another obstacle that may limit the effectiveness of viral vector-based vaccines is the generation of neutralizing antiviral antibodies. Administration of heterologous prime and boost viral constructs should decrease the formation of neutralizing antibodies and therefore enhance vaccine efficacy. In a phase II NCI-sponsored trial, patients with metastatic

TABLE 6.. Plasmid and viral vector vaccines

Vaccine preparation	Adjuvant Trial therapy phase	Stage(s)	Study Projected design¹ accrual
Intra-nodal infusion of plasmid DNA vaccine containing MART-1	None	I/II	IV
IM gp75 (TRP-1) DNA vaccine	None	I	III, IV
MART-1 adenovirus transduced DC	None	I/II	II–IV, recurrent
Vaccinia-TRICOM ⁴	None	I	IV, recurrent (measurable, unresectable disease)
Fowlpox-ER insertion sequence-gp100	Systemic IL-2	II	IV (measurable disease)
Fowlpox-tyrosinase or vaccinia-tyrosinase	± Low- or high-dose systemic IL-2	II	IV (measurable disease)

Ongoing trials from (www.ClinicalTrials.gov). Abbreviations: DC, dendritic cell(s); DTH, delayed-type hypersensitivity; ELISPOT, enzyme-linked immunospot; ER, endoplasmic reticulum; GM-CSF, granulocyte-macrophage colony-stimulating factor; n, number of patients; NS, not stated.

¹Trials: N, nonrandomized; R, randomized; C, controlled; U, uncontrolled; M, multi-center; T, trial.

²Stage is denoted “metastatic” (MET) in cases of metastatic disease not otherwise specified.

³Clinical responses include CR, PR, and minor responses (not mixed responses or disease stabilization).

⁴The TRICOM construct consists of genes encoding CD80 (B7.1), ICAM-1, and LFA-3.

melanoma are being treated with this heterologous prime-boost protocol. The vaccines consist of recombinant fowlpox or vaccinia virus encoding the tyrosinase transgene, and are administered by IM injection alone, with low-dose IL-2, or with high-dose IL-2.

Immunotherapy of Renal Cell Carcinoma

Renal cell carcinoma (RCC), along with melanoma, represents the prototypical malignancy for the application of immunotherapy. Although immunotherapy has been thought of as an adjunct to standard therapies for most solid malignancies, the relative insensitivity of RCC to chemotherapy, the occurrence of spontaneous regression,¹⁰⁹ and complete remissions observed in some patients treated with nonspecific immunotherapy have led to a focus on immunotherapy as the major therapeutic modality for RCC. Much of the history of immunotherapy for the

TABLE 6. (Continued)

Study design¹	Projected accrual	Primary outcome variable(s)	Study chair or P.I., group or sponsor
NMDT	6–18	Safety/Toxicity, clinical and immunologic response	Barbara Hickingbottom, CTL ImmunoTherapies Corporation
NDT	24	Safety, feasibility; clinical and immunologic response	Jedd Wolchok, MSKCC Memorial Hospital, ImClone Systems
NDT	6–36	Safety; clinical and immunologic response	J. E. Economou, Jonsson Comprehensive Cancer Center/NCI
NDT	3–12	Safety/Toxicity; clinical and immunologic response	H. L. Kaufman, Albert Einstein Cancer Research Center/NCI
NUT	65	Clinical and immunologic response	NS/NCI
RUT	73	Clinical and immunologic response	NS/NCI

treatment of RCC has involved nonspecific immunotherapy, but more recently, the discovery of tumor antigens expressed by RCC has opened the possibility that antigen-specific immunity may be applied to this malignancy.

Role of Nephrectomy in the Application of Immunotherapy

Nephrectomy may be necessary to control pain and bleeding in the minority of patients, and may be necessary to obtain tumor tissue to be used in the manufacture of a RCC vaccine and/or to obtain tissue to serve as targets in assays to test the activity of the immune response induced by immunization. More recently, it has become apparent that early nephrectomy may potentiate the effectiveness of immunotherapy. The data supporting this notion have come from several types of studies. First, large tumor bulk is immunosuppressive either due to interference with various aspects of immunity or serving as a site where T cells are trapped. Improvement in immunologic parameters including cell-mediated immunity has been observed in some patients with metastatic disease following nephrectomy.¹¹⁰⁻¹¹² Second, the primary tumor rarely, if ever, responds to immunotherapy.^{113,114} Third, responses of systemic disease seem to be lower when the primary tumor is left in place.¹¹⁴ Fourth, 2 clinical trials have now shown better median survival for patients treated with IFN- α

following nephrectomy compared with those treated with IFN- α who did not undergo nephrectomy.^{113,115} In the SWOG 8949 study,¹¹³ the median survival was 11.1 versus 8.1 months, favoring the patients undergoing nephrectomy. The difference in median survival between the 2 groups was independent of other factors such as performance status, metastatic site, and the presence or absence of a measurable metastatic lesion. In the EORTC 30947 study,¹¹⁵ the median survival was 17 months for the patients undergoing nephrectomy versus 7 months for those who did not. How the removal of the primary tumor actually improves the outcome (eg, by removal of a large fraction of inhibitory molecules, by removing a site of sequestration of T cells, or by some other effect on the immune system) is still not entirely known. It is also not known if this phenomenon is unique to renal cell carcinoma or could occur in other malignancies as well. Therefore, we currently advocate nephrectomy for good performance status patients with metastatic disease in whom the procedure can be performed with low morbidity. One argument against nephrectomy in the past was that some patients could not receive cytokines postoperatively because of surgical or tumor-related complications. However, this only occurred in 2% of patients in the SWOG study. For the rare patient for whom postoperative healing may be an issue, a trial of immunotherapy followed by resection if there is at least stabilization of disease may be considered. There are even rare instances in which metastatic disease will regress following removal of the primary tumor.

Unfortunately, immunotherapy with IFN- α has not been shown to provide a survival benefit when administered as an adjuvant to nephrectomy in patients with completely resectable renal tumors,¹¹⁶ and it is unknown whether immunotherapy can improve the outcome of patients with completely resected metastatic disease. The Cytokine Working Group is currently testing whether high-dose IL-2 may be effective in the adjuvant setting in a phase III trial that randomizes patients with completely resected stage III or IV disease to either observation or a single course of high-dose IL-2.

Nonspecific Active Immunotherapy with Cytokines

RCC has been treated predominantly with either IL-2 or IFN- α , but IL-12, IL-4, IL-6, and GM-CSF have also been studied. IL-2 has been administered in a variety of schedules, including high-dose bolus (600,000-720,000 U/kg IV every 8 hours for up to 14 doses),¹¹⁷ low-dose bolus (60,000-72,000 U/kg IV every 8 hours for up to 14 doses),¹¹⁸ low-dose s.c.,¹¹⁹ high-dose continuous infusion,¹²⁰ combined with

IFN^{121,122} and chemotherapy,¹²³⁻¹²⁶ inhaled,¹²⁷ or combined with LAK or TIL cells.¹²⁸ Reports by Fisher and colleagues¹¹⁷ and Rosenberg and colleagues,¹²⁹ each of which retrospectively analyzed the outcome of more than 200 patients with metastatic RCC treated with high-dose bolus IL-2, showed major response rates of 15% to 19%, with a roughly equal number of CRs and PRs. Both of these studies showed that CRs to high-dose bolus IL-2 were durable, with 70% to 80% remaining free of relapse at 10 years. Relapses after 20 months were very rare in patients with a CR. This demonstration of the durability of response to high-dose bolus IL-2 led to FDA approval in 1992 for the treatment of metastatic renal cell cancer. No other IL-2 regimens have exhibited this sort of response durability in RCC. A large, randomized trial reported by Negrier and colleagues showed that continuous infusion of high-dose IL-2 is ineffective, and although the addition of IFN- α to IL-2 in that study improved the response rate, the responses were of short duration and did not impact on survival.¹²¹

Whether high-dose bolus IL-2 is truly superior to low-dose IL-2 remains unclear. An ongoing study comparing HD bolus IL-2, low-dose bolus IL-2, and s.c. IL-2 conducted by the Surgery Branch of the NCI has not been completed, but preliminary results showed high-dose intravenous, low-dose intravenous, and s.c. outpatient rIL-2 regimens had response rates of 16%, 4%, and 11%, respectively.¹¹⁸ Although responses to high-dose IL-2 seemed to be more durable at the time of the preliminary analysis, no firm conclusions can be drawn until the study is completed. A Cytokine Working Group trial of high-dose bolus IL-2 versus low-dose s.c. IL-2 (5MIU/m²) plus IFN showed a greater overall response rate (25% versus 13%) and significantly higher CR rate for the high-dose arm.¹³⁰ Longer follow-up is needed to determine whether overall survival and/or 3-year progression-free survival are superior in the high-dose arm. For additional reviews, the reader is referred to the articles by Atkins¹³¹ and Margolin.¹³² At present, it is clear that select patients with metastatic renal cell cancer, including those with good performance status, clear cell histology, prior nephrectomy, slowly progressing disease, and no significant cardiac or pulmonary disorders, should be considered for high-dose bolus IL-2 in light of its proven ability to induce durable responses. There may still be a role for lower doses of IL-2 as an adjuvant to more specific immunotherapies.¹³³ An alternative form of IL-2 that binds preferentially to high affinity IL-2 receptors (BAY 50 to 4798)¹³⁴ is now in phase I studies to determine whether it may be as effective as standard IL-2, but induce less toxicity. A pegylated form of

IL-2 has been administered in a phase I study in combination with standard IL-2,¹³⁵ and a liposomal form has been developed as well.¹³⁶

IFN- α also has nonspecific effects in terms of stimulating immune effectors, but may also have direct effects on RCC¹³⁷ or antiangiogenic effects.¹³⁸ Phase III randomized studies have demonstrated a survival benefit for IFN- α . A combination of vinblastine and IFN had a better response rate (16.5% versus 2.5%) and overall survival (67.6 versus 37.8 weeks) than vinblastine alone.¹³⁹ IFN- α was associated with a better median survival (9 versus 6.75 months) than medroxyprogesterone.^{140,141} Unfortunately, negative results came from a study of IFN- α , IL-2, and tamoxifen versus tamoxifen alone, which showed no survival difference.¹⁴² Attempts to add other drugs to IFN have had variable results. The combination of 13-cis-retinoic acid plus IFN- α had a longer duration of response (33 months) compared with IFN- α alone (22 months), but there was no improvement in survival and the combination arm had a poorer quality of life.¹⁴³ A recent study of a combination of IFN- α with thalidomide versus IFN- α (ECOG E2898) has not been reported yet, although there has been concern about exacerbated neurologic toxicity from this combination.¹⁴⁴ As with IL-2, other formulations of IFN- α are in development. Pegylated forms of IFN- α -2a (Pegasys) and IFN- α -2b (PEG-Intron) that have a greater area under the curve (AUC) than standard IFN- α have been tested in phase I studies,^{145,146} and in previously untreated patients with RCC have response rates of 14% to 19%, but have the advantage of less frequent dosing. The Cytokine Working Group is currently testing the safety and efficacy of PEG-Intron combined with low-dose IL-2 in a phase I/II trial in patients with metastatic RCC. Other cytokines have been tested or are under development. IFN- γ was not better than placebo,¹⁰⁹ IL-4 had minimal activity,¹⁴⁷ and a combination of IL-6 and GM-CSF was not recommended for further development.¹⁴⁸ Of greater interest is the possible role of IL-12, which has shown activity in RCC when administered chronically as a twice-weekly IV injection.¹⁴⁹ A current phase I trial is examining whether the addition of low-dose IL-2 to this schedule of IL-12 has activity in RCC and melanoma.¹⁵⁰

Specific Active Immunotherapy: Vaccines

The experience with vaccinations for the treatment of RCC is limited to date, but may ultimately offer a more precise method for activating tumor antigen-specific immunity. In a phase I study of autologous tumor modified with the gene encoding the co-stimulatory molecule CD80, 4 of 19 responses were observed, although IL-2 was also given with the

vaccine, making it impossible to determine what portion of the responses could be attributed to the vaccine.¹⁵¹ In a study of GM-CSF-transduced tumor cells, immune responses indicated by positive DTH results were observed, although only 1 patient, who exhibited the strongest DTH response, experienced an objective response.¹⁵² Subcellular products of tumor cells may also be used in vaccines. Autologous tumor mRNA may be used to load DC for use as a vaccine.¹⁵³ A phase I study at our institution demonstrated the feasibility of this approach and induction of renal tumor specific immune responses in the majority of the immunized patients (J. Vieweg, paper submitted). Tumor lysate-loaded DC have been demonstrated to induce proliferative T cell responses against renal tumor antigens.¹⁵⁴ Of 12 subjects, 1 partial response and 5 patients with SD were observed. In another study of RCC lysate-loaded DC, 1 of 3 patients immunized had SD.¹⁵⁵ One SD was reported. Vaccination with fusions of autologous tumor cells and allogeneic DC has been shown to induce disease regression in 40% of a small cohort of 17 patients with metastatic renal cell cancer¹⁵⁶ (although this study has been the focus of critical re-review). Further studies are underway to determine whether this success is reproducible and to examine the efficacy of fusions made from autologous tumor cells and autologous DC. Finally, although few defined tumor antigens have been described for RCC, peptides corresponding to a mutation in the von Hippel-Lindau (VHL) gene have been described. Individuals with inherited mutations of the VHL gene are predisposed to renal cell carcinoma, and retinal and central nervous system (CNS) hemangioblastomas and pheochromocytomas, but at least one half of RCC have sporadic mutations in this gene. An ongoing study is assessing the feasibility of administering a peptide corresponding to a VHL mutation conjugated to the carrier molecule keyhole limpet hemocyanin (KLH) to patients with RCC. Epitopes have been found within the RCC-associated G250 protein that can be recognized by CD4⁺ and CD8⁺ T cells¹⁵⁷ and an epitope has been found that is derived from alternative open reading frames. Finally, an epitope within an alternative open reading frame of the intestinal carboxyl esterase mRNA appears to be presented by RCC and can be recognized by RCC-reactive TILs.¹⁵⁸

Passive Immunotherapy

Administration of cells with cytolytic activity directly into the tumor-bearing host is a more direct method of directing an immune response against tumors compared with vaccine strategies. A considerable amount of effort has gone into developing cellular therapies for RCC, first with LAK and then with TILs, which were thought to have greater tumor

specificity. Unfortunately, neither approach has been shown to be beneficial in large randomized studies.^{128,159-161} This is not entirely surprising for LAK cells, which are not tumor-specific and do not traffic particularly efficiently toward tumors. TILs should have included CTL clones that could recognize tumor-presented antigens. Nonetheless, they could not be grown in every individual, and it is possible that by the time the tumor is discovered, the TILs that are remaining are those that are not particularly tumor reactive. It is also possible that even with highly antigen-specific T cells, tumor-secreted molecules inhibit the activity of the cells that do enter the tumor microenvironment. To counter the failure of autologous T cells to be activated against in situ tumor, several strategies have been proposed. First, it is possible to genetically program T cells to recognize antigens on RCC. One approach used transfer of genes encoding a single chain FvG250 antibody chimeric receptor, specific for a RCC tumor-associated antigen.¹⁶² T cell proliferation and activation occur after binding of the fusion receptor to RCC expressing G250. A second approach uses in vivo stimulation and ex vivo proliferation of T cells for adoptive transfer. In particular, autologous tumor cells modified by gene transfer ex vivo to express the alloantigen HLA-B7 were used to immunize patients, and then the tumor vaccine draining lymph node lymphocytes were harvested and expanded ex vivo with anti-CD3 and IL-2.¹⁶³ The expanded T cells could then be adoptively transferred. Unfortunately, no objective tumor regressions were observed, suggesting that further modifications such as in vitro antigen stimulation be considered. A third approach is nonmyeloablative allogeneic stem cell transplantation. In this procedure, immunosuppressive doses of chemotherapy are administered, and then stem cells mobilized from a matched donor are infused. It is assumed that minor HLA antigen mismatches will contribute to a graft versus tumor effect. In the first fully reported study, 3 CRs and 7 PRs were observed in 19 patients with RCC refractory to IL-2.¹⁶⁴ Responses correlated with the development of graft versus host disease, and were seen only in RCC patients with the clear cell histology. In the second reported study, 4 of 15 patients had PR, but there were 4 treatment-related deaths.¹⁶⁵ A particularly appealing aspect of this approach is the ability to administer donor lymphocyte infusions following the transplant to augment the immune response. Further modifications to the donor leukocyte infusions (DLI) to increase their tumor specificity and decrease host specificity will likely be needed to improve the activity of this approach. In addition, this approach is only for the minority of patients who have an adequate match and who do not have rapidly progressive disease. The ability to use cord blood or matched unrelated

donors in these approaches would increase the number of patients to whom this approach may be applied.

Although the cellular arm of the immune system has received the most attention for the immunotherapy of RCC, antibody-based therapies have a role in other malignancies and may have a similar role in RCC in the future. Antibodies, unlabeled or conjugated to ^{131}I , that recognize the RCC antigen G250 have been tested in preliminary studies.^{166,167} Chimeric G250 antibody mediates antibody-dependent cellular cytotoxicity (ADCC), and this activity is enhanced with IL-2.¹⁶⁸ Antibodies targeting epidermal growth factor receptor (EGFR)¹⁶⁹ may also induce ADCC in addition to interfering with activation of tumor proliferation mediated by EGFR. Immunocytokines in which an antibody is conjugated to an immunostimulatory cytokine may also be able to direct cell-mediated immune responses to tumors. Other antibodies have been developed that interfere with molecules involved in the development, progression, or metastasis of RCC, such as the vascular endothelial growth factor (VEGF).

Immunologic Therapy for Breast Cancer

Breast Cancer Antigens. Immunotherapy of breast cancer, as for other malignancies, relies on the presence of antigens that are recognizable by immune effectors. The best known is human epidermal growth factor receptor-2 (HER2), which contributes importantly to oncogenic transformation through dysregulated intracellular signaling cascades. In normal cells, up to 20,000 to 50,000 copies of the HER2 receptor are present on the plasma membrane in a tissue-specific manner. However, tumor cells that overexpress HER2 may have up to 2 million copies of the receptor on their surface, making the receptor an attractive target for immune-based therapies.¹⁷⁰ Overexpression of HER2 in breast cancers is usually secondary to gene amplification—10 or more copies may be present in the amplified state.¹⁷¹ The structure of HER2 in malignant cells is the same as that seen in normal cells; no mutated forms of HER2 have been identified in human breast cancer. Overexpression of HER2 may be found in 20% to 30% of invasive breast cancers, and up to 50% of inflammatory breast carcinomas. Higher rates of HER2 expression are seen in ductal carcinoma in situ (up to 50% to 60% of cases). Almost all comedo-type carcinomas overexpress HER2 as well.¹⁷² HER2 overexpression in malignancy appears to be stable; the HER2 status of tumor at sites of local and distant metastases is the same as the primary cancer. Alluding to a role for HER2 in immunotherapy, specific immune responses against the HER2 protein can be detected occasionally in patients with breast cancer.

Up to 10% of patients develop circulating antibodies against HER2,¹⁷³ and both CTL¹⁷⁴ and Th cell responses¹⁷⁵ can be detected against HER2-overexpressing cancers. Nevertheless, the frequency, degree, and significance of these endogenous immune responses targeting HER2 remain unclear.

MUC1, a member of a family of heavily glycosylated mucin proteins found on the surface of epithelial cells, is more highly expressed on breast carcinomas as compared with normal tissue,¹⁷⁶ and importantly, changes in the glycosylation pattern of MUC1 increase the specificity and immunogenicity of this antigen.¹⁷⁷ In breast cancers specifically, a reduction in O-glycosylation of MUC1 results in greater exposure of core protein epitopes to the immune system.¹⁷⁸ Moreover, changes in the activity of glycosyltransferase activity cause flawed production of oligosaccharides lacking the multi-branched saccharide structure of normal breast cells. When sialylated, these abnormally exposed carbohydrate structures form sialyl-Tn antigens that themselves appear to be immunogenic. It has been established that MUC1 peptides can be processed and presented in conjunction with the MHC complex to the immune system for recognition¹⁷⁹ and that CTL bearing specificity for MUC1 can arise *de novo* in patients with cancer.¹⁸⁰ MUC1 can also be recognized by T cells in a non-HLA-restricted fashion.

Other identified antigens expressed by breast cancers include CEA (up to 50%), members of the MAGE family of proteins (particularly MAGE-1 and MAGE-3),¹⁸¹ prostate-specific antigen (PSA) and prostate-specific membrane antigen,^{182,183} SART (squamous antigen-rejecting tumor) antigens (particularly SART1 and SART3) produced by the majority of breast cancer cells,¹⁸⁴ and p53. Approximately 20% to 25% of breast cancers show mutations in the p53 tumor-suppressor gene, although there is substantial heterogeneity in the frequency and pattern of mutations among various geographically and racially diverse cohorts of women.¹⁸⁵ p53 mutations tend to correspond with the loss of estrogen receptor expression in more aggressive breast tumors.¹⁸⁶ Interestingly, antibodies directed against p53 can be found circulating in the sera of approximately 23% of breast cancer patients whose tumors exhibit mutant p53 expression,¹⁸⁷ suggesting a potential role for this protein as a target for directed immunotherapy against malignancy. However, since p53 is an intracellular protein, its ability to induce cytotoxic immunity is dependent on binding and presentation by MHC molecules during protein degradation. The generation of clinically significant immune responses against breast cancer cells producing mutant p53 remains to be evaluated. In addition, p53 is a protein expressed in normal cells, and mutant p53 epitopes can

vary greatly between individual cancers. Even so, vaccine strategies intended to induce immunity to mutant p53 are currently being investigated.

Challenges in Developing Immunotherapy for Breast Cancer

It has long been recognized that breast tumors are comprised of a diverse population of malignant cells, and correspondingly the expression of antigenic targets by breast cancers is also heterogeneous. The heterogeneous expression of breast cancer antigens may be traced to the genomic instability of neoplastic cells, and may limit the therapeutic effectiveness of immunotherapies for breast cancer that target a single or limited repertoire of tumor cell markers. Several series have documented the varied phenotypic profile of human breast cancer cells. In patients with poor prognosis inflammatory breast carcinomas, mucin and carcinoembryonic antigen expression varies in up to 20% of cases; HER2 overexpression is noted in 63% of cases.¹⁸⁸ Indeed, even the pattern of antigen expression by micrometastatic breast cancer cells to the bone marrow of patients cannot necessarily be deduced by phenotypic analysis of the primary tumor. Single cancer cells isolated from the bone marrow of breast cancer patients individually demonstrate heterogeneous expression of the tumor-associated cell-surface antigens HER2 (87%), CO17-A (78%), MUC-1 (79%), and Lewis^Y (79%), with further variability seen in the coexpression of these molecules by individual cells.¹⁸⁹ The intercellular heterogeneity of solid breast tumors and metastatic foci remains a significant challenge for immunotherapeutic strategies.

Even when target antigens are expressed, it appears that breast malignancies often employ several mechanisms to escape identification and destruction by immune surveillance. Many breast cancer antigens (HER2, CEA, etc.) are self proteins, albeit proteins expressed at higher levels than found in normal tissues. Rather than inducing immune reactivity, such self antigens may instead promote tolerance to malignant tumors. In addition, studies of the expression of major histocompatibility antigens (HLA class I and HLA-DR class II) on breast tumor cells demonstrate that fewer than 50% of tumors have high expression of MHC class I and class II antigens as compared with normal tissues.¹⁹⁰ Many advanced breast cancers have decreased or even absent cell-surface major histocompatibility complex class I.¹⁹¹ Failure to express MHC molecules by tumor cells portends the lack of antigen presentation, and a subsequent inability for CTL to recognize and destroy aberrant cells. Nonetheless, although cytotoxic T cells cannot recognize malignant cells that com-

pletely lack MHC class I expression, antibody-mediated therapies are not necessarily compromised by loss of MHC class I. In addition, tumor cells that lack MHC class I expression may also undergo destruction through other immune effector cells, since loss of class I appears to promote NK cell cytotoxic activities.¹⁹²

Breast tumor cells also appear capable of modulating the expression of antigens and antigenic epitopes to escape immune destruction. Cytotoxic T cells with both specificity and activity against MUC1 breast cancer antigens can be isolated from the peripheral blood of patients with breast cancer, indicating that these effector lymphocytes have encountered and subsequently been “primed” against MUC1. However, when ex vivo cytotoxic assays are performed using these MUC1-specific T cells against autologous tumor, variable degrees of target cell death are observed. Autologous tumor cells resistant to cell lysis were observed to have lost the expression of MUC1, or had altered the structure and antigenicity of glycoprotein, rendering them resistant to killing by cytotoxic lymphocytes.¹⁹³ This study suggests that despite initial antigen expression capable of inducing a population of specific effector T cells, breast cancer cells may then alter the pattern and degree of antigen production, allowing the tumor to “hide” from the immune system.

Despite immune system recognition of specific antigens, tumors as well may release soluble antigen “decoys” that can often neutralize the biologic effects of immune effector elements. For example, the extracellular domain of HER2 can be cleaved proteolytically from the intact oncoprotein, and soluble HER2 may often be found circulating in the sera of patients with HER2-overexpressing malignancies. In experimental systems, soluble HER2 is capable of blocking both the antiproliferative effects and antibody-dependent cell-mediated cytotoxicity effects of anti-HER2 monoclonal antibodies against HER2-overexpressing target cells.¹⁹⁴ MUC1 epithelial mucin is also shed by cancer cells and may be detected in the serum of patients; the effect of circulating MUC1 on immune responsiveness currently remains unclear, although there are suggestions that shed mucins possess immunosuppressive characteristics.¹⁹⁵

Finally, it appears that breast tumors possess the ability to induce a general suppressive effect on immune effector cells and the immune system as a whole, protecting the tumor while it grows and spreads. One proposed explanation for this lack of immune responsiveness is the production of soluble immunosuppressive factors such as transforming growth factor β , TNF- α , IL-6, IL-10, and histamine by breast cancer cells that may create a “protected zone” around the tumor.¹⁹⁶

Immunity and Immune Therapy Strategies Against Breast Cancer

Adoptive Immunotherapy with Natural Killer (NK) Cells. Human NK cells purified from peripheral blood mononuclear cells and subsequently expanded in the presence of IL-2 exhibit in vivo cytolytic activity against BT-20 breast cancer cell line spheroids (containing approximately 10×10^3 cells). Furthermore, on infusion into nude mice, certain populations of NK cells show in vivo activity against a xenograft model of human gastric and head and neck malignancies.¹⁹⁷

Much of the human breast cancer experience with NK cell adoptive immunotherapy has been in the context of high-dose chemotherapy with autologous stem cell support. The high rate of relapse following autologous transplantation for poor prognosis breast cancer has motivated interest in identifying novel therapies capable of eradicating residual tumor following the high-dose procedure. In a series of 43 women with breast cancer receiving high-dose therapy, the s.c. administration of IL-2 to stimulate the NK cell population during granulocyte colony-stimulating factor (G-CSF) mobilization of peripheral blood hematopoietic progenitor cells was well tolerated. Although the percentage of CD34⁺ stem cells decreased with IL-2 treatment, no adverse effects were observed in neutrophil or platelet engraftment times. Furthermore, IL-2 therapy increased the percentage of NK cells in the mobilized graft; collected mononuclear cells demonstrated increased in vitro activity against the MCF-7 breast tumor cell line. Unfortunately, the enhanced NK cell number in the autologous peripheral stem cell graft was sustained for only 2 to 3 weeks following re-infusion into the patients.¹⁹⁸ In another series of 15 human subjects who had undergone high-dose chemotherapy with autologous hematopoietic stem cell support, immediate posttransplant coinfusion of autologous NK cells with the activating cytokine IL-2 was well tolerated by all recipients, and did not appear to affect engraftment adversely.¹⁹⁹ Although this small study could not remark on the efficacy of NK cell therapy to improve outcomes for breast cancer patients, it does suggest a potential role for immune therapies in eradicating residual tumor after chemotherapy treatment. NK cells may represent a subset of immune effector cells with potential utility in adoptive immunotherapy for breast cancer.

Tumor-Infiltrating Lymphocytes

Although most insights into the biologic characteristics and activity of TILs have been derived from studies of patients with melanoma,

published reports have also described TILs isolated from breast cancers.²⁰⁰ TILs isolated and grown out from breast cancer biopsy specimens generally represent CD3⁺ lymphocytes bearing $\alpha\beta$ TCRs, although $\gamma\delta$ TCR-positive lymphocytes may also be observed occasionally.²⁰¹ Both CD4⁺ and CD8⁺ T cells can be identified in TIL populations from breast cancer specimens. However, in a review of TILs grown from 14 breast tumor biopsy specimens, a large percentage of the expanded cells exhibited a CD4⁺ surface phenotype associated with Th cells, not CD8⁺ expression identifying CTL.

Challenges to the use of TILs derived from breast cancers or T cells from tumor-draining lymph nodes include variable cytotoxic activity, depressed responses to mitogen stimulation, and variable cytokine expression, variable cytotoxic activity,²⁰²⁻²⁰⁴ and in some circumstances, they may suppress normal lymphoproliferative responses. In addition, experiences with expanding TILs from breast tumors in combination with IL-2 have been discouraging. In a preclinical study of breast cancer TILs, only 1 of 15 TIL plus IL-2 cultures demonstrated cytolytic activity against autologous tumor. TILs from only 3 of 11 patients secreted TNF- α or IFN- γ and GM-CSF specifically in response to autologous tumor.^{205,206}

Adoptive Immunotherapy With Activated T Lymphocytes

Although NK cells and TILs have had limited efficacy in breast cancer, these cells are relatively nonspecific for the tumor. Preclinical animal models support cellular adoptive immunotherapy with antigen-specific T cells as a potential treatment for human breast cancer.²⁰⁷ Human studies are limited, but suggest that clinical efficacy may be possible. Soda and colleagues²⁰⁸ activated CTL in vitro by culturing peripheral blood lymphocytes from cancer patients with inactivated autologous tumor cells. The activated lymphocytes were administered intravenously once every 2 weeks for 10 weeks. These investigators observed tumor regression in 4 of 11 patients. Unfortunately, not all patients had successful in vitro CTL production, demonstrating the complexity of these approaches.

Given the disappointing, albeit limited, experience with clinical responses of human breast carcinomas to therapy with autologous TILs, cellular adoptive transfer with allogeneic cytotoxic T cells has been proposed as an alternate cell therapy for inducing tumor regression. The human major histocompatibility complex nonrestricted killer T cell line TALL-104 (CD3⁺, CD8⁺, CD56⁺, CD16⁻) demonstrates cytolytic activity against breast carcinomas in murine models.²⁰⁹ Irradiated, non-dividing TALL-104 cells have been administered to 15 human patients

with metastatic breast cancer over the course of 5 days in a phase I trial. Serum levels of T cell activation markers such as IFN- γ , IL-10, soluble IL-2 receptor, and soluble intracellular adhesion molecule-1 rose following TALL-104 treatment; NK cell activity was also stimulated. Importantly, 2 patients displayed clinical improvement following therapy: liver metastases and ascites decreased in 1 patient, and another patient experienced dramatic improvement in bone pain.²¹⁰

Adoptive Immunotherapy With Antibodies

The clinical efficacy of trastuzumab, a humanized monoclonal antibody that specifically targets the extracellular domain of the HER2 protein, has been clearly established in the treatment of HER2-expressing metastatic breast cancer patients. In a pivotal multi-center, randomized phase III clinical trial comparing the use of trastuzumab plus chemotherapy, or chemotherapy alone as first-line treatment for HER2-positive metastatic breast cancer, the time to disease progression was improved markedly (7.6 versus 4.6 months, $P = 0.0001$) with the addition of trastuzumab.²¹¹ In addition, patients who received trastuzumab had a greater overall response rate to treatment (49% versus 32%, $P = 0.0002$), longer median response duration (9.3 versus 5.9 months, $P = 0.0001$), and importantly, an increased median survival was observed (25.4 versus 20.3 months after a median follow-up of 29 months, $P < 0.025$) compared with those who received chemotherapy alone.

The significant clinical activity of trastuzumab in the treatment of HER2-expressing breast malignancies has confirmed HER2 as a specific and effective target of passively transferred antibody therapies. Studies demonstrate that tumor regression occurs when the HER2 transmembrane tyrosine kinase protein is internalized and degraded on antibody binding, resulting in the inhibition of signal transduction pathways and cell growth.²¹² In addition, antibody-dependent cell-mediated cytotoxicity contributes substantially to the antitumor activity of trastuzumab in murine models.²¹³ Finally, the treatment of HER2-expressing tumor cells with trastuzumab sensitizes the malignant clones to destruction by HER2-directed CTL, presumably due to internalization, degradation, and subsequent presentation of HER2 protein by MHC class I molecules to T effector cells.²¹⁴ Clearly, HER2 represents a valid and clinically significant target of both the cellular and humoral immune system.

Monoclonal antibodies recognizing the nonglycosylated MUC1 peptide tandem repeat expressed by breast malignancies can induce antibody-dependent cellular phagocytosis and antibody-dependent cellular cytotoxicity against MUC1-expressing target cells.²¹⁵ Due to heterogenous

antigen expression by breast tumors, antibodies against MUC1 and other tumor antigens may eventually exhibit clinical value as a treatment for breast cancer either alone or in combination.

Bispecific Antibody Therapy to Augment Antigen-Specific Cell-Mediated Cytotoxicity

The recognition that antibody-mediated tumor destruction is often dependent on cellular toxicity mechanisms has led to the development of strategies intended to enhance CTL activity. Therapeutic bispecific antibodies are constructed to bind simultaneously a tumor antigen and also a unique marker expressed by T lymphocytes, physically bringing the cytotoxic effector cell and malignant target together. Bispecific antibodies targeting the glycosylated core of the MUC1 breast cancer antigen and the IgG Fc γ RI receptor expressed by cells that mediate antibody-dependent cellular cytotoxicity (eg, monocytes, macrophages) effectively induce antigen-specific target cell destruction in ex vivo assays.²¹⁵ Bispecific antibodies have also been generated that cross-link the HER2 breast cancer antigen with the CD3 T cell marker.²¹⁶ It is possible that in vivo administration of these agents may enhance the interaction of cytotoxic lymphocytes and breast cancer components, improving immune-mediated tumor cell death.

Protein and Peptide Vaccination

In a small phase I clinical trial, 19 HLA-A2⁺ patients with HER2-overexpressing cancers received a vaccine preparation containing 3 HER2-derived peptides identified previously as HLA-A2-binding motifs. For the majority of patients, vaccination significantly boosted the number of CD8⁺ T cells exhibiting HLA-restricted HER2-specific cytolytic activity as measured by in vitro chromium release assay against HER2-overexpressing targets. In select patients, these responses were long-lived and detectable more than 1 year after the vaccination procedure.²¹⁷ In a similar trial, when HLA-A2⁺ positive patients with HER2-overexpressing breast, ovarian, or colorectal adenocarcinomas were immunized with the HLA-A2-binding HER2-derived epitope p369 to 377, 3 of 4 subjects developed T lymphocytes capable of recognizing peptide-pulsed target cells. However, these T lymphocytes failed to demonstrate cytotoxic reactivity against HER2⁺ tumor cells.²¹⁸

Vaccination schemes have been designed that make use of immunostimulatory conjugates to increase reactivity against intrinsically weak tumor immunogens. For example, encouraging preclinical animal studies of immunization with the epithelial mucin MUC1 conjugated with the

large immunogenic protein keyhole limpet hemocyanin (KLH) and coadministered with the immune adjuvant QS-21 (a saponin fraction derived from the bark of the South American *Quillaja saponaria* Molina tree) has led to testing in human subjects.²¹⁹ On completion of standard adjuvant therapy for high-risk breast cancer, patients who were vaccinated with a MUC1-KLH conjugate plus QS-21 developed significant increases in both IgM and IgG anti-MUC1 antibodies that persisted at least 100 weeks after first immunization. Even so, despite these promising antibody responses, activation of CTL bearing specificity for MUC1 targets was negligible with this vaccination schedule. The small number of patients included in this study precluded assessment of clinical benefit.²²⁰ Another strategy designed to facilitate presentation of peptides by MHC class I and class II molecules involves conjugating MUC1 peptide to mannan (polymannose), which directs the immunogen to mannose receptors on APC. Patients who received this vaccine conjugate developed IgG antibody and Th cell responses against MUC1; however, only a small number of patients developed MUC1-specific CTL.²²¹

Humoral responses by B cells depend on interactions with activated antigen-specific Th lymphocytes. To enhance B cell immune responses against the breast cancer tumor antigen MUC1, a 7 amino acid sequence from the immunodominant core of the MUC1 mucin has been linked to the 111 to 120 peptide sequence of influenza hemagglutinin A/X-31, a known immunogenic MHC class II-binding determinant recognized by murine BALB/c Th cells. On immunization of BALB/c mice with this vaccine conjugate, induction of antibodies specific to the protein core of MUC1 was shown to be noticeably dependent on the presence of the influenza hemagglutinin A/X-31-derived amino acid sequence. Through poorly understood mechanisms, recognition of the MHC-peptide complex by Th cells appeared to confer immunogenicity to the MUC1 hapten. This experiment demonstrates that linkage of antibody- and cellular-specific immune epitopes is capable of augmenting humoral responses through T cell help.²²²

Carbohydrate Vaccination

The Theratope vaccine (synthetic sialyl-Tn conjugated to keyhole limpet hemocyanin) is the most extensively studied carbohydrate-based anticancer vaccine.²²³ When the STn-KLH vaccine is administered to patients with breast carcinoma, IgG antibody responses against STn can be detected consistently.²²⁴ Furthermore, patients with breast, ovarian, and colorectal cancer who generate the highest anti-STn IgG titers after immunization with STn-KLH demonstrate improved survival compared with patients who mount a much lower anti-STn antibody response.²²⁵

This observation suggests that anti-STn antibodies induced by STn-KLH vaccination may result in therapeutic benefit to cancer patients through direct effects on the tumor or via interactions with circulating STn. In addition, although carbohydrate antigens typically invoke antibody-based immune responses, there is evidence that the STn-KLH vaccine can also induce T cell responses.²²⁶ This cellular response to STn-KLH likely occurs by eliciting antibody responses that cause tumor cell death and the release of antigens that are subsequently presented to T effector cells by in situ DC. The final results of a recently completed phase III randomized study of Theratope STn-KLH versus KLH in patients with metastatic breast cancer following standard therapy will likely be reported this year.

Anti-Idiotypic Immunization

Preclinical work has been successful in generating monoclonal anti-idiotypic antibodies that imitate breast cancer antigens. Both the murine monoclonal antibody ACA14C5, which imitates a cell substrate adhesion molecule on breast cancer cells,²²⁷ and the 11D10 anti-idiotypic antibody that mimics a distinct epitope of the high molecular weight human milk fat globule expressed by some human breast cancers²²⁸ can induce antigen-specific humoral and cellular immune responses when injected into animals. When this anti-idiotypic antibody vaccine (also called TriAbTM) was administered to breast cancer patients following high-dose chemotherapy with autologous stem cell support, modest evidence of a specific T cell proliferative response was seen in a majority of subjects.²²⁹ These preliminary studies point toward further investigation to determine whether anti-idiotypic immunization can induce clinically significant antitumor responses in human breast cancer patients.

Viral Vectors Encoding Tumor Antigens

Viral vectors expressing tumor antigens theoretically provide several advantages for the induction of antitumor immune responses: tumor antigens can be expressed at high levels within the recipient, and the immunogenicity of the viral elements elicits an influx of inflammatory and APC to the inoculation site, leading to efficient presentation of both viral and tumor antigens to B and T cells. Vaccination with recombinant viruses expressing tumor antigens protects against disease in several animal models. For example, inoculation with vaccinia virus recombinants expressing the epithelial tumor antigen identified by the monoclonal antibody H23 (H23 ETA, found on 91% of human breast tumors) prevents rats from developing tumors when challenged with H23 ETA-expressing malignant cells.²³⁰ A vaccinia virus recombinant expressing

the extracellular domain of the rat neu oncogene-encoded protein (homologous to the human breast cancer HER2 antigen) fully protected animals from tumor challenge with neu-expressing Ha-ras-transformed NIH 3T3 cells.²³¹ The safety and efficacy of a live recombinant vaccinia virus expressing the human MUC1 gene along with the co-stimulatory cytokine IL-2 have been tested in human patients with advanced breast cancer. Among 9 subjects, vaccination was well tolerated, and no serious adverse effects were appreciated. Although 5 patients experienced an increase in antivaccinia antibodies, none of the patients had a significant increase in MUC1-specific antibody titers. MUC1-specific CTL could be detected in only 2 patients. The 2 patients in whom a cellular response to treatment was documented experienced the longest survival times among the cohort, although disease was treated with varying local and systemic therapy regimens on progression.²³² Viral vectors potentially offer benefit as both carrier and adjuvant for immunization against tumor antigens; it remains to be seen whether powerful antitumor immune responses can be directed against breast cancer using this novel technology.

DC Immunotherapy

DC can serve as a platform to deliver breast tumor antigens in a variety of forms, including peptides, proteins, DNA, mRNA, viral vectors, cell lysates, and tumor fusions. For example, preliminary studies show that dendritic/tumor fusion cells generated by either chemical fusion (polyethylene-glycol [PEG]) or electrofusion show equal efficacy in protecting mice against a murine mammary tumor challenge.²³³ Experience with DC vaccination for breast carcinoma patients remains limited. Administration of autologous DC pulsed with HER2- or MUC1-derived peptides was well tolerated by 7 patients in a preliminary study of vaccine treatment in advanced metastatic breast cancer. Following vaccination, peptide-specific CTL could be detected in circulation. Measurable cytotoxic immune responses occurred despite heavy pretreatment with chemotherapy in all patients, although significant clinical responses were not observed in this small study.²³⁴ We have recently completed a pilot study of vaccinating DC loaded with a HER2 intracellular domain protein in breast cancer patients with no evidence of disease following standard therapy. Among 6 patients who received this DC vaccine, no relapses have yet been observed (Morse and colleagues, Clin Breast Cancer. 2003;4:5164-5172).

Summary and Future Developments in Breast Cancer Immunotherapy

Several areas of development are expected in the next few years. First, the eagerly awaited results of the Theratope study, if positive, will give increased emphasis to the development of breast cancer vaccines. Second, integration of antibody and vaccine therapy is likely. Third, rational combinations of chemotherapy and immunotherapy will be described. Cyclophosphamide, paclitaxel, and doxorubicin administered with a GM-CSF-secreting, neu-expressing whole-cell vaccine, enhanced the antitumor effect of the vaccine in neu transgenic mice.²³⁵ Fourth, a better understanding of the effects of surgery on immune response may suggest ways to integrate immunotherapy with surgical interventions such as mastectomy or lumpectomy. Finally, as the technology for adoptive transfer of T cells improves, it is likely that adoptive immunotherapy will be attempted in the setting of high-dose chemotherapy and autologous stem cell transplant or following nonmyeloablative allogeneic stem cell transplantation.

Immunotherapy of Lung Cancer

Despite continuing progress in conventional approaches to the therapy of lung cancer, the long-term survival, even for patients with resectable disease, is disappointing. New strategies to eliminate microscopic residual disease in patients with resectable lung cancer, and to control or eliminate more advanced tumors initially treated with radiotherapy and/or chemotherapy, are clearly needed. Among these strategies is immunotherapy targeted against antigens expressed by lung cancers. Strategies employing all these types of immunotherapy are now being developed for the treatment of lung cancer as described in excellent, recently published reviews.^{236,237} The role of the surgeon in these approaches includes harvesting tumor tissue for use in the manufacture of tumor vaccines and/or to be used as targets for measuring the antitumor activity achieved by the various immunotherapies. In addition, because of the limited role for chemotherapy and radiotherapy in most resected lung cancer, the surgeon may be involved directly in the delivery of these immunotherapies in the postresection follow-up period.

Presence of Antitumor Immunity in Lung Cancer Patients

Antitumor antibodies and T cells have been detected in lung cancer patients, suggesting that an immune response does occur against tumor

and that it could potentially be harnessed for clinical benefit. The largest body of data exists for antitumor antibodies. Circulating antibodies against autologous tumor proteins have been identified in the sera of patients with lung cancer. Using protein extracts from autologous tumors, Winter²³⁸ detected serum antibodies against autologous tumor cell proteins in 58% of patients with small cell lung cancer (SCLC) and 75% (3 of 4) of patients with nonsmall cell lung cancer (NSCLC). Although identified antigens, such as p53 and HuD, were the target of the antibodies in a few patients, most were against unidentified proteins. SCLC patients with antibodies against autologous tumor cell proteins had improved survival rates compared with those in the antibody-negative group. These data suggest that an antitumor immune response may affect tumor growth and that the anonymous proteins detected by antitumor antibodies may serve as tumor antigens. Anti-Hu antibodies (anti-HuAbs), which can cause paraneoplastic encephalomyelitis/sensory neuropathy in SCLC, also are associated with a clinical benefit. In a group of patients with SCLC without the paraneoplastic syndrome, anti-HuAbs were associated with a better response to therapy and predicted longer survival.²³⁹ Others have found autoantibodies to be of prognostic value. Blaes²⁴⁰ examined antineural antibodies (AnAb) and antinuclear antibodies (ANA) in the sera of 61 patients with stage I-III NSCLC. Of these patients, 27.8% were antineural antibody positive, and 32.7% had antinuclear antibodies. Better survival rates were observed in antibody-positive patients. High serum concentrations of anti-KL-6/MUC1 were associated with a higher 1-year survival rate in patients with lung cancer. Thus, some autoantibodies may represent an effective immune response to the tumor, although there is no proof that these antibodies actually have clinical activity.

Not all antibodies are associated with clinical benefit. Antibodies to the tumor suppressor gene product p53 have correlated with shorter survival for NSCLC and SCLC in some studies,²⁴¹⁻²⁴³ but not others,^{244,245} and others have found them to identify a subgroup of patients with better outcome associated with or independent of therapy.^{246,247} An explanation for these findings could be that the antibodies are markers for patients with clinically relevant and aggressive p53 mutations rather than of any clinical antitumor activity. Decreases in tumor burden and thus antigen expression would therefore be associated with lower antibody levels. Alternatively, it is also possible that release of tumor antigen during tumor cell destruction would result in antibody/antigen complexes and a resultant decrease in free antitumor antigen antibody. It is also likely that some antibodies may have clinical activity and others may merely be markers for the disease. Regardless of the explanation, the presence of

these antibodies suggests that immune responses can be induced in the presence of lung cancer.

Detecting extant antigen-specific T cell responses in lung cancer patients has been more challenging. Lymphocytes with an activated phenotype have been detected in regional lymph nodes compared with peripheral blood. Lymphocytes from regional lymph nodes can be induced to recognize tumor antigens by stimulating them in vitro with tumor cells.²⁴⁸ When autologous tumor was mixed with regional lymph node cells with or without IL-2, proliferative responses to the tumor were found in 49% without IL-2, and in 56% with IL-2. These stimulated lymphocytes produced IFN- γ consistent with a Th1 helper T cell response. Peripheral blood lymphocytes (PBL) proliferated in response to tumor in 25% of the cases. Although this is an indication of tumor antigen-specific immunity, more direct evidence has been the discovery of restricted TCR V- β usage in the responding lymphocyte population. Restricted V- β usage is a marker for oligoclonality and presumptive evidence of an immune response.²⁴⁹ CTL that can recognize autologous tumor have also been identified in lung cancer patients.²⁵⁰ These results strengthen the view that local antigen-driven selection may occur, and support the hypothesis that an antitumor immune response may take place in some patients with NSCLC.

An easier task has been to demonstrate that tumor antigen-specific T cells can be induced within the peripheral blood, tumor specimens, or regional lymph node lymphocytes of healthy volunteers and lung cancer patients.^{251,252}

Tumor Antigens Expressed by Lung Cancer

Tumor antigens represent the ultimate way for immune effectors, such as T cells and antibodies, to recognize tumors. Numerous tumor antigens have been described for lung cancers. Several studies using predominantly RT-PCR have detected MAGE-1 in 11 to 36%, MAGE-2 in 30%, MAGE-3 in 38%-60%, and MAGE-4 in 13% of patients with NSCLC.²⁵³⁻²⁵⁶ In the most complete study to date, MAGE-A10 was expressed only by SCLC, and MAGE-A1, 3, 6, 12, and 4b were expressed by both SCLC and NSCLC.²⁵⁷ Of relevance to the use of polyvalent vaccines, 64.3% of NSCLC showed expression of at least 1 of MAGE-1, 2, or 3. Therefore, a vaccine targeting multiple MAGE antigens is likely to target most NSCLC. Indeed, peptide epitopes derived from MAGE-3 antigen have been found to be targets of CTL derived from the regional lymph nodes of lung cancer patients.²⁵⁸

Other members of the cancer-specific family of antigens, such as NY-ESO-1, have also been detected in lung cancers.²⁵⁹ Recently, the Wilms' tumor gene (WT1), broadly expressed in leukemias, has been detected in a high percentage of lung cancers and lung cancer cell lines.²⁶⁰ Because WT1 gene expression in adults is otherwise limited to the splenic capsule and stroma, the Sertoli cells of the testis, and granulosa cells of the ovary, it is an attractive target for the immunotherapy of lung cancer. Cytolytic T cells specific for an HLA-A2-restricted epitope of epithelial cell adhesion molecule (EpCAM) can be stimulated from the peripheral blood mononuclear cells (PBMC) of lung cancer patients, suggesting EpCAM may be a potential target for immunotherapy.²⁶¹ A murine anti-EpCAM antibody, engineered to have human amino acid residues in the exposed murine heavy chain variable region and in the murine light chain variable region linked to human immunoglobulin-G (IgG)-1 and κ constant region to be less immunogenic in humans, has been demonstrated in preclinical studies to be active against several human tumor cell lines.

Because epitopes recognized by T cells can be derived from intracellular, intranuclear, and cell surface-derived proteins, the search for antigens has included those involved in various cell functions such as transcription. The translation initiation factor, eIF-4G, for example, is expressed by squamous cell carcinomas and may be the target of immune responses in some patients.²⁶² Another broadly expressed tumor antigen, carcinoembryonic antigen (CEA), best known for its expression in colorectal cancers, is also expressed in more than 50% of NSCLC. HER2/neu has been detected by immunostaining in 4% to 60% of NSCLC, but only rarely by fluorescence in situ hybridization (FISH). Overexpression of HER2/neu, the transmembrane oncoprotein product of the C-erbB-2 oncogene whose activation results in tumor cell proliferation, is overall reported in 4% to 60% of NSCLC (adenocarcinoma more often than squamous cell carcinoma, not in SCLC, and generally only by immunostaining with overexpression by being rare).^{263,264} Interestingly, HER2/neu peptides that could be recognized by antigen-specific T cells have been isolated from NSCLC.²⁶⁵ Another member of the HER family, epidermal growth factor receptor, is overexpressed in virtually all squamous carcinomas and is also found in more than 65% of large cell and adenocarcinomas, but is not expressed in situ by small cell lung carcinoma.^{266,267} Sozzi²⁶⁸ reported that overexpression of EGFR and HER2/neu was found in 9 of 18 NSCLC and in 6 of 13 bronchial epithelium samples from patients with lung cancers. These findings

suggest that expression of tumor antigens may occur early during the transformation of bronchial epithelium into malignancy.

The mucin MUC4 has been cloned from tracheobronchial mucosa cDNA and reportedly is highly expressed in some human malignancies, including lung carcinoma. Forty-four percent of the lung cancer cell lines studied and 72% of the lung carcinoma tumor samples showed high levels of MUC4 mRNA expression.²⁶⁹ The MUC4 protein was detected by flow cytometry after paraformaldehyde and sialidase pretreatment in 50% of the cell lines and, by immunohistochemical studies without any digestion, in 67% of the tumors. In 29% of the patients, high levels of anti-MUC4 immunoglobulin-M (IgM) or IgG were detected, which suggests that MUC4 is sufficiently immunogenic to elicit humoral and cellular immunity specific for MUC4 in patients with malignant disease and should be useful as a target antigen. MUC1 expression has also been reported in NSCLC.²⁷⁰ Although better known for expression on colon cancer cells, 17-1A is expressed in 78% of primary NSCLCs, 57% of lymph node metastases, and 83% of bone marrow metastases.²⁷¹ This is an important finding because it may allow use of the therapeutic muAb 17-1A.

Immune Therapies for Lung Cancer

Nonspecific, Active Immunotherapy: Immunostimulatory Antigens.

Experimental approaches to immunotherapy for lung cancer were launched with the application of the nonspecific immune stimulator, BCG strain of *Mycobacterium tuberculosis bovis* to the treatment of resected lung cancer. In one of the first studies, McKneally and colleagues²⁷² administered a single postoperative intrapleural dose of BCG (to mimic an empyema) to 38 patients following pulmonary resection for lung cancer. They reported that preliminary findings in a randomized prospective clinical trial suggested that stage I lung cancer patients are significantly benefited by the treatment, whereas patients with more advanced disease were not. Since then, BCG has been tested by a variety of schedules and routes, including intrapleural, intratumoral, intradermal, or by aerosol. Although a few positive results were published, the results remained generally inconclusive. Two randomized trials failed to show any effect.^{273,274} Other attempts at nonspecific immunotherapy in lung cancer using BCG cell wall skeleton (CWS) and *Nocardia rubra* CWS have been published, with rather positive results, and also *Corynebacterium parvum*, with inconclusive results.

A Japanese team using a streptococcal preparation (OK-432) concluded it to be a potent biological response modifier against malignant tumors, which, when combined with chemotherapy, resulted in significant im-

provement in survival compared with chemotherapy alone.²⁷⁵ In patients whose lung cancer was completely resected and who had no evidence of disease, weekly intramuscular OK-432 injection for 3 years resulted in increased NK activity, lymphoblastogenetic reactivity to mitogens, IL-2 production, and skin test reaction to a streptococcal polysaccharide, none of which occurred in similarly injected patients, but who had persistent disease.²⁷⁶

More recently, SRL172, a suspension of heat-killed *Mycobacterium vaccae*, has been tested. Assersohn²⁷⁷ performed a phase II study in which patients with small cell lung cancer were randomized to receive chemotherapy (platinum or anthracycline-based) with or without SRL172 administered intradermally on day 0, weeks 4 and 8, and then 3 to 6 monthly. There was no difference in toxicity. There was a nonsignificant improvement in survival for the combination arm (median survival, 8.6 versus 12.9 months). The authors reported that a phase III study of chemotherapy in combination with SRL172 in SCLC is now underway. A similar study was performed in patients with NSCLC and mesothelioma.²⁷⁸ Patients with previously untreated NSCLC and mesothelioma were randomized to receive either chemotherapy alone, or chemotherapy administered with monthly intradermal injections of SRL172. The toxicity of chemotherapy was similar in the 2 groups. SRL172 caused mild inflammation at the injection site. In the group of patients randomized to receive chemotherapy combined with SRL172, there was a trend toward improved response rate (54% versus 33%), improved median survival (9.7 months versus 7.5 months), and improved 1-year survival (42% versus 18%). The precise cascade of immunologic events set off by SRL172 is unknown, but neither serum IFN- γ nor TNF- α levels changed with therapy. Another study assessed the use of SRL172 as an intratumoral/intrapleural injection in addition to intradermal SRL172 and standard chemotherapy for mesothelioma.²⁷⁹ Partial responses occurred in 37.5% (6 of 16) of patients. An increase in activation of NK cells and a decrease in the percentage of IL-4-producing T cells (Th2 cells) were found in all tested patients after treatment.

Another nonspecific immune stimulator is "transfer factor" (TF) extracted from the PBL of healthy volunteers. In a nonrandomized study, patients with resected NSCLC received monthly injections of TF.²⁸⁰ Compared with a historical group of untreated controls, the TF-treated group had better survival for patients in stages IIIA and IIIB, for patients with N2 disease, and patients with the large cell carcinoma subtype. In another study involving TF, patients with completely resected stage I NSCLC were randomized to TF plus *Nocardia rubra*-cell wall skeleton

(N-CWS) or no adjuvant therapy.²⁸¹ The 10-year disease-specific survival rates were significantly different in the TF + *Nocardia* group (85% versus 64%). Significant differences were observed in patients with no visceral pleural invasion or with T1 disease. The frequency of distant metastasis was significantly less in the TF + N-CWS group than in the control group.

Nonspecific adjuvants have been assessed as an adjuvant to radiotherapy and chemotherapy. In a group of patients with stage I-III squamous cell cancer responding to radiotherapy, Krestin (PSK) was administered following radiotherapy.²⁸² Compared with a nonrandomized group of responders to radiotherapy not receiving PSK, the 5-year survival rates of the treated patients were significantly greater (39% versus 17% for stage I/II; and 26% versus 8% for stage III). In a randomized trial of chemotherapy with ifosfamide compared with chemotherapy plus thymosin α 1 and low-dose IFN- α , there was a higher response rate (33% versus 10%) and time to progression for the combined therapy.²⁸³ Most notably, although CD4⁺, CD8⁺, and NK cell counts were significantly decreased after 2 cycles of chemotherapy, there were no differences in the chemo-immunotherapy-treated patients.

Nonspecific, Active Immunotherapy: Cytokines

The other major method for nonspecific active immunotherapy has been the use of cytokines, particularly IL-2, to activate immune effectors. Different IFN preparations have been evaluated in 4 trials of adjuvant therapy after response to chemotherapy for SCLC, but none of the trials showed a significant prolongation of survival.²⁸⁴

Although IL-2 has been shown to augment NK activity against K562 cells as well as autologous lung cancer cells, few patients with lung cancer have been treated on IL-2 protocols compared with the vast experience with melanoma and renal cell carcinoma. One study randomized patients with locally advanced or metastatic NSCLC to IL-2 (3 million IU/d s.c. for 6 days/wk for 4 weeks) and melatonin (40 mg/d orally every day, starting 7 days before IL-2) immunotherapy or with cisplatin and etoposide.²⁸⁵ A similar percentage of partial responses was achieved (20% to 25%), but the progression-free interval was significantly higher in patients treated with immunotherapy than in those treated with chemotherapy. The toxicity was lower in patients receiving immunotherapy. A follow-up study has not been published. Most studies that included IL-2 immunotherapy also administered TIL.

Despite promising results for nonspecific active immunotherapy, the

field has been moving toward more specific approaches that are expected ultimately to be more effective with an even greater margin of safety.

Nonspecific Adoptive Immunotherapy

Following the initial enthusiasm for adoptive immunotherapy with LAK cells, or TILs in renal cell carcinoma and melanoma, similar approaches were developed for lung cancer. Indeed, TILs could be expanded from 60% of NSCLC,²⁸⁶ which is similar to the success rate for renal cell carcinomas. A clinically meaningful number of cells (10 to 50×10^9 cells) was obtained, which included an oligoclonal population, and were capable of lysing autologous tumor.

Small clinical studies of this approach have been encouraging. Ratto²⁸⁷ administered TILs and IL-2 to 29 patients with resected stage III NSCLC. The challenges of this type of therapy were illustrated by the fact that in 5 patients, no TILs could be generated. In addition, dramatically different numbers of cells were obtained, with the number of TILs ranging from 4 to 70 billion cells. The median survival was 14 months, and the 2-year survival rate was 40%. Three patients remain alive and disease-free at more than 2 years after operation. Interestingly, most patients relapsed outside of the thorax, even if they had undergone an incomplete resection, suggesting more potent therapies are needed for the distant disease. These investigators updated their data subsequently for stage II and III NSCLC.²⁸⁸ Overall, the 3-year survival rate was significantly better for patients who underwent adoptive immunotherapy than for controls. Stage II patients had no benefit, stage IIIA patients had a benefit of borderline significance, and stage IIIB patients had a significant benefit. Again, for patients with stage III NSCLC, local relapse (but not distant relapse) was significantly reduced following the adoptive immunotherapy.

Suzuki and colleagues²⁸⁹ treated patients with multi-node-positive NSCLC (resected and incompletely resected) with combinations of cisplatin-based chemotherapy and adoptive immunotherapy with IL-2 and LAK cells or combinations of IFN- γ and OK432 every 2 to 3 months after surgery for 2 years. The 2-year survival rates for 5 completely resected cases and 6 incompletely resected cases were 80% and 66.7%, respectively. Kimura randomized 82 patients with stage IIIA disease who underwent curative resection to chemotherapy with MMC, cisplatin, and vindesine plus LAK plus IL-2 or chemotherapy or no therapy, although the chemotherapy-only arm was discontinued during the study. The 5-year survival rate was higher for the chemoimmunotherapy patients than for no therapy or chemotherapy alone (53% versus 33% versus 15%). A follow-up report from this group analyzed the data by comparing

those who received combined immunotherapy with chemotherapy or radiotherapy and those who received standard therapy only.²⁹⁰ The survival was greater for the combined modality group regardless of whether the resection was complete and regardless of the histology (squamous cell or adenocarcinoma).

Adoptive immunotherapy has generated interest even in less advanced disease, but has not been shown convincingly to improve the outcome overall. Yano²⁹¹ administered lymph node-LAK (LN-LAK) cells and IL-2 following lobectomy for primary lung cancer. Analysis of the PBL following treatment suggested an increase in CD4⁺ T cells and the NK and LAK cell activity also increased. When compared with a contemporary series of patients with resected lung cancer who did not receive the adoptive immunotherapy, there was no improvement in survival. These results are not surprising since the adoptively transferred cells are nonspecific. Animal models utilizing adoptive transfer of antigen-specific T cells have shown the ability to suppress the growth of implanted tumors expressing the antigen of interest.²⁹² Although there are no published studies using antigen-specific T cells adoptively transferred to lung cancer patients, this is an area for which the animal models support clinical trials.

Active Specific Immunotherapy: Autologous or Allogeneic Tumors

Because tumor cells express the entire panoply of antigens that may be targets for an immune response, it has been hypothesized that if they are combined with immune stimulants, that potent, broadly applicable immune responses may be stimulated. Tumors may be administered as whole/irradiated cells, lysates, or gene-modified cells. Clearly, the role of the surgeon in obtaining adequately sized pieces of viable lung tumor and ensuring its rapid delivery to the vaccine production facility cannot be emphasized enough. Nevertheless, in 1 study in which 50 patients with NSCLC and malignant effusion were enrolled, only 17 autologous lung cancer cell lines were established.²⁹³ To improve on the immunogenicity of the tumor cells, various manipulations, such as transfection with genes that encode immunostimulatory molecules, have been attempted. In a phase I/II trial for patients with stage IV or relapsed NSCLC, an allogeneic, irradiated lung adenocarcinoma line (Ad100) transfected with the co-stimulatory molecule B7.1 and HLA-A1 or A2 was administered as an s.c. vaccine.²⁹⁴ Preliminary results from 12 treated patients showed 1 patient to have a good partial response and remain stable for 6 months; 3 additional patients had SD lasting 7 to 12⁺ months. Three of these patients also showed significant increases (44- to 267-fold) in tumor-

specific CD8 CTL, measured by IFN- γ ELISPOT assay. Three of these 4 patients completed 3 courses (each course consisted of 3 s.c. vaccinations of 5×10^7 cells, 2 weeks apart). Padmanabhan and colleagues performed a phase I study of an allogeneic adenocarcinoma transfected with HLA-A1 or A2 and the co-stimulatory molecule B7.1 (CD80) in patients with stage III and IV adenocarcinoma of the lung.²⁹⁵ Of 11 patients, 3 had SD, and 1 had a PR. All patients developed an increase in antigen-specific CD8⁺ T cells secreting IFN- γ (ELISPOT assay). In some patients, levels as high as 1 of 100 CD8⁺ T cells were antigen-specific in this assay, which represents some of the highest levels observed in a lung cancer immunotherapy study.

Nemunaitis and colleagues²⁹⁶ performed a phase I/II study in which autologous lung cancers were harvested and gene modified to express GM-CSF using adenoviral vectors (called GVAX). This was clearly a challenging study since the success rate of tumor modification was 78% and there was a median dose of 1.3×10^7 cells (doses of 5 to 500×10^6 were planned). At the time of the report, 12 patients had been treated and 6 completed the immunizations. Of 3 patients with advanced measurable disease who completed the therapy, there was 1 complete response, 1 minor response, and 1 patient with SD. Because of the difficulty in reliably modifying autologous tumor, an alternative approach was begun in which bystander cells (K562) gene-modified to secrete GM-CSF are mixed with autologous tumor.²⁹⁷ The bystander cells produce much higher amounts of GM-CSF with less lot-to-lot variability. The enrollment to this study continues.

In summary, allogeneic cell lines modified to increase their immunogenicity represent an exciting approach to the treatment of lung cancer and represent a more reproducible, "off-the-shelf," and easier to deliver vaccine. Further studies are eagerly awaited.

Active Specific Immunotherapy: Immunization With Tumor Antigens

Because tumor cells may also cause inhibition of immune responses or contain antigens to which immune responses would not be desirable, recent emphasis has been placed on developing immunization protocols that use tumor antigens themselves. Tumor-associated antigens (TAA) derived from cell membranes of various tumors and found to induce antibody responses were tested in resected lung cancer patients. Takita²⁹⁸ reported on the 5-year results for a phase III study in which 85 patients with resectable (stages I and II) squamous cell lung carcinoma were randomized to specific immunotherapy (3 monthly doses of 500 mcg of

TAA emulsified with CFA), nonspecific immunotherapy (3 monthly doses of CFA emulsified in saline), or no immunization. The 5-year survival rate for the no immunotherapy group was 34%. For the TAA group the 5-year survival rate was 75%, and for the nonspecific immunotherapy group the 5-year survival rate was 53%. The median survivals for the 3 groups were 106, 71, and 38 months, respectively, which were significantly different. Tumor-derived exosomes are small 60 to 90-nm vesicles released by tumor cell lines in vitro and described to induce tumor-specific T cell responses when purified from cell culture supernatants. Electron microscopy studies of malignant effusions showed that tumor-derived exosomes are also present in vivo and contain antigen-presenting (MHC class I) molecules, antigen-binding (Hsp70) molecules, and cell-targeting proteins (lactadherin), and contain tumor antigens.²⁹⁹ Exosome-pulsed DCs may prove to be better than irradiated autologous tumor cells at inducing a tumor-specific CTL response. This was seen with ascites-derived exosomes from melanoma patients, which resulted in strong tumor-specific CTL cytotoxicity against autologous tumor cells and tumor-specific IFN- γ release.

Because these were relatively crude preparations and as more defined epitopes have been discovered, the field of immunotherapy has moved toward the use of these pure epitopes delivered in either adjuvants or presented by APC. Particularly important has been the improvements in the immunogenicity of these peptides by creating analogs with enhanced HLA binding or T cell avidity.³⁰⁰ MAGE-3-derived peptide-specific CTL could be induced from PBMC of HLA-A2-positive healthy donors and the regional lymph nodes of HLA-A2-positive lung cancer patients after 2 or more stimulations with MAGE-3 peptide-pulsed HLA-A2-positive APC.²⁵⁸ These MAGE-3 peptide-specific CTL lysed cells pulsed with MAGE-3 peptide, but not those pulsed with influenza matrix protein, ErbB-2, or wild-type p53. The peptide-specific lysis could be inhibited by monoclonal antibodies against MHC class I and HLA-A2.

Several clinical trials utilizing DC are underway in lung cancer patients currently. Preliminary evidence has shown that DC can induce CEA-specific³⁰¹ and p53-specific³⁰² T cell responses in vitro, which would have direct relevance to the treatment of lung cancer. We have demonstrated the induction of CEA-specific CTL immune responses by administration of an autologous DC vaccine loaded with total mRNA from a CEA-expressing tumor, into a patient with a lung adenocarcinoma.³⁰¹ In another clinical study, 2 of 10 HLA-A24 patients with CEA-expressing advanced digestive tract and lung cancers developed DTH to CEA-derived 9-mer peptide-pulsed autologous DC after intradermal and s.c.

vaccination.³⁰³ The CEA-derived 9-mer peptide was identified as the epitope of CTL restricted with HLA-A24. In the 2 patients who showed a positive skin response, disease remained stable for 6 and 9 months, respectively. In a third study, vaccination with DC loaded with a CEA-derived peptide, which was altered to be a more potent T cell antigen, resulted in 2 of 12 patients exhibiting tumor regression, 1 patient having a mixed response, and 2 having SD.¹¹⁴

T cells, from HLA-A2⁺ lung cancer patients and healthy donors and stimulated with DC transduced with an adenovirus wild-type p53 construct, similarly resulted in CD8⁺ CTL activity against HLA-A2⁺ tumor cells with accumulated mutant or wild-type p53, but not against HLA-A2⁺ tumor cells with undetectable p53 nor against HLA-A2⁻ tumor cells.³⁰² These CTL did recognize HLA-A2⁺ tumor cells with normal levels of p53 if the tumor cells were subsequently transduced with the adenovirus-p53 vector.

In a phase I trial, autologous DC were cultured from the PBMC of patients with MUC1-positive solid tumors, primed with mannan-conjugated recombinant MUC1 fusion protein (MFP), and reinjected into the patients intradermally.³⁰⁴ Three of 4 patients developed DTH-like responses after the third DC injection, and all 4 patients showed a strong Th1-type cellular response and significant levels of IFN- γ -producing cells reactive to MUC1 antigen, with no antibody response identified. Two patients had SD for more than 10 weeks, whereas 2 patients had disease progression.

Derivatives of DC, such as the bilamellar particle called dexosomes, are also under development. In a phase I study, dexosomes loaded with MAGE peptides were administered to patients following their standard chemotherapy.³⁰⁵ The immunizations were well tolerated. Increased DTH reactivity to some MAGE antigens was observed. Three of 9 patients remained stable following the immunizations. A phase II study of this approach in lung cancer patients is expected to open shortly.

Tumor antigen peptides may also be administered as the vaccine directly. In 1 clinical trial, HLA-A2⁺ patients with NSCLC and tumor expression of NY-ESO-1 were administered 8 weekly intradermal injections of a set of NY-ESO-1 peptides 157 to 165 and 157 to 167 with s.c. GM-CSF as adjuvant twice weekly.³⁰⁶ Preliminary results in 4 patients enrolled to date showed significant enhancement of NY-ESO-1 peptide 157 to 165 specific CD8⁺ T cell reactivity starting at week 4 in 1 patient and at week 6 in a second patient. NY-ESO-1-specific DTH became positive at week 8 in 1 patient, but negative thereafter. One patient had SD at the end of the study, whereas 3 patients did not have measurable

disease. MUC-1 peptides such as the 25-mer BLP-25, which has been lipid encapsulated, are under evaluation in patients with lung cancer.³⁰⁷

A different immunization approach is to use anti-idiotypic vaccines. Grant and colleagues³⁰⁸ studied immunizations with the anti-idiotypic MAb BEC2, which mimics the ganglioside GD3 expressed on the surface of most SCLC tumors, combined with BCG as an immune adjuvant. Fifteen patients who had completed standard therapy for SCLC received a series of 5 intradermal immunizations consisting of 2.5 mg of BEC2 plus BCG over a 10-week period. The vaccine was immunogenic since all patients developed anti-BEC2 antibodies, despite having received chemotherapy with or without thoracic radiation. Anti-GD3 antibodies were detected in 5 patients, including those with the longest relapse-free survival. The median relapse-free survival for patients with extensive stage disease was 11 months and had not been reached for patients with limited stage disease (>47 months). These impressive results were the basis for a phase III trial that is being conducted to evaluate BEC2 plus BCG as adjuvant therapy after chemotherapy and irradiation. A bivalent ganglioside vaccine, BMS248967, is also being studied at the phase II level.

Passive Specific Immunotherapy Against Lung Cancer: Monoclonal Antibodies

Although trastuzumab likely blocks binding of a ligand to HER2, it may also function by mediating ADCC. Trastuzumab has a modest cytotoxic effect against HER2/neu-positive NSCLC cell lines in vitro. Preliminary human studies^{309,310} with trastuzumab suggest a response rate of 21% to 40%. However, some phase II clinical trials adding trastuzumab to chemotherapy have shown synergistic effects with up to 42% (8 of 19) partial response and an additional 42% (8 of 19) SD,³¹¹ whereas others have not shown additional benefit.^{312,313} The reason for the lack of benefit may be due to the rarity of NSCLC patients with strongly HER2-positive (3⁺ by immunohistochemistry) disease or HER2/c-erbB2 gene amplification and who theoretically would have the greatest potential for benefit from trastuzumab.^{312,313}

Herbst³¹⁴ described the background and ongoing clinical trials for a variety of monoclonal antibodies (such as IMC-C225) that target EGFR in lung cancer. In a phase II study of IMC-225 administered with docetaxel in chemotherapy refractory NSCLC, 4 of 20 patients had a PR and 6 of 20 had SD.³¹⁵ Kelley³¹⁶ reported a phase II study of a murine MAb, 2A11, which binds gastrin-releasing peptide (GRP), which is secreted by and can activate SCLC in an autocrine loop. Thirteen patients

with previously treated SCLC received 2A11 at 250 mg/m² over 1 hour 3 times per week for 4 weeks. One of 12 (8%; 95% confidence interval, 0% to 38%) evaluable patients had complete resolution of radiographically detectable tumor lasting 4 months. Four patients (33%) had SD, and no toxic reactions were observed. The immunoconjugate BB-10,901, consisting of huN-901, a humanized murine MAb that binds to CD56 found on SCLC, linked to the maytansinoid cytotoxin DM1, a tubulin poison, has undergone phase I studies in patients with relapsed SCLC.³¹⁷ To date, most patients progressed after 1 or 2 cycles of therapy, but because it was well tolerated, a phase II study in less heavily pretreated patients was planned. Pretreatment with rituximab in patients with advanced stage carcinoma treated with similar immunotoxins may delay development of antitoxin-neutralizing antibodies, which cause rapid clearance of therapeutic immunotoxins.³¹⁸

Future Developments in Lung Cancer Immunotherapy

In addition to the search for more potent vaccines, it will be important to develop approaches that prevent evasion of lung cancers from immune effectors. For example, a common method for immune escape is down-regulation of HLA class I molecules. Administration of IFN- γ has been shown to up-regulate HLA class I on NSCLC and thus may be a useful adjunct. In addition, lung carcinoma cells are capable of killing the Fas-sensitive human T cell line Jurkat in coculture experiments.³¹⁹ Inhibiting fas-L expression by tumors might therefore be a useful adjunct to immunotherapies.³²⁰ Similarly, it may be necessary to interfere with the tumor antigen RCAS1, which may play a role in escape of tumors from immune surveillance (by inducing apoptosis in immune cells bearing the RCAS1 receptor) and which was positive in 47% of NSCLC specimens (and up to 80% of adenocarcinomas).³²¹

Recently, there has been considerable interest in the role of regulatory T cells in suppressing immunostimulatory T cells and in the down-regulation of T cell responses to tumor antigens. Woo³²² showed that lung tumors contain large numbers of these cells and that they have constitutive high-level expression of CD152 (CTLA-4). The CTLA-4 molecule antagonizes T cell activation by interfering with the co-stimulatory signals induced by the interaction of CD28 on T cells and B7 on DC. Furthermore, the CD4⁺CD25⁺ T cells mediate potent inhibition of autologous T cell proliferation. Together these results suggest that the CD4⁺CD25⁺ T cells found in lung tumors selectively inhibit the host immune response and therefore could contribute to the progression of lung cancer. Monoclonal antibodies that can block CTLA-4 or that can

deplete CD25⁺ T cells may therefore be of use in inhibiting the counter-regulatory response.

Finally, it will be necessary to find ways to combine chemotherapy and radiotherapy with immunotherapy. There have been few systematic evaluations of immunity in patients on chemotherapy. Nowak³²³ studied the effect of gemcitabine on antigen-specific antitumor immunity using a murine tumor cell line transfected to express influenza virus hemagglutinin (HA). Although T cells were decreased, B cells were more selectively depleted and IgG antibody responses to HA were significantly decreased. In contrast, in vitro T-lymphocyte recall responses to the class I- and class II-restricted dominant peptide epitopes of HA were enhanced in tumor-bearing, gemcitabine-treated mice. Thus, gemcitabine does not appear to be detrimental to specific antitumor cellular immunity and may be useful in combination chemo-immunotherapy protocols. In contrast, vaccination protocols requiring a humoral immune response for maximal efficacy may be compromised in patients treated with gemcitabine. Because gemcitabine is a frequently used drug in lung cancer, it will be necessary to further study the effect of it on specific immune responses.

Immunologic Therapies for Gastrointestinal Malignancies

Immunotherapy for Colorectal Cancer. As is the case for most other solid tumors, colorectal cancer possesses several ways to escape from control of endogenous antitumor mechanisms, including: down-regulation of HLA class I antigens allowing evasion of CD8-mediated cytotoxicity, decreased or faulty expression of Fas to escape Fas-mediated cytotoxicity, and production of immunosuppressive factors such as transforming growth-factor β (TGF- β) and IL-10.³²⁴⁻³²⁷ Various strategies of immunotherapy, both passive and active, have been employed to overcome these mechanisms and treat colorectal cancer.

Passive Immunotherapy With Antibodies

The use of antibodies for the therapy of colon cancer has been reviewed previously.³²⁸ The unconjugated monoclonal mouse antibody 17-1A (muAb 17-1A), an IgG1a antibody that mediates ADCC directed at a 38-kDa carcinoma glycoprotein, GA733-2,³²⁹ has been studied extensively in colon cancer. The major drawback to the use of such mouse antibodies is the almost universal development of human antimouse antibodies (HAMA). Several groups have shown that HAMA is associated with rapid clearance of antibody from the blood and consequent lack of therapeutic effect after HAMA development.³³⁰ To overcome this hurdle, genetically engineered humanized versions of MAbs have been

developed. The humanized version of muAb 17-1A has demonstrated the ability to lyse human colon cancer cells in vitro in the presence of human mononuclear cells.³³¹

Riethmuller and colleagues conducted a clinical trial studying 189 patients with Dukes' Stage C colorectal cancer.³²⁹ Patients were randomized to postoperative treatment with 5 injections of muAb 17-1A or to observation alone. Seven-year follow-up revealed that postresection MAb therapy significantly decreased distant metastases, but did not affect the rate of local relapses. Although this trial showed promise in the use of muAb 17-1A, it raised many questions about the antibody mechanism of action. For example, 80% of treated patients developed HAMA, and all of these patients were re-treated despite the presence of HAMA. However, development of HAMA did not seem to affect the outcome negatively. This observation suggests that either the full therapeutic effect of muAb 17-1A is seen after only 1 dose of drug, or that antibody tumor targeting is not necessary for therapeutic effect. In addition, the 17-1A Ag is expressed on normal tissues as well as on colorectal carcinomas, yet selective tumor versus normal tissue targeting has never been demonstrated rigorously. Thus, many questions regarding the mechanisms by which muAb 17-1A caused a significant decrease in distant metastases remain unanswered.

Radiolabeled MABs have been used to deliver focal doses of radiation to specifically targeted cells. This strategy has been employed with the monoclonal antibody, MAB 33, which recognizes a 43-kDa cell-surface antigen on normal human epithelium of the lower gastrointestinal tract. This cell surface antigen is expressed strongly on 95% of primary and metastatic colon cancers, but not on other tissues.³³² MAB 33 has been conjugated with ¹³¹I radioisotope to form a radioisotope conjugate capable of delivering targeted radioimmunotherapy to colon cancers while sparing normal tissues of intense radiation. The greatest difficulty in using radioisotope conjugates of antitumor antibodies is the physiologic barriers that impede transport of macromolecules from the vascular compartment to cell-surface tumor antigens. This leads to blood-borne radioisotope and significant bone-marrow toxicity. One approach to decreasing nonspecific background irradiation is the use of low-energy, short-range electrons such as ¹²⁵I. This strategy is based on the fact that A33 is internalized into an intracellular compartment near the nucleus after binding to the cell surface.³³³ Thus, the specificity of the cytotoxic mechanism depends on specific targeting to the cell membrane, followed by internalization of the radio-conjugate into cancer cells.

In 1 clinical trial, ¹³¹I-labeled MAB A33 was administered to 23 patients

with metastatic colorectal cancer who had failed conventional chemotherapy.³³² Patients received a single dose of antibody at varying dose levels from 30 mCi/m² to 90 mCi/m². Five of the 23 patients had some evidence of antitumor effects, but none qualified as a major response. Although there was variable antibody uptake in normal bowel, there was only mild bowel toxicity. Thrombocytopenia and neutropenia were the toxic dose-related side effects. The majority of these patients, however, had previously compromised bone marrow due to prior chemotherapy.

The low-energy electron-emitting radioisotope ¹²⁵I-labeled MAb A33 has been studied in a phase I/II trial based on preclinical evidence that low-energy emitting radio-immunotherapy will cause less bone marrow toxicity than the high-energy emitting radioisotope ¹³¹I- MAb A33.¹⁰ In this study, 21 patients with advanced colon cancer were treated with a single ¹²⁵I MAb A33 dose ranging from 50 to 350 mCi/m². Although antitumor effects were still modest, bone marrow toxicity was spared up to the highest attempted dose (350 mCi/m²).

An alternative approach to improve the targeting potential of antitumor antibodies is the creation of smaller antibody molecules. The first class of these agents to be created is a single-chain Fv (scFv) antibody, MFE23, that detects CEA.³³⁴ scFv antibodies consist of the antibody variable heavy and light regions linked with a short synthetic peptide to form a single molecule. Although the small molecular weight allows for optimal tumor penetration, further evaluation in clinical trials is necessary to demonstrate improved antigen targeting conclusively.

One final approach utilizing antitumor antibodies is coupling of bacterial or plant toxin to MAb. The most thoroughly studied of these immunotoxins is LMB-1, a MAb linked to *Pseudomonas* exotoxin directed against the Lewis-Y blood group antigen (Le^y), which is present on many solid tumors.³³⁵ Unfortunately, the therapeutic potential of such immunotoxins has been limited by the development of antibodies against the highly immunogenic toxin moiety, thus inhibiting a second treatment.

In a phase I study of LMB-1 immunotoxin, 38 patients with various solid tumors (23 with colorectal cancer) expressing Le^y antigen received varying doses with manageable toxicity.³³⁵ Objective antitumor responses were observed in 5 patients, 18 patients had SD, and 15 patients had disease progression. No uniform follow-up date was used in this study, and the duration of the therapeutic effect was unknown. The greatest obstacle to this approach was the fact that 33 of the 38 patients developed neutralizing antibodies against the toxin moiety, precluding the use of multiple doses of the toxin in most patients.

Active Immunotherapy: Anti-Idiotypic mAb Vaccines

Although monoclonal antibodies were discussed above as providing passive immunotherapy, monoclonal antibodies have also been found to have an indirect immunizing effect through the development of anti-idiotypic antibodies. For example, after infusion of MuAb throughout for co 17-1A, an anti-idiotypic antibody (Ab2) has been shown to develop.³³⁶ Parts of the variable region of Ab2 resemble the epitope that MuAb 17-1A recognizes. The anti-idiotypic antibody actually mimics the tumor-associated antigen and may subsequently induce the formation of anti-anti-idiotypic immune responses with T cells directed against the epitope targeted by MuAb 17-1A. Other tumor-associated antigens that have been used for anti-idiotypic immunotherapy include the anti-idiotypic monoclonal antibody 105AD7, which mimics the colon cancer tumor-associated antigen gp72, and the anti-idiotypic antibody 3H1 (CeaVac), a portion of which mimics CEA.^{337,338} In a phase Ib trial of 24 patients with advanced colorectal cancer, CeaVac induced formation of anti-3H1 antibodies (Ab3)³³⁷ detectable in the sera from 17 of 23 patients. Furthermore, 13 of the 17 responses were anti-CEA responses. Toxicity was minimal and the overall survival was 11.3 months, which is comparable to other phase II trials for advanced colorectal cancer treated by conventional chemotherapy.

In further study of anti-idiotypic CEA vaccines, 32 patients with resected Dukes' B, C, and D, and incompletely resected Dukes' stage D disease were treated with CeaVac.³³⁸ Fourteen of these 32 patients also received concomitant 5-FU. All 32 patients developed anti-CEA humoral and T cell-specific responses. The patients receiving 5-FU exhibited immune responses comparable to those patients not receiving chemotherapy. Only 3 of 15 with Dukes B or C colon cancer relapsed. In addition, 7 of 8 patients with completely resected Dukes' stage D disease remained disease-free at a median of 16 months. The authors of the study suggested that adjuvant CeaVac vaccination added clinical benefit. Phase III trials of adjuvant CeaVac vaccine are currently being designed for study in patients with stage II-IV disease.

The anti-idiotypic antibody Ab105AD7 was recently studied in a randomized, double-blind, placebo-controlled phase II trial of 162 patients with advanced colorectal carcinoma.³³⁹ Patients receiving vaccine were to receive 3 doses of 105AD7, but only 50% were compliant in receiving all 3 doses. The median survival from the randomization date was not significantly different between the vaccine and placebo groups. The authors suggested that the lack of demonstrable efficacy of 105AD7

vaccination was due to poor compliance and large tumor burden in the patient population.

Peptide-Based Vaccines

The immunogenic peptide epitopes for several tumor antigens expressed by colon cancers have been formulated into vaccines. MUC-1 is of particular interest because it is overexpressed and aberrantly glycosylated in colorectal cancer, making it more “apparent” to the immune system. Compelling data from preclinical animal models regarding the use of MUC-1 glycoprotein vaccination have led to several phase I vaccine trials. Goydos and colleagues vaccinated 63 patients (30 of whom had colorectal cancer) with 100 μ g of a 100 amino acid synthetic MUC-1 peptide.³⁴⁰ Patients received vaccine with adjuvant BCG at 3-week intervals for a total of 3 vaccinations. The vaccine was generally well tolerated, but there were local skin reactions at the vaccination site in several patients. Biopsy of the injection site revealed intense T cell infiltration in 37 of the 55 biopsied patients. In addition, there was a 2- to 4-fold increase in mucin-specific CTL precursors in 7 of 22 tested patients. Two patients with colorectal cancer had SD. This phase I study demonstrated that patients with adenocarcinoma are capable of generating a specific immune response to mucin peptide vaccination with only mild toxicity.

Karanikas and colleagues have boosted the immunogenicity of MUC-1 peptide by fusing MUC-1 to oxidized mannan (MUC-1 mannan-fusion protein or MFP).³⁴¹ Mannan increases presentation of MUC-1 peptide within the MHC-I complex, which induces an increased CTL response. This was clinically tested using MFP s.c. or intraperitoneal injections for patients with advanced colon cancer. Sixty percent of patients had high titer MUC-1 IgG antibody responses, with the intraperitoneal injection route yielding 10-fold higher increases in antibody formation over conventional s.c. injection. Specific anti-MUC-1 cellular immune responses were found in 28% of patients. This phase I trial established that the MFP vaccine is nontoxic and suggested that the intraperitoneal injection of colon cancer vaccines may confer benefit over s.c. injection.

A peptide fragment of the hormone gastrin has also been employed in vaccine therapy for colorectal cancer. Gastrin is a well-recognized growth factor, and the gastrin gene has been shown to be activated in colorectal cancer cells, which may promote cellular proliferation via an autocrine growth loop.³⁴² Gastrimmune (G17DT) is an antigastrin vaccine composed of the 9 NH₂-terminal amino acids of gastrin linked to the diphtheria toxin. It has demonstrated an antitumor effect in murine

models of colorectal cancer, and phase I/II trials of G17DT have been initiated. In a phase I/II clinical trial, 40 of 50 patients with advanced colorectal cancer who were treated with 3 injections of G17DT had a measurable antibody response rate.³⁴³ The antibodies had a high affinity and competed with gastrin-17 for receptors on colon cancer cells. Prospective, placebo-controlled trials of G17DT are now underway for colon and gastric cancer.

Recombinant viral vaccines expressing specific peptide epitopes provide another vehicle for active immunotherapy. Recombinant vaccinia virus encoding full-length cDNA of human CEA (rV-CEA vaccine) was the initial viral vaccine used for colon cancer.³⁴⁴ Three phase I vaccine trials examining the use of a recombinant vaccinia virus expressing CEA (rV-CEA) have been completed. These trials established the safety of the rV-CEA vaccine in humans. The first study by Tsang and colleagues identified a specific epitope of CEA that binds the major histocompatibility complex HLA-A2 and generates CEA-specific T cell responses.³⁴⁵ This 9-mer peptide was named CEA-antigen peptide-1 (CAP-1). No patients in this trial had an objective antitumor response. One hypothesis for the lack of response was that all patients developed strong immune responses to the live viral vector, precluding additional immunizations after the first injection. To overcome this obstacle, Marshall and colleagues employed an approach in which patients received vaccination with rV-CEA alternating with vaccinations using a second, less immunogenic recombinant viral vector, the avian vaccinia virus (ALVAC-CEA).³⁴⁶ Unlike the vaccinia virus, the ALVAC-CEA vector does not replicate in mammalian cells. This strategy using alternating vaccinations with 2 different recombinant vaccines against the same epitope is called a prime-and-boost regimen. In a phase I trial in which 18 patients received either rV-CEA, followed by 3 ALVAC-CEA vaccinations (VAAA) or 3 ALVAC-CEA vaccinations, followed by an rV-CEA booster (AAAV), the diversified schedule appeared to be superior to vaccination with either vaccine alone. In addition, the VAAA schedule was found to be superior over the AAAV schedule for generating CEA-specific T cell responses. Further trials utilizing the diversified prime-and-boost regimen as adjuvant therapy in colon cancer patients with smaller tumor burdens are being planned.

Immunization strategies utilizing DC have also been studied on a limited basis in colorectal cancer. In a study of 12 patients with advanced gastrointestinal malignancies (3 with colon cancer), autologous, ex vivo generated DC, pulsed with MAGE-3 peptide, were administered every 3 weeks for 4 total vaccinations.³⁴⁷ Minor tumor regression was encoun-

tered in 3 patients, and peptide-specific CTL responses were observed in 4 of 8 patients. Other DC immunotherapy strategies have utilized DC loaded with CEA peptide or modified CEA peptide.^{348,349} Morse and colleagues have completed a phase I trial in which 21 patients with CEA-expressing malignancies received vaccination with autologous DC loaded with CAP-1.³⁴⁹ Autologous DC were expanded in vitro by culturing with GM-CSF and IL-4. One patient had a minor response and 1 had SD. There has also been interest in mobilizing DC in vivo. Morse and colleagues have shown that administration of the bone marrow stimulatory cytokine Flt3 ligand (Flt3L) to metastatic colon cancer patients increases the percentage of DC in peripheral blood as well as increases DC infiltration into peritumoral regions.³⁵⁰ Fong and colleagues performed another study in which patients were immunized with DC loaded with CEA peptide.³⁵¹ Unlike the earlier study in which DC were expanded ex vivo, systemic administration of Flt3L was used to expand DC in vivo. This study also utilized an altered version of the CEA peptide. This altered peptide, CAP-1(6D) (aspartate substituted for asparagine), yields increased potency at inducing CTL activity against CEA in vitro. Two of 12 vaccinated patients experienced dramatic tumor regression, 1 patient had a mixed response, and 2 patients had SD. Finally, hints of tumor antigen-specific immune responses have been found in preliminary studies using DC loaded with tumor antigen in the form of CEA mRNA or autologous whole tumor mRNA.³⁵²

Whole Tumor Cell and Tumor Lysate Vaccine Approaches

Whole tumor cell and tumor lysate vaccine approaches, which have been studied in clinical trials, include the use of autologous irradiated colon cancer cells plus BCG, and autologous irradiated colon cancer cells infected with the Newcastle disease virus. Three large studies have evaluated the use of autologous, irradiated whole tumor cell vaccines in the treatment of colon cancer. The first study randomized 80 patients with Dukes' stage B2-C3 colorectal cancer to surgical resection alone or surgical resection plus 3 vaccinations of autologous whole tumor cells plus BCG.³⁵³ There was no significant difference in survival or disease-free survival between the vaccine and control group. Of note, all patients with rectal cancer in this study received postimmunotherapy radiation. Since radiation may cause a diminution of the immune response, a subset analysis for each colon and rectal cancer patient versus control group was performed. There was a significant improvement in survival and disease-

free survival in colon cancer patients who received adjuvant immunotherapy, whereas there was no benefit in rectal cancer patients.

A similar ECOG study randomized 412 patients with stage II and III colon cancer to postoperative observation or vaccination with autologous tumor cells plus BCG.³⁵⁴ Patients received 3 weekly intradermal injections of autologous cells with the first 2 injections accompanied by BCG. Development of immunity to the tumor was measured by serial delayed cutaneous hypersensitivity skin testing with autologous tumor cells compared with normal colon cells. No statistically significant differences in survival were detected between the 2 arms. However, when disease-free survival was compared between patients who developed an effective immune response against the vaccine versus patients who did not, patients with a DTH response of more than 5 mm of induration had improved survival compared with patients who developed a DTH response of less than 5 mm. Furthermore, survival correlated with the diameter of induration on DTH. The 5-year survival rate for patients with an induration of less than 5 mm was 45%. The 5-year survival rate was 74% for patients with induration of 5 to 10 mm and 85% for patients with induration of more than 10 mm. These data suggest that the clinical outcome depends on the degree of immune response to vaccination.

Vermorken and colleagues performed another study of autologous whole tumor cell vaccine using BCG as an adjuvant.³⁵⁵ Based on previous data showing that DTH responses waned 6 months after the last vaccination,^{353,354} this study vaccinated patients with a 6 booster vaccination 6 months after the initial 3 vaccinations. Two-hundred-fifty-four patients with stage II and III colon cancer were randomly assigned to postoperative observation or vaccination. The risk of recurrent disease was reduced by 49% for patients receiving vaccination compared with patients in the observation cohort. When the data were analyzed by stage, the largest impact was for patients with stage II disease, and there was no significant benefit for patients with stage III disease. Hanna and colleagues performed a meta-analysis of all 3 studies and concluded that the use of postoperative adjuvant whole tumor cell vaccine plus BCG provides significant clinical benefit in patients with stage II disease.³⁵⁶

The first clinical trial using xenogenically altered colon cancer cells was a nonrandomized phase II trial of 23 colorectal cancer patients who underwent vaccination of autologous tumor cells infected with Newcastle Disease Virus (NDV) after complete surgical resection of liver metastases.³⁵⁷ Patients received adjuvant vaccine every 2 weeks for a total of 5 vaccinations plus 1 additional booster vaccine 6 months later. At minimum follow-up of at least 18 months, only 61% of vaccinated

patients developed a tumor recurrence compared with an 87% recurrence rate in the control group.

Ockert and colleagues performed a nonplacebo controlled study in which 48 patients with Dukes' stage B2-C colon cancer received either autologous tumor cells infected with NDV or autologous tumor cells plus BCG.³⁵⁸ The 2-year survival rate was 97.9% in the group who received tumor infected with NDV compared with 66.7% in those who received tumor cells plus BCG. Neither of the above studies were randomized and more rigorous studies of xenogenically altered tumor cells are necessary for further evaluation.

An alternative method of augmenting the immunogenicity of tumor cell vaccines is the introduction of genes encoding immunostimulatory molecules. Autologous colon cancer cells transduced with several cytokine genes have been studied, including colon cancer cells transduced with IL-2, IL-12, GM-CSF, and GM-CSF plus IL-4.³⁵⁹⁻³⁶² The transfer of genes encoding cytokines and immunomodulators has achieved success in preclinical animal models, but has yet to be studied extensively in clinical trials. One study examined injection of a liposomal vector containing a plasmid encoding the HLA-B7 gene and β 2-microglobulin gene directly into liver metastases of HLA-B7-negative patients.³⁶³ Plasmid DNA was detected in 14 of 15 patients, and 7 of the 14 who were effectively transfected displayed HLA-B7 protein expression by fluorescence-activated cell sorting analysis. Examination of liver biopsy specimens revealed that 8 of 15 patients developed specific anti-HLA-B7 CTL, 14 of 15 had detectable NK cells, and 9 of 15 had LAK cells. No objective clinical responses were seen in this phase I trial, but it did establish that liposomal gene transfer is nontoxic and feasible.

Because of the difficulty establishing some tumors in culture, as well as inefficient gene transfer to tumor cells, Sobol and colleagues have utilized a vaccine technique using autologous tumor cells mixed with fibroblasts genetically modified to express IL-2.³⁶⁴ Patients with colorectal carcinoma received a mixture of autologous irradiated tumor cells with genetically engineered fibroblasts expressing IL-2. Before therapy, low frequencies of tumor-specific CTL precursors were detected in 4 of 7 patients. After therapy, there was a 5-fold increase in the frequency of tumor-specific CTL in 2 of 6 evaluable patients. The treatment was well tolerated by all patients, and further studies using this approach are planned.

Immunotherapy for Pancreatic Cancer

Passive Immunotherapy: Monoclonal Antibody Therapies. In addition to its use in colon cancer, MuAb 17-1A has been tested in pancreatic

cancer. However, early studies have shown limited immune responses without any survival or disease-free or overall survival benefit.^{365,366} Larger European clinical trials are currently in progress.

The monoclonal antibody BW 494/32 recognizes pancreatic cancer surface proteins and initiates antibody-dependent cytotoxicity. A prospective randomized trial including 61 patients with resected pancreatic cancer demonstrated a trend toward prolonged survival for patients receiving the antibody. The difference, however, was not statistically different.³⁶⁷ In vitro application of BW 494/32 in the presence of the cytokine GM-CSF demonstrated increased pancreatic cancer cell lysis. Perhaps new clinical trials utilizing new preparations including cytokines will provide improved results.

Epidermal growth factor (EGF) plays an important role in cell growth and differentiation, and its receptor has been shown to be overexpressed in pancreatic cancer.³⁶⁸ Binding of EGF receptor by different antibodies has been shown to inhibit pancreatic cancer in mice models.³⁶⁹ Although this almost certainly is due to blockade of the receptor, it is possible that immunologic effectors could be involved as well. In a phase I/II trial in 26 patients with unresectable pancreatic cancer, auto-EGF receptor antibodies and separate injections of TNF- α (to enhance ADCC) were administered.³⁷⁰ The results demonstrated the safety and efficacy of this approach. Increasing doses of antibody resulted in primary tumor inhibition and prolonged median survival, including 1 patient with a complete tumor remission at 3-year follow-up.

Another new promising antibody approach is the use of fusion proteins. For example, by combining the Fab fragment of the pancreatic cancer cell carbohydrate antigen (C242) with the superantigen staphylococcal enterotoxin A, the resulting fusion provides a large nonspecific immunologic stimulus in a specific targeted area.³⁷¹ Phase I trials have defined the safe dosing range for this approach, and early trials in patients with metastatic disease have demonstrated some partial responses of malignant metastatic lesions to the liver.^{371,372}

Active Immunotherapy: Vaccine Therapies

Peptide-Based Approaches. As pancreatic cancer tumor-associated antigens have been identified, there has been a resurgence in vaccine development as a therapeutic approach to pancreatic carcinoma. Detection of circulating peptide-specific CTL precursors in pancreatic cancer patients suggests that peptide vaccines may be used to stimulate an immune response.³⁸⁵ Targets that have been employed for peptide-based

vaccines include cancer cell surface antigens, mutated oncogenes, and tumor suppressor genes.

Like other epithelial malignancies, pancreatic cancers express MUC-1 aberrantly. CTL cloned from pancreatic cancer patients could recognize MUC-1 expressing targets *in vitro*.³⁷³ To date, there have been few reported clinical trials in patients with pancreatic cancer in which MUC-1 was targeted. As described above, a phase I study of MUC-1 peptides plus BCG included patients with pancreatic cancer, and immune responses specific for MUC-1 could be detected.³⁴⁰ One of the pancreatic cancer patients had SD following the immunizations. CEA is also a target in pancreatic cancers, and a few pancreatic cancer patients have been included in the clinical trials of DC vaccines or CEA-expressing viral vectors described above, although there have been no clinical responses in these patients.^{346,349,374}

The k-ras mutations are present in 90% of pancreatic cancers,³⁷⁵ and the novel proteins that are encoded by k-ras point mutations at codons 12, 13, and 61 are potential immunogens against which to target vaccine therapy. Various studies have demonstrated the safety of using mutated k-ras in vaccine trials, but the immune responses have varied. A phase I study utilized a vaccine consisting of a 13 amino acid sequence corresponding to k-ras mutated at codon 12. Patients with advanced pancreatic cancer underwent 3 monthly *s.c.* vaccinations with Detox adjuvant.³⁷⁶ The vaccines were well tolerated without any serious reactions. Three of 10 patients had a CD4⁺ and/or HLA-A2-restricted CD8⁺ CTL response after vaccination. The CD8⁺ CTL were capable of lysing an HLA-A2-matched tumor cell line carrying the corresponding mutant, but not the wild-type ras gene. This *in vitro* stimulation of specific T cell corresponding to the mutated peptide could potentially be applied for adoptive immune therapy. A separate study of pancreatic cancer patients with metastatic disease used the same vaccine preparation and administration schedule, but combined the vaccination with either IL-2 or GM-CSF. SD was noted in 33% of patients (3 of 9) who received the vaccine with IL-2, and 20% (2 of 10) of patients who received the vaccine with GM-CSF had no progression of disease.³⁷⁷ Based on the dismal natural history of pancreatic cancer, establishing SD could be considered successful.

Another trial using intradermal injections of peptide corresponding to a k-ras mutation along with GM-CSF was performed with pancreatic cancer patients after surgical resection (*n* = 10) or with advanced disease (*n* = 38). Patients received vaccination weekly for 4 weeks, then received booster vaccination at weeks 6 and 10. Immune responses were measured by local DTH reactions, proliferative T cell responses from peripheral

blood, and survival. Twenty-one of 43 patients exhibited a positive DTH response after vaccination. Proliferative T cell responses were seen in 17 of 43 patients. The mean survival for resected patients was 25.6 months. Of 34 patients with advanced disease, 11 maintained SD after vaccination. All 11 patients with SD exhibited an immune response to the vaccination, but not all patients with an immune response had nonprogression of disease. Of those patients who did demonstrate an immune response, the improvement in average survival was statistically significant (148 days versus 61 days, $P = 0.0002$).³⁷⁸ Further trials using k-ras mutation vaccinations after surgical resection of pancreatic cancer are currently in progress.

Several other pancreatic cancer-associated antigens have been identified and are being evaluated both in vitro and in vitro as potential immune targets. These antigens include the oncogenes HER-2/neu and CaSm, tumor suppressor gene p53, carbohydrate antigens CA19 to 9 and CA242, and glycoprotein GA733. Further work is needed to fully characterize the roles that these antigens may play in immunotherapy for pancreatic cancer.

Tumor Lysate and Whole Cell Vaccine Approaches

In a preclinical model, human DC were pulsed with tumor lysate of an HLA-matched pancreatic cancer cell line. These DC were activated with TNF- α and PGE-2, then cultured with mononuclear cells. After 4 stimulations, CTL-mediated cytotoxicity specific for the pancreatic cell line was seen.³⁷⁹ This work demonstrated the feasibility of using tumor lysate-pulsed DC to generate a pancreatic cancer-specific CTL response in vitro and suggests a potential clinical vaccine application.

Irradiated tumor cells provide another possible agent for vaccination. Despite being able to isolate and grow pancreatic cancer cells, they have not been largely immunogenic. To increase the immunogenicity of tumor cell vaccines, cytokine and co-stimulatory genes have been transduced into pancreatic cancer cell lines.^{380,381} A phase I dose-escalation trial evaluated the safety and immune response of an irradiated allogenic pancreatic tumor cell line vaccine that was transfected to secrete GM-CSF.³⁸² Fourteen patients with resected pancreatic carcinoma received different doses of vaccine 8 weeks after pancreaticoduodenectomy. Patients then underwent 6 months of adjuvant chemoradiation therapy and were reassessed for status of their disease. Six of the original patients were still in remission and they received additional monthly vaccinations for 3 months. The main side effects of vaccination were local reactions at the injection site. Three of 7 patients who received at least 1×10^8

vaccine cells demonstrated DTH reactions to autologous tumor cells after vaccination. Furthermore, these 3 patients had prolonged disease-free survival compared with nonresponders, remaining free of recurrence at 25 months follow-up. Further trials evaluating whole cell tumor vaccines with GM-CSF for both resected and metastatic pancreatic cancer patients are in progress.

Immunotherapy for Gastric Cancer

Immunotherapy of gastric cancer has been hindered by a general lack of tumor-associated antigens, and thus most research has focused on nonspecific modulators of the immune system.

Nonspecific Immunotherapy for Gastric Cancer

Polysaccharide K (PSK), a protein-bound polysaccharide extracted from the *Coriolus versicolor* strain of *Basidiomycetes*, which is thought to activate the immune system by competing with immunosuppressive factors³⁸³ and inducing several immune-potentiating cytokines,³⁸⁴ has been studied extensively in Japan. Several investigators studied this immunomodulator throughout the 1980s in Japan.³⁸⁵⁻³⁸⁷ This experience culminated in the establishment of the Study Group for Immunotherapy with PSK to further test the survival benefit of this immunomodulation. Addition of PSK to an adjuvant regimen of 5-FU and mitomycin C in margin-negative T2 and T3 gastric cancer led to a survival improvement from 60% (5-FU and mitomycin-C alone) to 73% at 5 years.³⁸⁸ Subgroup analysis of which tumor characteristics predict favorable responses to treatment found that those with an HLA-A2 haplotype had a better response to PSK.³⁸⁹ The reasons for differences in response based on HLA haplotype are unclear, but may involve tumor-associated antigens. Further research is necessary to define such antigens.

Another agent that has been tested extensively in Japan is picibanel (OK-432). OK-432 is a heat-inactivated extract from *Streptococcus pyogenes* A3, which is thought to modulate the cellular immune system by inducing the production of ILs, IFNs, and TNF. This immunomodulatory adjuvant has been administered in several ways. Systemic administration in conjunction with 5-FU and mitomycin C led to an increase in the 5-year survival rate from 29.8% to 45.3% over 5-FU and mitomycin alone in patients with stage III gastric cancer.³⁹⁰ Other investigators have studied intratumoral injection of OK-432 endoscopically before surgical resection. This method led to no survival benefit when the entire cohort was analyzed, but subgroup analysis showed a survival benefit for those with stage III disease.³⁹¹ Finally, OK-432 has been administered intra-

peritoneally to try to prevent recurrence. Combined with systemic dosing of mitomycin C and tegafur-uracil, intraperitoneal OK-432 led to a 40% increase in freedom from peritoneal recurrence.³⁹² In addition to its use in the adjuvant setting, OK-432 has been administered to patients with advanced gastric cancer. One group in Japan combined intraperitoneal OK-432 with IL-2 in patients with malignant effusions secondary to gastric cancer. Responses were seen in 81% of the 22 patients treated with this regimen.³⁹³

Immunotherapy of Primary Brain Tumors

Primary brain tumors continue to pose a significant challenge to both medical and surgical neuro-oncologists. More than 17,000 new primary brain tumors are diagnosed annually in the United States,³⁹⁴ and the incidence is increasing.³⁹⁵ Unfortunately, the most aggressive subtypes, glioblastoma multiforme (GBM) and anaplastic astrocytoma, are also the most common histologic types.³⁹⁶ High grade gliomas are highly infiltrative, and complete surgical resection is impossible without unacceptable damage to eloquent regions of the normal brain. Adjuvant treatment strategies, including radiotherapy and chemotherapy, continue to be associated with a poor prognosis. Furthermore, lack of specificity of both radiation and chemotherapy for malignant cells has led to unacceptable CNS or systemic toxicity with the most effective regimens.³⁹⁷ The median survival of patients with newly diagnosed GBM is only approximately 9 months, with only 10% of patients alive at 2 years.³⁹⁸ Following recurrence, patients succumb to the disease with median progression-free and overall survivals of only 9 and 25 weeks, respectively.³⁹⁹ Given these outcome data, our center and others have aggressively investigated innovative therapeutic strategies such as immunotherapy to treat patients with these tumors.

Regional Delivery of Radiolabeled Monoclonal Antibodies

As a result of the blood-brain barrier and other physiologic barriers such as increased interstitial pressure within the tumor that discriminate against larger molecules, treatment of CNS tumors with MAbs delivered systemically results in relatively ineffective concentrations at the tumor site. For example, human imaging studies using a MAb demonstrated that after systemic intravenous administration only from 0.0006% to 0.0043% of the total injected dose localized to the tumor.^{400,401} Intraarterial delivery directly into the carotid artery has been similarly disappointing.⁴⁰² As a result, surgical intervention to allow regional delivery of these agents

through the placement of drug reservoirs into surgically created resection cavities (SCRC) or the stereotactic placement of intralesional infusion catheters has become a necessary adjunct to the treatment of brain tumors with these large therapeutic agents. Regional therapy is a particularly attractive modality for the treatment of primary brain tumors because neoplastic disease in these cases is isolated to the cranial compartment so systemic therapy is not even necessary.

Delivery of radiolabeled MAbs into SCRCs bypasses the blood-brain barrier and delivers the therapy directly to the tumor while minimizing systemic exposure. In a recent promising phase I/II clinical trial, bolus injection of ^{131}I -labeled 81C6 MAb, targeting the extracellular matrix protein, tenascin, into closed SCRCs resulted in posttreatment median survival times of more than 60 weeks in patients with recurrent brain tumors.⁴⁰³ Subsequent clinical trials have also suggested a potential survival benefit of ^{131}I -labeled 81C6 injected into SCRCs for patients with newly diagnosed malignant gliomas as well.^{403,404} The median survival for all patients in this study was 86.7 weeks, and was 79.4 weeks for those with GBM. Unfortunately, many patients with malignant brain tumors remain ineligible for this promising mode of immunotherapy because their tumors are either not safely resectable or a closed SCRC cannot be created.

Convection-Enhanced Delivery of Targeted Toxins and Unarmed Antibodies

In contrast to radiolabeled MAbs, which target specific proteins on the cell surface, but exert a regional therapeutic, but also toxic, effect as the result of the radioligand, several newer biologic agents have enhanced specificity based on their need for direct cell contact for cytotoxic activity. However, diffusion of these high molecular weight agents into the brain, even after regional delivery, is severely limited because the rate of diffusion is inversely related to the size of the agent, and is usually slow relative to tissue clearance.⁴⁰⁵ Thus, diffusion results in heterogeneous distribution of such agents with steep concentration gradients between the point of delivery and the advancing tumor border. This restricts the distribution of these therapeutic agents to within a few millimeters of the site of delivery, and lethal tumor cell populations that exist a few centimeters beyond the contrast-enhancing tumor border escape exposure to the therapeutic agent.

Delivery of high molecular weight agents vast distances throughout the intracerebral compartment can be achieved by using relatively high pressures to very slowly infuse the drug directly into and around the

tumor site using a technique called convection-enhanced delivery (CED).⁴⁰⁵⁻⁴⁰⁸ In contrast to diffusion, CED is dependent only on hydrostatic and oncotic pressure gradients that naturally exist or that can be induced within the extracellular spaces of the brain. CED has been shown to enhance the distribution of MABs and other large molecules in the normal rat, cat, primate, and more recently human brain (J. H. Sampson and colleagues, unpublished data) by an order of magnitude relative to diffusion, and has produced a homogeneous distribution of the agent throughout the infused region. Using flow rates from 0.5 to 6.0 $\mu\text{L}/\text{min}$, the apparent volume of distribution has exceeded the volume of infusion by 5- to 10-fold in animal models.⁴⁰⁵⁻⁴⁰⁹ Thus, CED allows infusion of immunotherapeutic constructs to unresectable portions of tumor and saturates tumor cells invading the brain far from the site of infusion.

Protein toxins produced by bacteria and plants are 1 example of cytotoxic agents that have been delivered effectively by CED. These toxins can be coupled to targeting ligands that bind to neoplastic cells directly and are internalized to produce a potent cytotoxic effect. Two such toxins are the *Pseudomonas* and *Diphtheria* exotoxins. The mechanism of action of targeted toxins may have important advantages over that of other traditional chemotherapeutic agents as well. First, they are effective against hypoxic cells. Second, these cytotoxins are far more potent and may require that only 1 toxin molecule reach the cytosol to kill a single tumor cell, whereas most conventional chemotherapeutic drugs often require more than 10^4 molecules/cell to cause cell death.⁴¹⁰ The conjugation of these lethal cytotoxins with a tumor-specific targeting molecule creates a potent and specific antitumor agent.

One example of a targeted toxin used clinically for patients with brain tumors is TP-38. TP-38 is a recombinant chimeric protein composed of the epidermal growth factor (EGFR) binding ligand, TGF- α , and a genetically engineered form of the *Pseudomonas* exotoxin. Replacing the native binding domain of the exotoxin with TGF- α selectively targets the toxin to cells expressing the EGFR. Because human malignant gliomas and many malignant tumors that metastasize to the brain overexpress EGFR,^{411,412} yet EGFR is expressed at only very low levels in normal brain, a therapeutic window exists for targeting the EGFR in malignant brain tumors.⁴¹³

The dramatic therapeutic potential of TP-38 has already been demonstrated in mice. Mice EGFR-expressing brain tumors treated intratumorally with 0.03 μg and 0.1 μg of TP-38 had 100% and 90% long-term

survivors, respectively, as compared with saline-treated controls that all died with a median survival of 19 days.

A phase I/II dose-escalation clinical trial is currently underway to assess the potential toxicity and efficacy of TP-38.⁴¹⁴ Twenty patients with recurrent malignant gliomas have been enrolled in this study. TP-38 is delivered by CED directly into the tumor via 2 surgically implanted, stereotactically placed catheters. Two of 15 (13.3%) patients with residual tumor at the time of therapy have demonstrated radiographic responses. One patient, treated for a multi-recurrent bifrontal GBM with 40 mL of TP-38 (25 ng/mL), has had a nearly complete response. She is alive, well, and off corticosteroids more than 83 weeks after being treated with TP-38. Another patient, treated with 40 mL of TP-38 (100 ng/mL), has had a partial response with greater than 50% shrinkage of his tumor 24 weeks after TP-38 therapy.⁴¹⁴ The overall median survival after TP-38 therapy for all patients is currently 21.6 weeks.

Another ligand that has been investigated for targeting these toxins is transferrin. The transferrin receptor is overexpressed on any rapidly dividing cell including glioma and other cancer cells due to the increased iron requirement of these cells. However, this receptor is not generally present on most normal cells in the brain. By conjugating *Diphtheria* toxin to transferrin, a selective targeted toxin conjugate is produced that binds to the transferrin receptor on glioma cells and selectively kills these cells. This immunoconjugate (TF-CRM107) has also proven to provide a significant dose-dependent antitumor effect in a mouse model of intracranial glioma.⁴¹⁵ In a recent phase I trial of this transferrin-*Diphtheria* conjugate, patients with tumor volumes ranging from 2 to 50 cm³ were treated with infusion volumes ranging from 5 mL to 180 mL. There were no infusion-related deaths or life-threatening toxicities.⁴⁰⁸

Transient neurological worsening was evident in only 3 of 44 infusions. These adverse events were restricted to patients with significant pretreatment edema and mass effect and always resolved with completion of the infusion or temporary treatment with steroids or hyperosmotic therapy. Only 4 patients had a seizure during the infusions; all of these also had seizures before the infusions and only 2 of these required adjustments in antiseizure medications. Currently, further clinical trials are investigating the efficacy of this promising therapy.

Another example of immunotherapeutic agents that require direct cell contact to be effective antitumor reagents is unarmed antibodies. Several unarmed MAbs have had clinical success in treating systemic cancers including Herceptin,⁴¹⁶ anti-HER2/*neu* MAb used against breast cancer, and Rituximab, an anti-CD20 MAb effective against B cell lymphomas

expressing this antigen. Recently, successful intratumoral CED of unarmed MABs for therapy of intracranial tumors has been reported as well. For example, a recent study has demonstrated the efficacy of intratumoral CED of Herceptin against HER2/*neu*-expressing tumors in a murine model of intracranial metastatic breast cancer.⁴¹⁷ Unarmed MABs have also been investigated in experimental models of primary brain tumors. Direct intracerebral delivery of an unarmed MAB specifically targeting a tumor-specific mutation of the EGFR (EGFRvIII), which is overexpressed on almost one half of all malignant gliomas,⁴¹⁸ increased the median survival by 274% in a murine model.⁴¹⁹ The antitumor effect in this model system was likely mediated by ADCC. This work suggests a potential therapeutic effect for unarmed anti-EGFRvIII humanized MABs in human clinical trials.

Adoptive T Cell Therapy

Adoptive T cell immunotherapy for primary brain tumors has several potential advantages. First, T cell recognition of tumor antigens is highly specific, which, as mentioned above, allows for a maximal antitumor effect with minimal related toxicity to the normal surrounding brain. Second, once activated, a T cell response persists as tumor-specific memory T cells that potentially provide protection against recurrence of disease even long after the treatment is complete. Unfortunately, most attempts at systemic T cell immunotherapy for primary brain tumors have been largely unsuccessful.^{420,421} Additionally, attempts at intralesional T cell immunotherapy have also been disappointing.⁴²²⁻⁴²⁴ Although anti-tumor T cell response can be demonstrated in the brain, gliomas are thought to release immunosuppressive cytokines that may be responsible for the decreased response of primary brain tumors to T cell immunotherapy. Additionally, T cell-stimulating cytokines, such as IL-2, which have proven to be a necessary adjuvant to successful T cell immunotherapy of systemic tumors, cause peritumoral edema in brain tumors, which is a dose-limiting toxicity.⁴²⁵ A recent strategy has been to extract T cells from tumor-draining lymph nodes that have been specifically activated and stimulate them *ex vivo* before returning them to the host to maximize the antitumor response *in vivo*. Tumor specimens from the initial surgical resection are taken to the laboratory and grown in culture, isolated, and then irradiated. The irradiated cells are then suspended in a solution with GM-CSF and administered as an intradermal vaccination to the patient. After several days, the lymph nodes draining the site of vaccination are removed and the antitumor-activated T cells are harvested. These tumor-specific T cells can be expanded through the use of bacterial superanti-

gens⁴²⁶ or through the activation of the CD3 T cell receptor with anti-CD3 MAbs and cytokines.⁴²⁷ The stimulated T cells are then injected back into the patient systemically. A recent phase I clinical trial examined this treatment strategy in 12 patients with malignant gliomas.⁴²⁸ In all, the treatment was generally well tolerated, with the only major toxicity being an increase in seizure frequency in 2 of the 12 patients. One patient developed a DTH reaction at the site of vaccination. Although this was a very small patient sample, the clinical observations were promising. Eight of the patients had residual tumor following resection before the T cell therapy; of these, 4 had radiographic evidence of tumor regression following T cell transfer. Currently, a larger phase II study is underway to determine whether this is an effective treatment modality.

DC-Based Active Immunotherapy

Although primary brain tumors express tumor-specific antigens, an effective CTL response against the brain tumor obviously is lacking. This may be the result of local immunosuppressive cytokines expressed by the tumor, the decreased presence of T cells and APC in the CNS, or inefficient presentation of antigen. One antitumor immunotherapeutic strategy is to enhance and activate the native CTL response against intracranial tumors by using DCs pulsed with tumor antigen. DCs are highly potent APC and have shown promise as an effective treatment modality for several other systemic cancers as well.

Following several promising animal trials of glioma-antigen-pulsed DCs, several groups have begun human clinical trials of DC-based treatments for glioma. In the first published phase I trial, 9 patients underwent surgical resection and radiation therapy for gliomas.⁴²⁹ DCs from the patients' peripheral blood were harvested and grown in culture with IL-4 and GM-CSF. Following surgical resection, the patients' tumor cells were grown in culture, tumor-specific surface peptides were extracted, and the DCs were incubated with tumor peptide. These tumor antigen-pulsed DCs were then reintroduced to the patients' systemic circulation. The treatment proved relatively safe, and encouraging survival statistics were presented.

A potential limitation of specific concern for the immunotherapy of brain tumors is the potential for inducing cross-reactive autoimmunity. In light of the documented expression of normal and fetal brain antigens on human glioma cell lines⁴³⁰ and brain tumor tissue,^{431,432} active immunization with untested or unselected antigens risks inducing an uncontrolled autoimmune response against normal CNS antigens similar to experimental allergic encephalomyelitis (EAE). The clear susceptibility of humans

to the induction of EAE was discovered accidentally when patients were vaccinated against rabies with spinal cords from rabbits that were infected with the rabies virus. Given the range of protocols that routinely use immunization with CNS tissue for the production of lethal EAE in nonhuman primates and the documented susceptibility of humans to EAE, the induction of such autoimmune responses as a result of immunization strategies is of particular concern. In response to this concern, a clinical trial has been initiated that uses DCs pulsed with PEP-3, a peptide fragment spanning the tumor-specific mutation of the EGFR (EGFRvIII). Using a tumor-specific peptide antigen such as PEP-3 may reduce the risk of autoimmune complications. Immunologic monitoring data from this trial has confirmed that PEP-3-KLH-pulsed DCs can elicit a potent immune response in patients with gliomas without inducing autoimmunity.

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