British Journal of Cancer (1998) 77(5), 683–693

Review

Biological therapy: approaches in colorectal cancer

Strategies to enhance carcinoembryonic antigen (CEA) as an immunogenic target

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In the UK, almost 20 000 people die each year from colorectal cancer. Despite a potential curability rate of 70% or greater, the overall survival at 5 years is just over 30%; a figure that has changed little over the last 4 decades despite advances in adjuvant and therapeutic chemotherapy and radiotherapy (King's Fund Forum, 1990).

Moreover, there is evidence that over half the patients operated upon for cure have occult metastatic disease at the time of initial surgery (August et al, 1994). Recent novel immunocytochemical techniques using immunobead polymerase chain reaction (PCR) for detection of the tumour-associated antigen carcinoembryonic antigen (CEA) have permitted the identification of single malignant cells in peripheral blood samples, bone marrow aspirates and peripheral stem cell harvests through the recognition of unique hybrid gene transcripts. (Schlimok et al, 1990; Lindeman et al, 1992; Hardingham et al, 1993; Johnson et al, 1995).

Although the presence of such cells in the bone marrow at the time of preliminary colon resection appears to be associated with a worse prognosis (Riethmuller and Johnson, 1992) the number of cells that correlates with a poor outcome is unknown, and the relationship between the development of secondary disease and circulating tumour cells remains poorly understood (Osborne et al, 1991). Phage cloning and hybridization have taken advantage of the limited but specific genetic alterations in developing large bowel neoplasms to detect *ras* mutations in colorectal cancer cells in the stool (Sidransky et al, 1992). The demonstration of small tumour burdens of this type in which cells are exposed in unshielded mesenchymal locations may provide relatively novel immunotherapeutic and chemoimmunotherapeutic targets and identify surrogate end points in treatment that may prove superior to crude survival time.

Immunotherapeutic strategies in advanced colorectal cancer have generally met with little success. Conventional treatments have largely relied on either interleukin 2 (IL-2) or adoptive IL-2-stimulated tumour infiltrating lymphocytes (TILs), with only sporadic reports of tumour regression (Rosenberg et al, 1989; Kradin et al, 1989). Recently, there has been a resurgence of interest in immunotherapy (and the potential of gene therapy) in advanced colorectal cancer and in an adjuvant setting. The adjuvant use of the murine monoclonal IgG_{2a} antibody, 17-1A directed against the CO 17-1A surface epitope of CEA (found in up to 80% of colorectal carcinomas) has resulted in an improvement in disease-free survival and a reduction in locoregional recurrence rates of almost 30% in patients with Dukes' C carcinoma compared with untreated controls (Riethmuller et al, 1994). Carcinoembryonic antigen (CEA), a surface-expressed tumour-associated antigen, is a well-characterized glycoprotein represented in high density on most malignant tumours of the gastrointestinal tract (Muraro et al, 1985). The immunogenicity of CEA as a potential target antigen in colorectal cancer is at present unclear, with variable reports of inducible humoral and cell-mediated responsiveness to CEA epitopes in patients with different stages of disease. There is much that remains unknown regarding the natural immunological response to a native antigen such as CEA both in terms of its antigenic processing and its potentially immunodominant epitopes.

This review assesses the role of CEA as a 'natural' autoantigen along with strategies that render epitopes of CEA potentially immunogenic. This may be achieved by the use of xenogeneic, chimaeric, humanized or wholly human monoclonal and polyclonal antibodies and with anti-idiotypic therapy. The advantages and limitations of each strategy and their potential role in the treatment of advanced colorectal cancer are discussed.

Received 11 June 1997
Revised 9 September 1997
Accepted 12 September 1997

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MECHANISMS OF TUMOUR ESCAPE FROM IMMUNOLOGICAL RECOGNITION

The variability of tumours permits their escape from immune recognition. An improvement in the understanding of the immunobiology of cell-mediated anti-tumour defences as well as a better knowledge of tumour recognition molecules expressed on the surface of many tumours has permitted the development of new anti-tumour strategies to stand alongside conventional chemotherapy and radiotherapy in colorectal cancer.

Isolated tumour cells are able to be eliminated by several conventional immunological mechanisms, most notably antibody dependent cellular cytotoxicity (ADCC) (Steplewski et al, 1983; Adams et al, 1984) complement-dependent cytolysis (Herlyn and Koprowski, 1981) and apoptosis (Trauth et al, 1989). Knowledge of cell surface regulatory molecules expressed on tumour cells may enhance natural apoptosis and tumour regression (Wyllie et al, 1980).

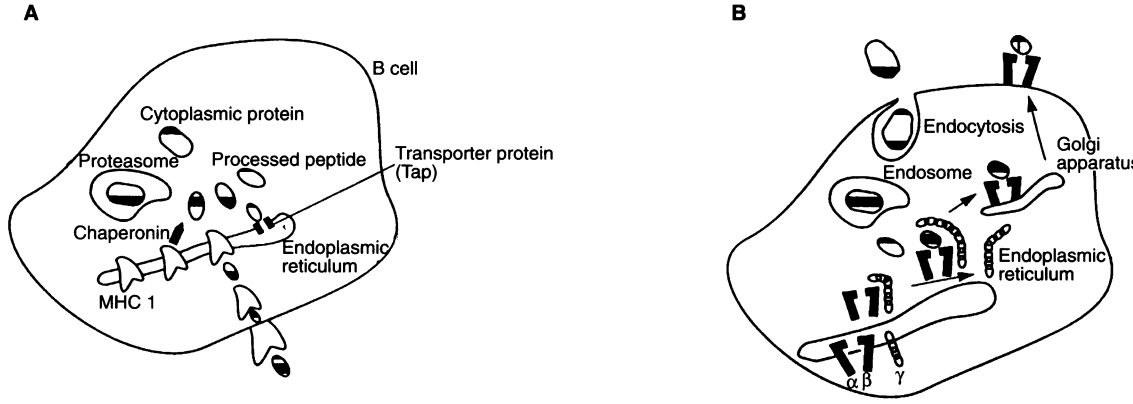

Figure 1 Mechanism of MHC molecule antigen processing. (**A**) Class 1 MHC antigen processing. Proteasomes digest cytoplasmic protein into processed peptides (eight or nine amino acids in length). These peptides adhere to the endoplasmic reticulum by polymorphic transporter proteins (Tap-1 and Tap-2). Chaperonin molecules detain empty MHC class I molecules in the endoplasmic reticulum for association with processed antigen and transfer to the cell surface. (**B**) Class II MHC antigen processing. Foreign antigen is endocytosed and after processing (peptides 15–25 amino acids in length), the peptide is aggregated in the Golgi apparatus with α, β and γ components of the MHC class II molecule formed in the endoplasmic reticulum. After complexing with foreign peptide, the γ-chain is degraded and the αβ heterodimer/processed antigen is expressed on the cell surface for Th TcR recognition. The mechanism of transport of the complex from the Golgi apparatus to the cell membrane is unknown

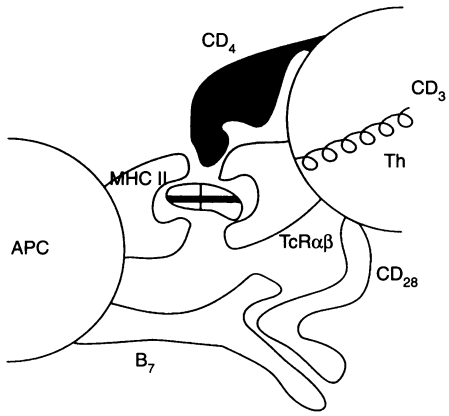

Figure 2 Mechanism of T-cell receptor (αβ) complex interaction with antigen presenting cell. T cells have a dual specificity for MHC molecules and processed antigen. The TcR αβ is complexed with CD3, which has an intracytoplasmic component for signal transduction after occupancy with processed antigen. The CD4 molecule secondarily interacts with MHC class II to produce local cytokines (IL-2 and IFN-γ) for Th cell support and transformation of B cells. CD 28 initiates signal transduction independently to augment local cytokine production using B7 as a ligand molecule

The finding of unique tumour-associated antigens (TAAs) in solid malignancies has presented a range of important targets for adoptive humoral and cellular immune therapies. Early attempts to define TAAs in murine models by immunizing mice against either spontaneous tumours or chemically and virally induced tumours were confused by general species reactivity to normal transplantation antigens. The development of syngeneic mice with identical histocompatibility antigen expression systems permitted cutaneous but not tumour transplantation, implying the presence of tumour-specific antigenicity. Most work on tumour immunology has centred on cytotoxic T-cell (CTL) activity since the demonstration that the ability to reject tumours can be adoptively transferred by lymphocytes and not by serum.

The recognition of foreign tumour antigens requires a presentation to immunocytes in the form of target fragments linked to the major histocompatibility complex (MHC) class I and class II molecules interacting with the T-cell receptor (TcR) mechanism. Intracellular antigens, such as oncogene products and extracellular (or foreign) antigens, are handled differently by the immune system with the development of parallel but separate mechanisms for dealing with these foreign challenges. Intracellular antigens produce processed peptides (usually eight or nine amino acids in length), which are generally presented to CD8+ cells (T-suppressor/cytotoxic lymphocytes) by MHC class I molecules. Extracellular antigen is processed for presentation as 15–25 amino acid length peptides to CD4+ cells (T-helper cells) by the MHC class II molecules found on specialized (so-called professional) antigen-presenting cells, such as dendritic cells, macrophages or B lymphocytes. The mechanisms of MHC-restricted antigen processing are shown in Figure 1.

Figure 2 shows the complex activation process of the T cell receptor.

Tumour cells have developed several mechanisms to inhibit immunological attack for survival advantage in hostile locations.

The failure of a tumour to induce a specific rejection response may partly be a result of the poor expression of foreign TAAs, such as CEA. Knowledge of the intricate mechanisms involved in foreign antigen presentation has permitted the development of potential genetic targets to overcome heterogeneity of surface antigen expression. The discovery of co-stimulatory signals has created a model of lymphocyte and specifically TcR activation (Bretscher and Cohn, 1970), whereby processed antigen may be linked either to the MHC complex or to a co-stimulatory ligand in its presentation to the T cell.

Co-stimulatory signals implicated in the activation of the TcR include tyrosine kinases, interleukins, the B7 family (Chen et al, 1993), the ICAM group, lymphocyte function-associated antigens, vascular cell adhesion molecules (VCAM-1) and heat stable antigens (HSA).

Studies of immunological tolerance have also assisted in the understanding of mechanisms of tumour escape from immune

surveillance and lysis. The exposure of both the T- and the B-cell lineage to TAAs may result in clonal selection, with proliferation of immunocompetent effector cells, clonal anergy with down-regulation of immunogenic capacity or effector maturation arrest (Nossal and Pike, 1980; Goodnow et al, 1991). The concept of a population of down-regulated T- and B-cell repertoires with a threshold affinity for silencing was advanced by Nossal (1983) as one of 'immune ignorance'. T-cell tolerance of this type, which is part of normal thymocyte maturation of medullary CD4+ and CD8+ cells, permits the acquisition of TcR molecules with a high affinity for self-MHC and self-epitope recognition (Miller and Moralan, 1992; Shortman, 1992). How important this model is, however, outside in vitro systems is not known. Immune ignorance as opposed to T-cell deletion or anergy appears to be a more complex phenomenon and is secondary to a differential inability of lymphocytes in the periphery to recognize antigenic motifs in restricted tissue sites.

B-cell tolerance is clearly physiologically important too, as it prevents the development of a range of naturally produced auto-antibodies against cross-reactive self-epitope. This type of auto-antibody phenomenon, although common, is fortunately transient. The decision between clonal anergy and clonal ignorance is probably dependent upon the affinity of the B-cell receptor for the antigen concerned as well as the antigenic molar concentration. Anergy is favoured in states of very high antigen concentration, strong antigen cross-linking and high antibody affinity.

Widespread extracellular antigen expression (of CEA, for example) may therefore have already induced substantial T- and B-cell tolerance of the types mentioned. As a result, any anti-tumour vaccine based upon a native protein needs to either couple the epitope for recognition with another highly immunogenic carrier molecule (a so-called adjuvant) or use secondary strategies to enhance its immunogenicity.

Intratumoral variation may also provide an avenue for tumour escape from immunological attack. Previously, the main markers for tumour heterogeneity were morphological, biochemical and karyotypic, but increasingly there is recognized to be both molecular biological and immunohistochemical variability within tumour cell subpopulations that may affect immunotherapeutic and chemotherapeutic response. The multistep nature of colorectal carcinogenesis postulates potential mechanisms for intratumoral heterogeneity. At its simplest level, differences in tumour differentiation and tumour DNA ploidy may be reflected in differences in outcome. Tumour aneuploidy has been shown to correlate with overall prognosis in ovarian, renal cell, thyroid, adrenal and breast cancer (Rodenburg et al, 1987; Hamming, 1988; Oosterwijk et al, 1988; Haak et al, 1993; Hedley et al, 1993).

Finally, tumour heterogeneity may be a reflection of the underlying host immune defence systems. Potential mechanisms for immunological escape by tumour cells include changes in the structure of crucial molecules, such as MHC activation ligands, regulators of complement activation, lytic enzyme neutralizers and adhesion molecule receptors.

Disturbances in B-cell-mediated responsiveness may occur through other mechanisms, such as insufficient neoantigen presentation, relative immunological isolation (in areas such as the central nervous system or in ocular tumours), tumour-produced suppression by local inhibitors (prostaglandins and transforming growth factor beta) and drug-induced immunosuppression. In this sense, the immune system contributes to the phenotypic heterogeneity of the tumour.

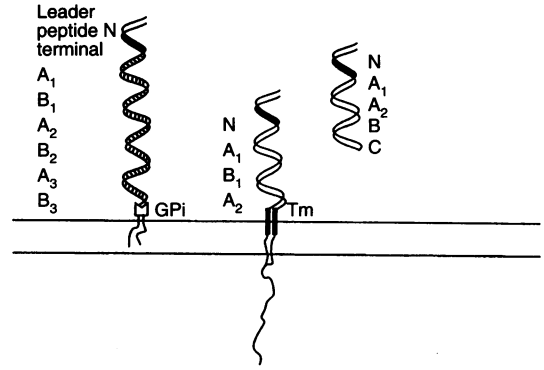

Figure 3 Representation of the carcinoembryonic antigen family. Ig-like domains are shown as ribbons. The black N domain is Ig v-like and the striped domains are Ig C-like. Ig C regions are repeated extracellularly into discrete domain regions A1–3 and B1–3. In the CEA molecule the membrane attachment is by a glycosyl phosphatidyl inositol anchor. Biliary glycoproteins (BGP) are membrane attached by a hydrophobic transmembrane region with a relatively long intracytoplasmic tail for signal transduction. Pregnancy-specific globulins (PSG) are actively secreted from the cell. GPi, glycosyl phosphatidyl inositol hook; Tm, ... transmembrane attachment

CEA AS AN IMMUNOGENIC TARGET: STRUCTURE AND FUNCTION

Knowledge of the molecular structure of CEA defines recognition epitopes as immunological targets and assists in the design of monoclonal antibody therapy directed against cell-based TAA. This is particularly important in a molecule such as CEA, in which extensive carbohydrate moieties mask epitopes.

Molecular cloning at the cDNA level has permitted the identification of at least 29 CEA-related genes that are tightly clustered on a 1.2-Mb region located on the long arm of chromosome 19 (Tynan et al, 1992).

Comparisons of related family members have shown high sequence conservation (80–95% of N domain exons within subgroups and 65–70% homology between subgroups). The CEA gene along with that for non-specific cross-reacting antigen (NCA) and the biliary glycoproteins (BGP) comprise the CEA subgroup and the pregnancy-specific globulins (PSG) the other group of related genes. The basic common domain structure of the CEA subgroup (Figure 3) incorporates a variable number of internal repeating subunits that have equivalent secondary and tertiary structure to the immunoglobulin C_2 domains. The N domain of the molecule is structurally homologous, with the immunoglobulin V-like domain rendering the CEA family within the immunoglobulin superfamily of molecules (Williams and Barclay, 1988).

CEA is attached to the cell membrane by a glycosyl phosphatidylinositol (GPi) hook that is structurally distinct from the transmembrane attachment of the PSG group member proteins (Hefta et al, 1988; Thompson and Zimmerman, 1988; Ferguson, 1991; Thompson et al, 1991).

The homology of CEA with basic immunoglobulin molecules gives a clue as to the functional role of CEA-related antigens. Those containing C_2 domains are involved in cell adhesion. CEA-transfected cells have been shown to have adhesive properties through both homophilic and heterophilic interactions. Selective binding between carbohydrate moieties on CEA and lectin molecules on bacterial fimbriae may permit CEA to regulate gut–bacterial binding and control lymphocyte homing during gut

inflammation (Leusch et al, 1990). As a result, the regular luminal shedding of CEA may serve to control luminal bacterial load and translocation across the gut.

Many studies with regard to differential expression of CEA-related proteins during development have been produced in animal models that do not normally possess endogenous CEA-like species. The specificity of both polyclonal and monoclonal antibodies to CEA for immunohistochemistry has been enhanced by the use of cDNAs in expression vector systems producing a series of stable transfectant eukaryotic clones expressing most of the major CEA-related protein products (Arakawa et al, 1990; Berling et al, 1990; Hefta et al, 1990).

Over 50% of the CEA molecule is glycosylated with at least 28 separate putative sites of carbohydrate attachment. The position and density of these sites affect the exposure of potential antigenic recognition surfaces and, because of the heavy glycosylation of the molecule, crystallization for diffraction studies and structure interpretation have been difficult (Bates et al, 1992). Knowledge of these binding sites will permit the engineering of antibody molecules and other immune targeting agents based on CEA epitope structure that have slower off rates and that facilitate tumour retention by the antibody molecule (Boehm et al, 1996).

Limitations should be placed on extrapolation of data regarding the inherent immunogenicity of CEA within such models. For this work, it has been necessary to introduce CEA gene-regulatory plus indicator segments into transgenic mice as well as to produce gene inactivation by homologous recombination in induced colonic tumours that subsequently express human CEA (Eades-Perner and Zimmerman, 1995).

Recently, the novel anti-CEA monoclonal antibody PR1A3 has been successfully used in radioimmunoscintigraphy for the detection of CT-negative and CEA-negative recurrent colorectal cancer as well as to investigate the cell-based epitope domain of CEA (Granowska et al, 1989, 1993; Durbin et al, 1994). This antibody was originally produced in mice against components of normal human colonic epithelium and has been demonstrated to be highly sensitive by immunohistochemistry for nearly all human colorectal carcinomas, regardless of differentiation. It is highly specific with only minor cross-reactivity in normal respiratory epithelium (Richman and Bodmer, 1987). The significance of this antibody is its inability to bind circulating and purified CEA or CEA released from tumours and sequestered in lymph nodes. This epitope is not expressed by bacteria transfected with CEA fusion genes, implying the importance of post-translational modification and/or conformational changes in CEA once shed. The use of CEA-BGP chimaeric peptides has localized the PR1A3 epitope to the C terminus of the protein and binding is entirely reliant upon the presence of a small spacing peptide between the GPi anchor mechanism and the BGP recombinant construct (LM Stewart and D Snary, personal communication). This spacing peptide is believed to lift the B3 domain away from the cell membrane and permit antibody binding to the epitope. The exact mechanism of abrogated binding to circulating antigen is unknown, but may involve partial domain loss, the formation of steric hindrance by dimers of CEA or disruption of the epitope on release from the cell.

Immunotherapeutic strategies making use of murine (and humanized) PR1A3 will have the advantage of providing surrogate responses to cell-based TAA, avoiding circulating complex formation. At present, a phase I/II trial using murine PR1A3 in chemo-relapsed colorectal cancer has commenced at our institution.

HUMORAL AND CELL-MEDIATED RESPONSES TO CEA

There is conflicting evidence that CEA functions as a natural immunogen in patients with advanced epithelial malignancy. It has traditionally been supposed that CEA is likely to have induced a state of tolerance and that this is compounded by the relative anergy evident in patients with advanced disease (Monson et al, 1986).

Some studies have consistently demonstrated the presence of circulating specific anti-CEA antibodies by indirect haemagglutination (Gold, 1967), radioimmunoassay (Gold et al, 1972; MacSween, 1975) and affinity chromatography (Pressman et al, 1980). Moreover, specific immune complexes directed against CEA, when present, have been shown to inversely correlate with overall survival and disease stage (Kapsopulou-Dominos and Anderer, 1979; Staab et al, 1980; Mavligit et al, 1983; Ura et al, 1985; Konstadoulakis et al, 1994). Other groups (Collatz et al, 1971; LoGerfo et al, 1972; Sorokin et al, 1973) have been unable, however, to demonstrate CEA-specific antibodies in the sera of patients with different gastrointestinal neoplasms. Very early studies have shown CEA as non-stimulatory for autologous lymphocytes in in vitro blastogenesis assays (Lejtenyi et al, 1971; Hollinshead et al, 1972; Mavligit et al, 1973*a*), although these reports are difficult to interpret as there are a mixture of potentially anergy-inducing factors inherent in these experiments. Differences in tumour antigen extraction technique, inactivation of CEA during the extraction process and the potential need for presensitized lymphocytes in stimulation assays may all affect the outcome of results (Mavligit et al, 1973*b*).

Recently, molecular cloning techniques have identified a range of tumour-specific peptides (largely in malignant melanomas) that are recognized by autologous MHC-restricted human T cells. It is uncertain whether these peptides are actually normally processed in vivo or whether cytotoxic-specific T-cell repertoires to these agents naturally exist (Slingluff et al, 1993; Wolfel et al, 1994).

Given the recent evidence of natural CTL reactivity against the normal tyrosinase enzyme system in melanoma patients, it is likely that CEA may be sufficiently immunogenic either alone or antigenically enhanced to function as a cancer vaccine (Anichini et al, 1993). Similar MHC-restricted CTLs have been demonstrated in ovarian and renal carcinoma, sarcoma, squamous cell carcinoma of the head, neck and lung and glioblastoma (Miyatake et al, 1986; Slovin et al, 1986; Ioannides et al, 1991, 1993; Finke et al, 1992). It remains unclear why natural tolerance to these peptides is not fully established or why T cells become reactive to self peptides on melanoma, for example, but the same peptides are not recognized by the lymphocytes of patients bearing other cancer histologies.

The potential options for using CEA as a direct immunizing antigen include the use of recombinant vaccinia virus–CEA constructs, polynucleotide CEA vaccination and anti-idiotypic antibodies. Secondary strategies rely on recombinant CEA and CEA-derived peptide booster therapy to maintain specific anti-CEA response.

RECOMBINANT VACCINIA CEA (rV-CEA)

The strategy here is that a relatively weak immunogen is presented with a highly immunogenic viral protein and that the resultant

immune reaction is directed in part against the inserted gene product (Kaufman et al, 1991).

The insertion of stable eukaryotic genes into vaccinia vectors is only a recent development (Edwards and Rutter, 1988). The vaccinia virus is capable of co-presentation of antigen, and constructed vaccinia viruses have been shown to protect animals against infectious disease and tumour challenges (Bennick et al, 1984; Moss et al, 1984; Bernards et al, 1987; Lathe et al, 1987; Moss and Flexner, 1987; Estin et al, 1988). It has been shown that vaccinia virus vectors are stable and that inserted gene products from human colon cancer cell libraries are expressed and normally post-translationally modified (Coupar et al, 1988). Preliminary work has shown the induction in mice of specific anti-CEA antibodies, with reduction in the growth pattern of syngeneic murine colon carcinoma deposits transduced with the human CEA gene.

RV-CEA also induces CEA-specific lymphoproliferative and CTL responses as well as delayed-type hypersensitivity reactions. (DTH) (Kantor et al, 1992*a*). The virus insert approach has also been successfully used in mouse and primate tumour models against the melanoma-associated antigen p97, which is weakly expressed on normal cells, and this has resulted in subsequent protection against tumour challenge with cells expressing the human *p97* gene product (Estin et al, 1988; Hu et al, 1988). Recombinant vaccinia (and other virus) products will not be perfect, however, as there are cross-reactive epitopes for CEA-like species, such as NCA, normally expressed on human (and primate) granulocytes. The hope for clinical use is that immune responses principally occur to the immunodominant epitope located on CEA and that immunotolerance may be greater to the more widely distributed NCA antigen (Nap et al, 1988).

The results of the use of this approach in a rhesus monkey model that displays primate MHC and in which NCA cross-reacting antigen is expressed on normal monkey granulocytes show that it induces proliferative DTH response to intradermal challenge with CEA, proliferative blastogenesis to CEA and also primate-directed antibody-induced lysis of CEA-bearing tumour cells using human effector lymphocytes. The treatment has been shown to be relatively free of side-effects (Kantor et al, 1992*b*). In humans, recombinant vaccinia viruses have been shown to be safe, stable and to have acceptable immunogenicity, even when the individual has been previously exposed to a vaccinia virus as occurs after routine smallpox vaccination (Karzon, 1985; Chelyapov et al, 1988).

A phase I clinical trial has been reported by Hamilton et al (1994) in 26 patients with gastrointestinal, lung and breast cancers using 10^7 plaque forming units (p.f.u.) of rV-CEA at monthly intervals for 3 months. T-cell responses to the vaccinia virus were observed, but there was no response to soluble CEA in blastogenesis assays.

Canarypox (Avipox group) has also been engineered to express the human cDNA of CEA. This virus is restricted, however, in its replication hosts, although it is likely to result in enhanced immunoresponsiveness in those patients previously exposed to smallpox vaccination or when local reactivity to repeated rV-CEA proves to be unacceptable (Hodge et al, 1997).

Antigenic peptides reflecting potential class I epitopes of CEA have recently been selected by screening for matches to consensus motifs of HLA-A2 and A3 binding peptides as the most commonly expressed HLA alleles. The CEA peptides (so-called CAP peptides) identified have been incubated in a T-cell binding assay in which up-regulation of surface HLA-A2 on the T cells was quantified by flow cytometry using an anti HLA-A2 antibody label (Nijman et al, 1993).

Specific T-cell lysis has been generated against autologous EBV-transformed B cells presenting the CAP-1 peptide motif but not against autologous non HLA-A2 EBV-transformed B cells pulsed with the same peptide. Tumour cell lysis of lines transduced with CEA serve as targets for these effector cells, implying that autologous B cells present and process these antigens in an MHC-restricted fashion. Allogeneic SW403 HLA-A2-positive cell lines also expressing CEA function as equivalent targets and non HLA-A2 allogeneic carcinoma cell lines (SW 1417 and HT-29) that do not express substantial CEA are not lysed. This is the first study to demonstrate peptide based CEA-specific CTLs and evidence of MHC-restricted CEA epitope processing by B cells. (Conry et al, 1995*a*).

This type of therapy still requires substantial work. The importance of non-human CEA-like and human CEA-transduced systems in natural immunity is unclear. Epitopes that are immunologically relevant in tumour biology must be able to be stably and consistently coexpressed with dominant immunogenic viral peptides. Tachyphylaxis associated with such approaches still needs to be overcome, but it is evident that troublesome cross-reactivity does not appear to be a clinical problem.

CEA POLYNUCLEOTIDE VACCINATION

This form of active specific immunotherapy may be provided by both DNA and RNA and has certain advantages over tumour cell vaccines. It avoids potentially replicating virus and the need for adjuvants and appears stable in terms of gene product expression and induction of CEA-specific T-cell repertoires. Intracellular synthesis of the TAA favours MHC class I display and large quantities of the vaccine can in theory be produced and standardized for clinical use (Wolff et al, 1992; Conry et al, 1994, 1995*b*, 1996*a*).

The use of this form of immunization avoids potential recombinational events that may produce replication-competent viruses or the inadvertent incorporation of viral genomes into the host chromosomal complement. Both of these events may have serious consequences from the standpoint of the activation of oncogene sequences. The direct delivery of naked DNA therapy will also reduce the likely event of insertional mutagenesis. Polynucleotide vaccination uses the full length of h CEA cDNA driven by a CMV promoter and induces anti-CEA humoral and CEA-lymphoproliferative responses in mice. It has not been shown, however, to result in murine protection against syngeneic challenge with CEA-transduced colorectal cancer cells, unless administered by the intramuscular route. Stable gene expression systems use murine intramuscular plasmid injection, and DNA-coated bead projectiles have also been developed (Wolff et al, 1990; Yang et al, 1990).

The level of immune responsiveness with DNA vaccination appears equivalent to that induced by rV-CEA, although there is greater dose and schedule dependency. The amounts of gene gun dosage required appear to be minute (Eisenbraun et al, 1993; Pertmer et al, 1997).

The system of direct intramuscular plasmid injection needs improvement as mouse myocytes expressing CEA tend to die after about 10 days. The mechanism whereby the mouse myocyte functions as a semiprofessional antigen presenting cell is at present unknown, however myocytes have been shown to up-regulate MHC expression after γ-interferon stimulation, and their immunostimulant capacity is enhanced by co-transfection with B7

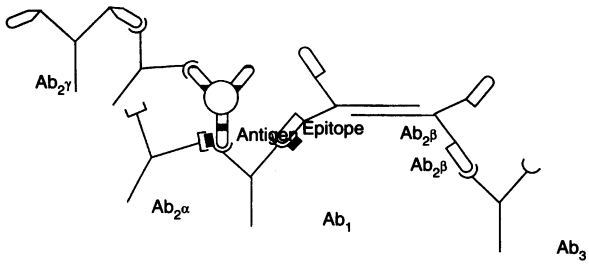

Figure 4 The immune network in tumour biology. A range of anti-idiotypic antibodies are produced in response to the AB_1 molecule. $Ab_2\beta$ antibodies recognize the antigen binding site of Ab_1 and are 'internal images' of the epitope. $Ab_2\alpha$ antibodies recognize idiotopes that lie outside the antigen binding site of Ab_1. $Ab_2\gamma$ antibodies recognize a portion of the antigen binding site of Ab_1 but do not carry the internal image of the antigen. An idiotype cascade results in the production of Ab_3 antibodies that resemble Ab_1 in their binding site sequence and that secondarily recognize the antigen

Table 1 Techniques to enhance monoclonal antibody tumour targeting

Strategies to enhance tumour localization
Vascular endothelial monoclonal antibodies
New labelling techniques
Different antibody isotypes
Genetically engineered antibodies
High-affinity antibodies
Unlabelled antibody pre-dosing
Antibody cocktails
Fractionation
Strategies to increase radioimmunoconjugate clearance
Antibody fragments
Pre-targeting approaches
Metabolizable chelates
Second-clearing antibodies
Plasmapheresis
Other localizing strategies
Regional administration
Biological-response modifiers to increase TAA expression
Increases in tumour vascularity

plasmids, suggesting a role as formal antigen presenters (Goebels et al, 1992; Hohlfield and Engel, 1994; Conry et al, 1996*b*). It may be that an inflammatory response against the plasmid construct evokes a secondary recruitment of professional antigen-presenting and other effector cells.

Successful CEA polynucleotide vaccination strategies have now been conducted in non-human primates (Wang et al, 1993). An initial phase I dual-specific plasmid DNA (CEA and hepatitis B surface antigen) study in patients with metastatic colorectal cancer has recently been jointly approved by the Recombinant DNA Advisory Committee and the NIH in the USA (Conry et al, 1996*c*). The use of naked mRNA vaccination offers several advantages when injection of DNA potentially encoding tumour growth factors risks incorporation of delivered genetic material into the host genome. This approach has been used with CEA mRNA transcripts transfected into CEA-negative cell lines using cationic liposome vectors. In this instance, the immunization schedule to maintain CEA expression needs to be more intensive than that used for naked DNA. Most mice, however, develop anti-CEA antibody responses when challenged with CEA-expressing tumour cells. Work is progressing using single-stranded RNA vectors for eukaryotic transfection that undergo self-replication after transduction but that are non-infectious (i.e. they do not contain the gene regions encoding viral-packaging proteins). So far, representatives of the Togaviridae have been used, notably poliovirus, Semliki Forest virus and Sindbis virus (Ansardi et al, 1994; Conry et al, 1995*c*; Zhou et al, 1995).

The in vivo delivery of plasmid DNA encoding a relevant TAA, although immunologically effective in animal models, has several problems in humans. The principal difficulty is the reduced efficiency of non-human primate and human expression of plasmid DNA in muscle (Jiao et al, 1992). This may require the co-delivery of either a cytokine gene or co-stimulator cDNA for enhancement of the CEA polynucleotide.

DESIGNER MONOCLONALS, ANTI-IDIOTYPIC ANTIBODIES AND COLORECTAL CANCER

Jerne's proposal of a cascading network of idiotype–anti-idiotype antibodies after antigenic immunization (Jerne, 1974) has been widely adopted as a model for the induction of V-domain interactions as part of a humoral response to available TAAs. This immune network is shown in Figure 4. The primary antibody (Ab_1) reacting with the main antigenic epitope contains components on its V domain that function as secondary 'epitopes' and that are recognized as antigenic by a second line of antibody molecules referred to as anti-idiotypic (Ab_2) antibodies. These Ab_2 molecules are serologically and stereochemically divisible into several subgroups: $Ab_2\alpha$ molecules, which identify idiotopes outside the antigen binding site; $Ab_2\beta$ molecules, which function as internal images of the original antigen; and $Ab_2\gamma$ molecules, which are capable of partial antigen/Ab_1 blockade but which are not antigenic internal images. Anti-idiotypic antibodies potentially induced by TAA exposure may serve as natural agents for use in passive immunotherapy because of their ability to act as surrogate antigen vaccines, being capable of stimulating anti-anti-idiotypic antibodies (designated as Ab_3), which functionally mimic the steric structure of the Ab_1 molecule and which directly attack the primary antigen.

Such anti-idiotypic cascades are involved in immune regulation in several ways. Ab_2 antibodies are capable of neutralizing circulating Ab_1 as well as binding to surface immunoglobulin receptors on activated B cells, thus interfering with TcR function and T-/B-cell interaction.

Monoclonal antibodies are used in patients with colorectal cancer for radioimmunolocalization of recurrent or metastatic disease, in radioimmunoguided surgery and as specific immunotherapy, inducing anti-idiotypic cascades and CTL reactivity when directed against well-characterized epitopes of CEA. The latter approach is undergoing a revolution with the use of humanized and bifunctional antibodies, F(ab′) and single Fv fragments as well as with human anti-idiotypic antibodies. In addition, therapies may be conjugated with toxins or radiopharmaceuticals.

There are many barriers to monoclonal antibody usage in solid malignancy. Heterogeneity of TAA and MHC expression may limit antibody binding. Physical factors most notably related to distorted tumour vascular architecture and increased intratumoral interstitial pressure limit the diffusion and biodistribution of macromolecules to the periphery of tumour deposits.

As tumour deposits enlarge, available surface area for transvascular molecular exchange diminishes in the majority of tumour types (Jain et al, 1988). Further, as the interstitial pressure

particularly in the centre of most tumours exceeds that of normal tissue, convection of macromolecules through the interstitial space, which is primarily dependent upon pressure gradients between the vascular and extravascular spaces, will work against the movement of antibodies towards the tumour matrix (Jain, 1987).

Approaches to overcome these difficulties are shown in Table 1. Lower-molecular-weight fragments of the primary antibody or the regional administration of antibody may improve local intratumoral concentration, but are often associated with accelerated elimination. The pharmacokinetics of these agents as well as that of bifunctional antibodies with hypervariable murine anti-idiotypic domains linked to TcR cell surface recognition molecules, remains to be elucidated.

One of the greatest difficulties with the use of murine monoclonal antibodies is the development of a human anti-murine antibody (HAMA) response to the Fc portion of the primary mouse antibody administered. The extent of this response particularly to repeated murine exposure will limit the therapeutic effect of monoclonal treatment, shorten antibody half-life, enhance clearance of antibody and potentially induce a serum sickness reaction in treated patients. The nature of the HAMA response is polyclonal with anti-isotypic and anti-idiotypic reactivity and may even affect the administration of human and humanized antibodies. This type of heterophilic antibody response will also interfere with assays that routinely use murine monoclonals, most notably standard CEA assay (Morton et al, 1988).

The finding of significant HAMA responses has resulted in the production of a range of designer antibodies, such as chimaeric antibodies, V_H domain molecules, antigen-binding peptides and recombinant antibody fusion proteins (Mayforth and Quintans, 1990; Fell et al, 1991; Winter and Milstein, 1991).

Genetically engineered antibodies that lack Fc reactivity could be used when Fc function is not desired, such as in radioimmunolocalization to diminish background (Bird et al, 1988). Single-chain antigen-binding fragments (sFv) and recombinant sFv peptides, which consist of V_L and V_H domains joined by peptide linkers and expressed in large quantity by *Escherichia coli*, are being developed for imaging purposes, although problems exist both with reduced affinity compared with the parent molecule and steric hindrance of the linker peptides. Many of these newer peptides are also relatively unstable. Despite chimaerization, anti-idiotypic antibodies that recognize the murine V region are potentially still a problem (Bruggemann et al, 1989). Although humanization of antibodies reduces their immunogenicity, the antigen–antibody binding affinity of the parent antibody may not be reproduced.

Although idiotypic–anti-idiotypic cascades can be demonstrated in patients with tumours after xenogeneic monoclonal antibody therapy, their exact significance is not known. The further advantage of such immune therapy, however, in solid tumours is their relative ease of production for general use, without the need for custom-made therapies using autologous tumour cells or autologous-stimulated TILs. The recent development of genetic recombinant libraries expressing specific epitope domains that are entirely human will enhance the ability to expand the repertoire of immunotherapies against a variety of unique TAAs, with large-scale production of antibody for use in conjunction with either conventional chemotherapy or progenitor cell support (Bona, 1989).

One of the main advantages of using anti-idiotypic antibodies in tumour therapy is in states in which the primary antigen is either weakly expressed or is frankly non-immunogenic. They may, in theory, break immune tumour tolerance to weak determinants and be useful against antigens that are difficult to characterize or synthesize. The use of human anti-idiotype therapy rather than either monoclonal or polyclonal xenogeneic anti-idiotype therapy avoids troublesome interspecies reactivity and the induction of inappropriate and non-specific human T-cell repertoires. Human therapies are likely to mediate more efficient complement-dependent cell lysis and ADCC (Chattopadhayay et al, 1992; Koido et al, 1995). For anti-idiotypes, the problems of oversecretion of complexing antibody, HAMA responsiveness, the induction of down-regulating idiotypic cascades and the difficulty of matching bizarre tumour cell idiotopes that are not shared between tumour types still remain. Human anti-idiotypic monoclonal therapy has resulted in survival benefit in patients with advanced malignant melanoma, and advanced colorectal carcinoma has demonstrated improved outcome when compared with historical controls (Robins et al, 1991*a*; Mittelmam et al, 1992).

Further, CTL activity of both peripheral blood and mesenteric node lymphocytes against autologous tumour has been demonstrated in a small number of patients with rectal cancer after immunization with human anti-idiotypic antibody when lymphocytes did not initially respond in vitro to autologous biopsy material (Austin et al, 1991; Durrant et al 1994*a* and *b*; Robins et al, 1991*b*). Similar findings producing anti-CEA antibodies have been shown in cynomolgus monkeys using the murine anti-idiotypic antibody 3H1, which mimics an epitope on CEA normally absent on adult colonic epithelium (Bhattacharya-Chatterjee et al, 1990; Chakraborty et al, 1995).

There is as yet comparatively poor prediction of the relative immunogenicity of anti-idiotypes necessary for T-cell responsiveness in tumour systems (Raychaudhuri et al, 1990; Tsang et al, 1995). Recently, cytokines have been used in combination with monoclonal antibodies to enhance MHC (Rosa and Fellous, 1988) and CEA (Kantor et al, 1989) expression as well as to improve residual tumour radioimmunodetection (Nieroda et al, 1995). Interferon-γ has been shown to increase CO 17-1A-directed ADCC by human effector cells against colorectal cancer cell lines (most notably SW 116) (Steplewski et al, 1986); however, phase II studies in patients with advanced colorectal carcinoma combining the monoclonal antibody 17-1A with interferon-γ, although safe for clinical use, have shown inconsistent anti-idiotypic responsiveness and poor clinical responsiveness (Blottiere et al, 1990). Granulocyte–macrophage colony-stimulating factor (GM-CSF) (Sieff et al, 1985), which stimulates differentiation and maturation of the monocyte–macrophage lineage, enhances in vitro ADCC function, stimulates delayed-type hypersensitivity and encourages professional antigen presentation, has also been used in combination with monoclonal antibody therapy (Morrissey et al, 1987). Recent reports assessing its use in combination with 17-1A in advanced colorectal cancer have shown clinical remissions, although the therapy is marred in some patients by the presence of immediate-type allergic responses to the murine monoclonal after repeated exposure. This has necessitated reduction of the monoclonal antibody dose (Raganhammar et al, 1993, 1995). Further, the ultimate development in most patients of neutralizing anti-GM-CSF antibodies after combination therapy may result in significant failure of the normal peripheral lymphocyte expansion seen during colony-stimulating factor (CSF) treatment.

This may have a signicant bearing on the type and level of

sustainable immune response during monoclonal antibody treatment. This is particularly evident in non-immunosuppressed patients capable of mounting an auto-immune reaction against endogenous colony stimulating proteins. This may render conventional CSF therapy in such patients relatively ineffective (Wadhwa et al, 1996). It is clear that unconjugated anti-idiotypic therapy induces specific humoral and cell-mediated responses against syngeneic and histocompatible colorectal cancer cell lines. At present, the dosage scheduling and the need for combination therapies in patients with advanced disease has yet to be determined. Clinical responses to date are sporadic.

FUTURE STRATEGIES OF BIOLOGICAL THERAPY AND COLORECTAL CANCER

The prospects for gene therapy in colorectal cancer include the correction of abnormal oncogenes implicated in the development of colorectal tumours, the augmentation or replacement of tumour-suppressor genes, such as *p53*, and strategies to interfere with tumour-related growth factor and growth factor receptor genes.

Ancilliary approaches will include genetically directed immunopotentiation of effector lymphocytes either to improve TIL capacity or to encourage TIL homing to tumours. The possible exploitation of techniques directed at newly discovered angiogenic factors controlling tumour neovasculature represents an exciting potential therapy (Baillie et al, 1995).

Tumour cells themselves may also be transduced with cytokine and cytokine receptor genes to render them suitably immunogenic.

The gastrointestinal tract represents a unique portal for potential gene therapy, although new techniques of delivery, such as the use of liposomal carriers, biodegradable microspheres and attenuated *Salmonella* spp. carriers, are required for consistent gene expression in such a hostile environment.

The Fearon–Vogelstein model of colorectal tumorigenesis (Fearon and Vogelstein, 1990) represents a challenge to modify the natural history of colonic tumours and premalignant disease through genetic intervention, as it is recognized that many mammalian cells have the cellular machinery required for successful integration of foreign genetic material into the parent genome (Capecchi, 1989). Many of these approaches in colorectal cancer are still theoretical, and much work needs to be done before they can become clinically valuable. The approach to increase the immunogenicity rather than the antigenicity of the tumour cell itself and to abrogate a tumour-induced immunosuppressive microenvironment remains a significant challenge for the future.

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