Chapter Four

DISCUSSION
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There are three major themes that dominate the field of cancer immunology. The first has to do with a continuing fascination with the concept of immunosurveillance, the idea that the immune system has the capacity to distinguish and under certain circumstances destroy cancer cells. A vigorous search goes on for tumor antigens that serve as targets for humoral or cellular immune recognition in the syngeneic or autologous host. The second theme deals with another long-standing objective of cancer immunology, development of a comprehensive map of the antigenic structure of cancer cells and their products. The third and perhaps the predominant theme of cancer immunology is centered on the development of effective cancer therapies based on immunological principles and techniques.

4.1 Strategies used to study tumor antigens

It is necessary to isolate the antigenic protein from an immunogenic tumor in order to study its properties. A scheme of protein purification usually exploits the variations in the physical and chemical properties among different proteins. The properties of proteins which make them distinct from other proteins are used to resolve them. Proteins can be usefully separated on the basis of several criteria which include charge, molecular weight, specific interactions and differential solubility. In order to purify a new protein whose biochemical and physical characteristics are unknown, the protocol is based more on the precedent and empirical factors rather than on the chemical and physical parameters.

The purification of a tumor antigen involves three major steps, dissociation of the antigen from membrane or cytosolic fractions, assay of the antigen and isolation of the antigen. Transplantation assays have been traditionally used to identify tumor antigens. Briefly, the partially or completely purified fractions are used to immunize naive animals and after immunization, these animals are challenged with the tumor cells and observed for the development of the tumor. These assays, first described by Foley (1953), and later used by Prehn and Main (1957), showed that methylcholanthrene induced sarcomas of inbred mice were highly immunogenic and that each tumor was immunologically distinct. Though
expensive and time consuming, these assays to date remain the most reliable method for
tumor antigen identification. To characterize these antigens and the type of immune response
they elicit, there has been much interest in devising assays that supplement or replace the
transplantation rejection assay. The approaches used include assays for humoral immunity
(most extensively the search for cytotoxic antibody), and in vitro and in vivo assays for
cellular immune reactions, such as tests for cytotoxic T cells, macrophage migration
inhibition assays, skin tests for delayed hypersensitivity (in the case of guinea pig or rat
tumors) and Winn assays with lymphocytes and macrophages. Though these assays have been
useful in supplementing and reducing the time factor in the identification of tumor antigens,
one of these can replace the transplantation tests for reliability in identification of a tumor
antigen.

Although subcellular fractions and solubilized extracts are active in transplantation tests and
in vitro assays, few groups have made a sustained effort to isolate and characterize the tumor
rejection antigens of chemically induced tumors. Kahan and his group (Pellis and Kahan,
1975 and LeGrue, 1985) have focused on butanol extracts of antigenically distinct C3H/f
sarcomas as the starting material for their purification efforts. They have identified a 36 kDa
component (as a part of a 200 kDa complex) that induces resistance to tumor challenge. Sato
et al., (1987) also used butanol extracts to solubilize antigens from two nitrosourethane-
induced BALB/c colon tumors and found that the resistance-inducing activity resides in a
30kDa component (also part of a larger complex). Plasma membranes derived from two
transplantable sarcomas of BALB/c origin, MethA and CMS5, elicited a potent tumor
rejection response comparable in strength and specificity to immunization with whole
irradiated tumor cells. This activity was purified by lectin-affinity and ion-exchange
chromatography, and resided in a 96 kDa cell surface glycoprotein (gp96) in both tumors
(Srivastava and Old, 1988). Amino-terminal sequencing revealed that the gp96 molecules
from MethA and CMS5 were related. Based on this sequence information, oligonucleotide
probes were prepared and used to isolate cDNA clones from libraries of MethA CMS5 and
BALB/c spleen. In addition several clones have been isolated from cDNA expression libraries
using anti-gp96 antibodies.
Vlock et al., (1991) purified a 66 kDa melanoma associated antigen identified by autologous antibody. This antigen was isolated from spent tissue culture medium in which the cells were grown. The antigen in the spent medium was separated from serum albumin using an isoelectric focusing column followed by HPLC ion-exchange chromatography. Further contaminating albumin was removed by blue agarose affinity chromatography. This antigen was shown to be an unusually acidic glycoprotein of pl 2-3. During the past few years, it has been possible to generate tumor-specific CTLs in vitro from peripheral blood, draining lymph nodes and metastatic tumor deposits of human cancer patients. These are mostly CD8+ cells whose target cell recognition is restricted by class I MHC molecules. The existence of tumor-specific CTLs in cancer patients evidence for an ongoing, though insufficient, immune response to tumors. It is believed that augmentation of the immune response may result in more effective anti-tumor immunity. In murine tumor systems, it is possible to protect a naive animal against subsequent tumor challenge by vaccination with irradiated tumor cells, and it is possible to eradicate metastatic tumor deposits by adoptive transfer of immune lymphocytes.

Most antigens recognized by cytotoxic T cells are short peptides bound to class I MHC molecules. These peptides have been produced by the degradation of proteins that are produced inside the cell. A major focus of tumor immunologists is therefore, the identification of the subset of MHC-associated peptides that are selectively expressed on tumor cells and that act as epitopes for tumor specific CTLs. Strategies for the identification of peptide epitopes recognized by class I MHC restricted, tumor-specific CTLs have utilized patient derived CTLs that specifically recognize the tumor. One strategy is to evaluate the existence of peptide epitopes derived from proteins whose expression is already known to correlate with the tumor. In this approach, cells are transfected with expressible genetic fragments encoding the candidate protein, or they are pulsed with synthetic peptides derived from the protein sequence, before they are evaluated for recognition by T cells.

An alternative strategy does not make any assumptions about the nature of the candidate proteins, but instead uses tumor-directed T cells as a primary screen. In one variation of this
approach, genomic or cDNA libraries are made from the tumor, and subsets of these molecular clones are transfected into cells that express the appropriate MHC molecule, but not the tumor-specific epitope. Molecular clones that encode the relevant epitope are identified after multiple rounds of screening with progressively smaller subsets of the library.

In a second variation, peptides associated with MHC molecules on the tumor cells are directly extracted, fractionated and then added back to a non-tumor cell that expresses the appropriate MHC molecule. Again, progressive fractionation of the peptide extract is used to reduce the number of candidate peptides, which are then identified and sequenced. While conventional Edman sequencing has been employed for this purpose, the use of tandem mass spectrometry affords a more sensitive and versatile approach. In an elegant piece of work, Cox et al., (1994) used tandem mass spectrometry to identify and sequence a tumor antigen peptide associated with the MHC HLA-A2.1 of a melanoma cell line. HLA-A2.1 restricted cytotoxic T cell lines were used to assay for the tumor antigen peptide. Melanoma specific CTLs had an exceptionally high affinity for this nine residue peptide.

4.2 Molecular cloning strategies
Recently, molecular cloning techniques are being increasingly used to isolate and identify tumor antigens. Oligonucleotide cDNA probes synthesized based on the N-terminal sequence of the biochemically purified tumor antigens have been used to screen cDNA libraries of the tumors to obtain cDNA clones of these antigens. Coney et al., (1991) used this technique to obtain cDNA clones of a tumor antigen from an ovarian carcinoma cell line IGROV1. This tumor antigen defined by monoclonal antibodies was shown to be a human folate binding protein. A 66 kDa glycoprotein antigen, pE4, expressed in a rat carcinoma cell line and defined by a monoclonal antibody was cloned using oligonucleotide probes derived from the N-terminal sequence of the protein. Complete cDNA sequence of the clone revealed it to be 416 aminoacid protein with homology to a human receptor for poliovirus. A major 2.2 kb transcript was detected in all the rat tumor cell lines tested. However it was found to be expressed only in trace amounts in normal adult rat tissues.
4.3 Cloning of melanoma antigens

Boon and colleagues at the Ludwig Institute for Cancer Research, Brussels pioneered a method for the identification and isolation of tumor antigens recognized by T cells from melanoma patients. They used a genetic approach to identify and clone T cell defined tumor antigens. By mutagenizing clonal mouse tumors \textit{in vitro} with \textit{N}-methyl-\textit{N'}-nitrosoguanidine (MNNG) they produced variants of the parent tumors which were unable to form progressive tumors in syngeneic animals (Boon, 1983). These variant cell lines were named as “tum−” in contrast to the original “tum+” cell line. They showed that the failure of tum− variants to form tumors is a consequence of an immune rejection response. Mice that have rejected a tum− variant usually present a higher degree of resistance against a challenge with the same variant than against any other tum− variant derived from the tumor cell line (Boon and Van Pel, 1978). This was the first evidence that most tum− variants express new transplantation antigens that appear to be specific for each variant. In rejecting tum− variants mice acquire an immune memory that enables them to resist challenge with the same variant even when they receive immunosuppressive irradiation concurrently. This immune memory can be transferred adoptively with T lymphocytes. When spleen cells of DBA/2 mice that have rejected a tum− variant are stimulated \textit{in vitro} with the same variant, very active populations of CTLs are produced. These CTL clones recognize antigens that appear to be strictly specific for the immunizing tum− variant. These antigens were therefore named tum− antigens. Cloning of the tum− antigen genes was achieved by using DNA transfection procedures and selection of the antigen expressing clones by their ability to stimulate the proliferation of the antigen specific CTL clones. Cosmid libraries of the tum− variant genomes were made and transfected into the parental tum+ cell line. The resultant transfectants were selected for their ability to stimulate the proliferation of the specific CTL clones. The tum− sequences were retrieved from a positive transfectant by directly packaging the DNA of the transfectant into \textlambda{} phage components. Boon and colleagues have identified about 15 antigens from melanoma patients using this technique. These genes encode a variety of peptides that can combine with various MHC molecules to produce different tumor rejection antigens. Prominent among them are \textit{MAGE-1}, \textit{MAGE-3}, \textit{BAGE} and \textit{GAGE} genes which encode the antigens MZ2-E, MZ2-D, MZ2-Ba and MZ2-F respectively.
4.4 Cloning of other tumor antigens

Tumor antigens defined by either polyclonal or monoclonal antibodies have been cloned by using the antibodies as probes to screen expression cDNA libraries of the tumors. The tumor associated antigen L6, which is expressed on human lung, breast, colon and ovarian carcinomas was cloned by transfecting COS cells with a cDNA expression library of the tumor cell line and screening by panning with a monoclonal antibody recognizing the antigen (Marken et al., 1992). The murine counterpart of this antigen was subsequently cloned by using another monoclonal antibody. In another study a melanoma associated antigen was cloned by screening an expression cDNA library of a melanoma cell line A375 constructed in the vector λgt11, with sera from melanoma patients. (Hayashibe et al., 1991). This clone designated D-1 was found to be 1029 bp long and was found to be expressed in human melanoma, neuroblastoma, erythroleukaemia, B lymphoid and T lymphoid cell lines but not in a renal carcinoma, PBL, and cultured skin fibroblasts. The fusion protein produced by the D-1 clone showed significantly higher reactivity with sera from melanoma patients than from healthy controls. The D-1 clone did not show any homology to any of the known sequences in the database. The structural properties of D-1 are different from those of previously described melanoma associated antigens. It is suggested that D-1 represents a novel melanoma antigen and that this approach can be used to identify and isolate novel tumor antigens.

4.5 Observations with the AK-5 tumor model

As described in section 1.13 of chapter 1, the AK-5 is a highly malignant tumor cell line when grown intraperitoneally. However, when injected subcutaneously, it forms solid tumors and regresses spontaneously in about 80% of the animals. Animals that reject the solid tumor have a high titre of anti-tumor antibody in their serum. This antibody is cytotoxic to AK-5 cells in a complement dependent assay. The anti-AK-5 antibody when preincubated with membrane fractions of AK-5 cells fails to lyse the AK-5 cells in the complement mediated assay. It was demonstrated earlier that immune NK cells from animals that reject the tumor require the participation of anti-AK-5 antibody in order to mediate ADCC in vitro. With an aim towards identifying the antigen(s)' responsible for such a strong humoral response, an
immunoscreening approach was used where a cDNA expression library of AK-5 constructed in the vector λgt11 was screened with the autologous anti-AK-5 serum. This strategy was adopted based on two important considerations. It is possible that there is more than one tumor specific antigen in the AK-5 tumor and this protocol would allow the isolation of the most immunogenic antigen. Second, the subsequent analysis of the antigen at the molecular level and its regulation would be greatly facilitated following its cloning.

This approach yielded the clone designated as 1(1) which reacted strongly with the anti-AK-5 serum (fig.1). Western blot of the fusion protein produced by the 1(1) clone using the anti-AK-5 serum indicated that the cDNA clone indeed encodes an antigen recognized strongly by the anti-AK-5 serum. In addition to anti-AK-5, two monoclonal antibodies raised against AK-5 cells in syngeneic animals (Khar et al., 1992), which also lyse AK-5 cells in a complement dependent lysis assay recognize this fusion protein (fig. 7 and fig. 8). These results strongly indicate that the protein produced by the 1(1) cDNA is a tumor specific antigen of AK-5.

We next wanted to check whether the antigen produced by the 1(1) cDNA is able to evoke active immunity in syngeneic animals. This would prove that the protein does carry antigenic epitopes specific to the tumor. When immunized into syngeneic animals, the fusion protein was found to evoke a strong humoral response in 100% of the immunized animals as tested by western blotting. To check that the antibodies are specific to the antigen part of the fusion protein and not to the GST portion, the antibodies against the fusion protein were adsorbed to GST bound to the glutathione Sepharose column. This was done to clear the serum of any GST reactive antibodies. In order to check the levels of the anti-fusion protein antibody in the sera of the immunized animals a modification of the ELISA protocol described by Avrameas and Ternynck (1971) was used. This modification utilizes tumor cells as the antigen immobilized on polystyrene plates (Khar et al., 1992). The levels of the antibody binding of the sera from immunized animals to AK-5 cells is comparable to that of anti-AK-5 and is significantly higher than that of the control antibodies. This result strongly indicates
that anti-AK-5 serum and the antibodies raised against the fusion protein recognize the same protein in AK-5 cells.

The real test for a tumor rejection antigen is the transplantation assay. Immunization with a purified or recombinant protein should protect animals from challenge of the tumor from which the antigen in question was isolated for it to be of any use in immunotherapy trials (Srivastava and Old, 1985). In order to check whether the antigen produced by 1(1) cDNA can provide protective immunity in syngeneic animals, the animals were immunized with the fusion protein as well as the control GST protein and subsequently given intraperitoneal challenges of about 1.5 million AK-5 cells per animal and monitored for survival. There was a significant difference in the total number of survivors between the fusion protein immunized and the control protein immunized groups. Though the difference in the percentage of survivors between the control and immunized groups was significant, immunizations with the recombinant antigen protected only 58% of the animals. Passive immunizations of syngeneic animals with the anti-AK-5 serum did not protect these animals from intraperitoneal challenges of the tumor. This could be due to the fact that complete rejection of the AK-5 tumor requires the participation of NK cells activated in the presence of AK-5 or cytokines like IL-12, TNFα, and IL-2 (Kausalya et al., 1994; Khar et al., 1993; Hegde et al., 1994). Therefore, from these results it appears that antibodies to the tumor rejection antigen are necessary but not sufficient for the complete rejection of the AK-5 tumor.

Having established the 1(1) protein as a tumor specific antigen of AK-5, the next step was to identify its molecular nature and its normal function in the cell. Northern blot analysis of the AK-5 total RNA was done using 1(1) cDNA as a probe. 1(1) cDNA picks up a message of approximately 2.5 kb in AK-5 cells. The message is also present in normal macrophages but absent in either normal rat liver or ZAH, a rat hepatoma cell line. Both the levels and the size of the transcript are unaltered in AK-5 as compared to normal macrophages. This result indicates that there is neither gross alteration of the gene for 1(1) in AK-5 as compared to normal tissues nor is it overexpressed (fig.11). This result is also borne out in the
Southern blot experiment where AK-5 tumor DNA and genomic DNA isolated from liver and normal macrophages was digested with three different restriction endonucleases and run on an agarose gel, transferred to nylon membranes and probed with the 1(1) cDNA. The result (fig.13) shows that there is no difference in the DNAs isolated from the three tissues in any of the restriction digests. Both the northern and Southern data indicate that there is no gross alteration of the gene for 1(1) in AK-5 cells.

In order to address the question whether 1(1) was expressed in tissues other than AK-5 and macrophages, northern blot analysis of various tissue RNAs was done using the 1(1) cDNA. As described in chapter 3, the message for 1(1), in addition to the cells of the immune system, is found in brain and testes. In order to know whether the 1(1) cDNA encodes a known protein, DNA sequencing of 1(1) was done from both ends of the insert using the M13 forward and reverse primers. Initial sequencing of the 1(1) cDNA using both primers yielded a sequence of about 250 bp from both ends of the insert. Homology search in the EMBL database was done and the search did not reveal any homology of the 1(1) cDNA with any of the known cDNA or protein sequences in the database. This indicates that the 1(1) cDNA encodes a novel protein. In order to obtain the complete nucleotide sequence of 1(1), deletion clones of 1(1) cDNA were generated using exonuclease III enzyme. Sequencing of the deletion clones and alignment of the sequences obtained from each deletion clone yielded the complete sequence of the 1(1) cDNA. As shown in fig.15, the cDNA is 950 bp long and the deduced amino acid sequence as revealed by the PC Gene sequence analysis program is 103 amino acids long. The rest of the sequence comprises of 3' untranslated regions. There is a poly A tail at the 3' end of the sequence. All these data indicate that 1(1) is a partial cDNA clone and probably encodes the C-terminus of the antigen.

Repeated attempts at cloning a bigger fragment of 1(1), using both the anti-AK-5 antibody as well as the 1(1) cDNA failed. All the clones obtained using both strategies yielded clones containing the same sized insert as 1(1). This problem could have been due to the fact that the AK-5 cDNA library was overamplified. It is well established that repeated amplifications of cDNA libraries result in the loss of clones that are either slow growing or under-
represented and accumulates clones that are over-represented. In order to circumvent this problem it was decided to construct a new cDNA library of AK-5 in the vector λZapII. Using a commercially available cDNA cloning kit, the new library of AK-5 constructed in the λZapII vector was found to contain 74% recombinants with a titre of $4.3 \times 10^5$ pfu/ml. This library was amplified once and used for screening with radiolabeled 1(1) cDNA as a probe. A positive clone, designated as A1, was picked up and after plaque purification and subcloning was found to contain two inserts. One of the inserts was of the same size as 1(1) and the other was slightly larger, about 1.1 kb.

As described in the section 3.15 the possibility that the two clones are unrelated and are present in the same clone as a result of a cloning artifact was ruled out by northern analysis using the unique fragment of the A1 cDNA. The results as shown in fig.18 and described in section 3.15 indicate that both 1(1) and the unique fragment of A1 pick up the same message. This result unambiguously proves that the 1(1) and A1 fragment are derived from the same gene. These results also indicate that the unique fragment of A1 probably encodes the N-terminal end of the antigen. In order to confirm this possibility and to find out about the nature of the antigenic protein sequencing of the unique fragment of the A1 clone was attempted. Deletion clones of the subcloned fragment were made as described for 1(1) cDNA and the sequence obtained from various deletion clones was collated to yield the complete cDNA sequence of the antigen.

Homology search of the EMBL database using the FASTA program revealed that the sequence encoded by the unique fragment of the A1 clone has 70.85% identity with a human cDNA clone c-leg08 derived from the brain. The c-leg08 is a partial cDNA sequence obtained from a three month old female human muscular atrophy patient. The total mRNA from the brain was isolated, oligo-(dt) primed and directionally cloned into the HindIII and NotI sites of the lafmid BA vector. The sequencing was done using a single read fully automated sequencing method (Auffray et al., 1995). The deduced aminoacid sequences of A1 and c-leg08 showed 68.54% identity. In a stretch of 73 residues 61 are identical indicating that these sequences are the rat and human homologues of the same gene. This
is consistent with the tissue specific distribution of the message for 1(1) where it was shown to be expressed in the brain (fig.12). Sequence analysis of the complete A1 cDNA was done using the PC Gene sequence analysis software. The total length of the putative protein encoded by the cDNA is 443 residues. A potential glycosylation site was found at the N-terminal end on both the antigen and c-leg08 protein on the 13th residue which is asparagine. Computation using the algorithm ‘RAOARGOS’ revealed a potential membrane spanning helix from the 203rd to the 234th residue. This algorithm predicts the deduced aminoacid sequence as an integral membrane protein. These analyses together indicate that the antigen encoded by A1 may be a membrane protein.

Using the program ‘ANTIGEN’ two antigenic determinants were detected from residues 403 to 414 and 438 to 443. All the three determinants fall in the sequence encoded by 1(1) and hence may explain its high antigenicity as determined not only by reactivity to anti-AK-5 serum and the anti-AK-5 monoclonal antibodies but also its ability to evoke a strong immune response in syngeneic animals.

4.6 Most tumor antigens are normal proteins

It was an early belief that tumor-specific antigens are a special set of proteins that are induced as a result of malignant transformation and elicit tumor immunity. After about two decades and several hundred publications, it turns out to be substantially incorrect. It now appears that there are no tumor specific molecules, but only tumor specific epitopes of common molecules. Tumor-specific antigens were earlier sought primarily among cell-surface proteins. Recent advances in our understanding of antigen presentation by MHC class I antigens have rendered that dogma untenable and have replaced it with the new dogma: any alterations in a coding gene are potentially recognizable by the cellular immune system and all proteins are potential tumor antigens.

It is becoming accepted that a large set of self antigenic determinants do not induce self-tolerance (Sercarz et al., 1993) and that these peptide determinants furnish target structures for autoimmune attack (Lanzavecchia, 1995) and could provide potential targets for immune
responses directed against tumors. The focus of most tumor immunologists is on how to initiate, maintain, and regulate anti-tumor immunity, which could translate into effective treatment in cancer clinics. Are there in fact certain tumor-related determinants that can be rendered into crucial targets of attack by the immune system? The pertinent focus is on suitable antigen processing and subsequent presentation by major histocompatibility complex (MHC) class I and class II molecules expressed by tumor cells. Any of the peptides that is bound in the groove of an MHC molecule on tumor cells provides a potential target determinant for attack by the immune system. The peptides bound by the MHC molecules of all cells, including tumor cells, are derived from endogenous cellular (or viral) proteins, and the antigen-processing machinery of the cells manages to display certain antigenic determinants to the host T cells. Tumor cells are distinct in that they possess additional oncoproteins that either are overexpressed owing to dysregulation or are mutated and have thereby conferred the tumor phenotype to these cells, to developmental antigens re-expressed during the process of tumorigenesis, or to passenger mutations in non-oncogenic proteins that result from the loss of mechanisms that maintain genomic stability. Using T cell clones that are specifically able to kill the tumor as detectors for immunogenic tumor-derived determinants bound to MHC molecules of the tumor, several investigators have shown that T cells indeed recognize peptides from endogenous normal (and occasionally mutant) self-proteins.

As mentioned earlier in section 1.9, Boon and colleagues in their pioneering studies drew a page from bacterial genetics and cloned genes encoding tumor antigens recognized by CD8+ T cells specific for human melanomas (Boon, 1992). MAGE-1 (Melanoma antigen 1) to MAGE-3 were the original family of human melanoma-specific antigens that were molecularly defined this way and were followed by isolation of determinants on tyrosinase, gp100, and Melan-A\textsuperscript{MARTI} recognized by melanoma reactive host T cells. The MAGE gene family is not expressed in any normal adult tissue except testes, but is expressed in a large proportion of other human tumors (including small cell lung carcinoma, breast cancers, and colon carcinoma) and perhaps represents developmental antigens reexpressed during the process of tumorigenesis. Tyrosinase, gp100, and Melan-A\textsuperscript{MARTI} are normal self proteins.
specific to the melanocyte lineage and T cells specific for determinants on each of these antigens can be found in a large majority of melanoma patients (Houghton, 1994; Pardoll, 1994). It is thus becoming clear that there is no special group of proteins that can be dubbed as tumor antigens, and the distinction between self-antigens and tumor antigens is rapidly vanishing.

4.7 Self-peptides can induce an immune response in the tumor context

Can the immune system mount a T cell response against all the peptides bound to MHC molecules of a tumor cell? Part of the answer lies in the availability of the T cell repertoire membership directed against MHC-bound determinants and the proportion of T cells rendered tolerant in each individual. It is now thought that only well expressed self-determinants are efficient in tolerance induction. In addition, on every self antigen, there are sequestered determinants that do not succeed in inducing tolerance but, under the circumstances of severe inflammation and its attendant cytokine milieu, can be displayed in an immunogenic context. Among the diversity of self-reactive T cells that evade negative selection, tumor-specific members can be mobilized under conditions of heightened awareness by the immune system in which MHC molecules, surface adherence molecules, and costimulators are up-regulated and become available for possible killing interaction along with newly displayed, previously "cryptic" self-antigenic determinants (Sercarz et al., 1993; Lanzavecchia, 1995). Autoimmune disease on one hand and the existence of tumor-specific cytolytic T cells recognizing self-peptides in the cancer patient on the other are a testimony to the enormous potential resources inherent in the positively selected T cell repertoire, directed to the cryptic self. For example, T cells specific for peptides of self-protein tyrosinase can be isolated from normal individuals, which can attack and kill melanoma cells from HLA matched cancer patients (Visseren et al., 1995). What may be critical is the density of the up-regulated peptide-MHC complexes, as well as the expression of the costimulatory signals that influence activation of the otherwise silent tumor-specific T cell repertoire existent in the cancer patient.
It is well established that the immune system gets propelled only upon ligand recognition in the context of heightened expression of costimulatory molecules, adhesion molecules, and MHC molecules on antigen-presenting cells (APCs) or in the context of "danger" (Matzinger, 1994). Adjuvants can transform a weak stimulus into one that signals danger, the only state the immune system has evolved responsiveness. One reason for this could be the induction of macrophages to produce IL-12, a cytokine that is a potent activator of T helper type 1 (Th1) cells (Mosmann et al., 1991) and cytotoxic CD8+ T lymphocyte cells (CTLs).

In an experiment by Norguchi et al., (1995), a mutant peptide of p53 injected along with IL-12 was able to destroy completely an established tumor in a tumor bearing host, whereas the same peptide or IL-12 alone was totally ineffective in killing the tumor.

IL-12 has been shown to be required for the regression of the AK-5 tumor. Subcutaneous transplantation of AK-5 tumor induced the expression of IL-12 message by day 6-8 in the splenocytes of syngeneic rats. Analysis of serum samples from tumor-bearing animals showed the presence of circulating IL-12 around the same time. Interaction of immune cells with antibody-tagged AK-5 cells in vitro also triggered the expression of IL-12 message and protein. Coculture of AK-5 cells with splenocytes from animals that had rejected the tumor, showed the presence of IL-12 message and protein as detected by northern and western analysis. However no message was seen when anti-AK-5 antibody was omitted from the culture or when splenocytes from normal animals were used. The data from these experiments indicate that the AK-5 tumor induces the production of IL-12 in the tumor bearing host or in vitro (Hegde et al., 1994). Further, NK cells from normal animals when activated in the presence of IL-12 were able to kill AK-5 cells as efficiently as NK cells from tumor bearing animals in ADCC reactions in the presence of anti-AK-5 antibody. In addition to IL-12, levels of TNFα (Khar et al., 1993), IL-2 and IL-4 have also been shown to be increased during the regression of the AK-5 tumor (Kausalya et al., 1994). Given the heterogeneity among tumors, different cytokines might be effective in the elimination of different tumors.
The effectiveness of heightened expression of costimulatory molecules and cytokines in anti-tumor immunity is also evident from studies using whole tumor cell vaccines. Genetic manipulation of tumors designed either to enhance the presentation of tumor antigens or to provide enhanced costimulatory signals to T cells has been a route adopted by immunologists to increase immunogenicity of whole tumor cells. Many cytokine genes have been introduced into tumor cells, including IL-1α, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12, granulocyte-macrophage colony stimulating factor (GM-CSF), IFN-α, IFN-γ, and tumor necrosis factor (TNF) (Colombo and Forni, 1994). The target cells for these cytokines are diverse, ranging from CD8+ CTLs to B lymphocytes to NK cells, and for many cytokine transduced tumor vaccines a wide range of effector cell types are found at the vaccination site. Vaccination with tumor cells transduced with GM-CSF induced long lasting anti-tumor immunity, involving both CD4+ and CD8+ T cells. This effect was attributed to the ability of GM-CSF to promote differentiation of dendritic cells, which are the most potent APCs for activating both class I and class II restricted T cells (Dranoff et al., 1993). The variable regions of the immunoglobulin molecules expressed on malignant B cells are tumor-specific, but are weak immunogens. By fusing a tumor derived epitope of one such idiotype to GM-CSF, Tao and Levy (1993) have been able to convert it into a strong tumor antigen capable of protecting syngeneic mice from tumor challenges. Tumor cells transfected with B7 genes to provide enhanced costimulation were clearly more potent immunogens than parent tumors (Moudgil and Sercarz, 1994). Preliminary evidence in our laboratory indicates that the message for B7 is upregulated in AK-5 cells upon coculture for a short time with immune splenocytes. In addition, NK cells from immune animals have been shown to express the CD28 molecule. Thus, the interaction between B7 on AK-5 cells and CD28 on the effector cell may play an important role in regression of the AK-5 tumor (unpublished observations).

That self-peptides associated with tumors actually induce T/B cell responses in cancer patients was shown by Cheever whose group demonstrated that patients with breast cancer have an existent immune response to HER-2/neu (Disis et al., 1994). T cells specific for peptides of HER-2/neu have also been demonstrated by Peoples et al., (1995) in patients with breast and ovarian cancers. HER-2/neu is a growth factor receptor, which is overexpressed
in 25%-30% of patients with breast cancer as well as some patients with colon, pancreas, gastric and ovarian cancer. Tumor cells from breast cancer patients display a 40- to 50-fold excess of this molecule relative to the very low levels in normal tissue, making this a target self-molecule of choice for raising a possible therapeutic anti-tumor autoimmune response. In patients with overexpressed HER-2/neu, both T cells and immunoglobulins reactive to HER-2/neu could be demonstrated, thus indicating that self-tolerance has been circumvented. Rodent studies have identified vaccine regimens capable of inducing HER-2/neu immune responses in naive hosts. Rat and human HER-2/neu proteins are highly homologous (89%). Immunization of rats with 15-mer peptides identical to the natural sequence of both rat neu and human HER-2/neu proteins elicited immunity specific for both the proteins (Peoples et al., 1995).

Another strategy has been to generate tumor cells that can directly present tumor peptides and specifically activate tumor specific CD4+ Th lymphocytes. Numerous studies have shown that transfection of tumor cells with class II MHC molecules can enhance the immunogenicity of these tumors. This approach is based on the assumption that the effectiveness of CD8+ T cells is dependent on sufficient help from tumor-activated CD4+ T cells, and that optimal immunological memory can be generated if both CD4+ and CD8+ T cells are stimulated (Kern et al., 1986). It was reasoned that tumor cells transfected with syngeneic MHC class II genes could present endogenously synthesized tumor peptides in the context of MHC class II molecules, thereby directly presenting antigen to CD4+ Th cells (Ostrand-Rosenberg et al., 1990). A variety of mouse tumors have been transfected with syngeneic MHC class II genes, and the resulting transfectants are very effective vaccines against subsequent challenge with wild type class II- tumors. The induced immunity is dependent on CD4+ T cells, strongly suggesting a role for Th cells in the immune response. As mentioned earlier in section 1.13, the AK-5 is a macrophage-like tumor cell line and possesses all the characteristic features of an antigen presenting cell including class II MHC molecules and ability to phagocytose bacteria (Khar, 1986). Therefore it may not be too far fetched to presume that the AK-5 cell is able to process and present peptides derived from endogenous proteins in the context of
MHC class II molecules and thereby stimulate the CD4\(^+\) T cells, explaining its high rate of spontaneous regression in syngeneic animals.

Humoral as well as the T cell-mediated arms of the immune response are important in the fight against tumors, which has been amply demonstrated by Fagerberg et al., (1995). Their results from a Swedish study of colorectal carcinoma patients treated with idiotypic antibody (mAb2 recognizing a mAb against a cell surface glycoprotein antigen (GA733) overexpressed by colorectal carcinoma cells) showed that there was a very positive clinical outcome in each case. Reithmuller et al., (1994) previously reported that treatment with this antibody alone prevents micrometastases in such patients, and in two cases, when administered along with GM-CSF, complete remission resulted. Survival of many tumors in the environment of measurable anti-tumor cellular activity underlines the importance of regarding the growing tumor as more than an aggregate of tumor cells. The tumor can help to establish its own microenvironment by altering the expression of MHC molecules and peptide transporter molecules or the levels of other activities affecting immune induction, such as antigen masking and antigen shedding, thus preventing the display of peptide determinants on their cell surface.

4.8 Concluding remarks

It is obvious from the growing literature in the field of tumor immunology that for the complete regression of a tumor all the components of the host immune system need to be activated to sufficiently high levels. It is presumed that the humoral arm of the immune system may not play a major role in the response against tumors. Data from our laboratory show that contrary to the available literature, antibody responses do play an important role in the rejection of a highly malignant but immunogenic tumor cell line. AK-5 is a spontaneous tumor and its regression in syngeneic animals is also spontaneous. We have so far shown that natural killer cells, anti-tumor antibody and cytokines like IL-12, IL-2, IFN-\(\gamma\) and TNF\(\alpha\) are the major mediators of this response.
Our data presented here show that one of the antigens isolated from AK-5 evokes a humoral response in syngeneic animals and also affords partial protection against the tumor. It is possible that there are other antigens in AK-5 and that the humoral response seen is a combined effect of these. The structure and function of this antigen is as yet unknown. It has significant homology to a human gene expressed in the brain which probably points to an important function. Further work in elucidating the structure and normal function of this gene may throw light on the mechanisms involved in the generation of this and possibly other antigens of AK-5. As a step towards this end we attempted the cloning of the complete cDNA of the antigen. The A1 clone obtained in this process is still not complete. Though it contains most of the cDNA, the 5' end of the gene is still missing in this clone. In order to obtain the full length cDNA of the antigen we are in the process of screening the AK-5 cDNA library as well as a rat genomic library constructed in the vector EMBL3. A few putative full length cDNA clones and genomic clones have been isolated and are being analyzed. Also, studying the mechanisms involved in the regression of AK-5, would lead to newer insights into the natural mechanisms of immune responses against tumors and help in the development of newer immunotherapeutic strategies.