Chapter One

INTRODUCTION
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1.1. Origin of tumor antigens

A neoplastic cell develops from its normal progenitor as a result of changes in some members of a restricted set of cellular genes. As a result of transformation, cellular properties can be altered considerably and changes such as anchorage independence, loss of contact inhibition (Stoker and Rubin, 1967), increased uptake of essential nutrients (Bhargava et al., 1976), low serum requirements in culture (Gospodarowicz and Moron, 1976), invasiveness and ability to metastasize (Poste and Fidler, 1980) are frequently observed. The transformed phenotype is characterized by various biochemical and functional alterations which can be traced back to the genetic level. Gross rearrangements in the chromosomal composition have been reported which are easily detected using chromosomal preparations. Stable changes at the fine structural level, which cannot be detected using comparatively less sensitive techniques are also known, and these may turn out to be crucial with respect to regulation.

As a consequence of these changes the protein profile of a tumor cell is altered considerably with respect to its normal tissue counterpart. Analysis of protein preparations from various transformed cells has generated a vast amount of data regarding the biochemical and immunological characterization of new proteins which are expressed on the cell surface or intracellularly upon neoplastic transformation. These molecules which have the ability to elicit an immune response in a tumor bearing host are experimentally defined as tumor specific antigens. The first evidence for the existence of tumor antigens came when Foley (1953) demonstrated that methylcholanthrene induced sarcomas of mice were highly immunogenic and protein preparations from these tumors were able to protect the mice from tumor challenges. These tumor antigens were distinct to the individual tumors and no cross reactivity was found among them.
Ever since, the interaction between the tumor cell and the host immune system has attracted the attention of cancer biologists and efforts were begun in earnest to understand the principles governing the basis of these interactions. The ability of a tumor antigen to evoke an immune response varies depending upon the tissue of origin and the initial carcinogenic event.

Quantitative variation in the response evoked covers a large spectrum spanning from the highly immunogenic viral antigens (Terethia et al., 1984) to the weakly immunogenic tumor antigens of the spontaneous tumors (Dubois et al., 1980; Hellstrom and Brown, 1979). The type of response evoked also varies considerably, from humoral to cellular or both.

Based on the causal agent, type of cell and the molecular characterization of the antigen expressed, tumor antigens can be classified into the following groups:

1. Antigens unique to a tumor.
2. Antigens unique to a class of tumor.
3. Antigens unique to the transforming agent.
4. Antigens shared by more than one type of tumor.

These classes will be dealt with in the following pages.

1.2. Antigens unique to a tumor

This kind of antigens are produced as a result of carcinogenic attack such as a chemical carcinogen like methylcholanthrene (Pellis and Kahan, 1975) or a physical agent like UV radiation upon which the DNA of the host cell undergoes various changes such as point mutations (Sibille et al., 1990; Monach et al., 1995; Cheever et al., 1993), gene rearrangement (Pawelec et al., 1992), deletions (Bishop, 1987), etc., which eventually result in the expression of neoantigens.
A point mutation resulting in a single base pair change can give rise to a novel epitope on a protein which can be recognized as foreign by the immune system. This epitope may be conformational or structural. The p198 gene of the mouse mastocytoma p815 codes for a tumor antigen which upon sequencing has been shown to differ from its normal counterpart by a single base pair change in exon 7 of the gene which resulted in the replacement of an Ala with Thr residue and in the generation of a new antigenic peptide (Sibille et al., 1990). The p91A gene from the same tumor was also found to be altered by a point mutation from G to A which changed an Arg to His at residue 274 (De Plaen et al., 1988). The L9 tumor antigen of the UV induced tumor 613 2A is a ribosomal protein and was found to contain a single base pair change which converted leucine to histidine. The peptide derived from the protein carrying the change in residue is sufficient for it to stimulate T cells (Monach et al., 1995). Many malignancies harbor mutated ras proto-oncogenes encoding the 21kDa protein with single aminoacid substitutions. These aberrant p21 ras proteins are potential tumor specific antigens in that CD4+ class II MHC-restricted T cell response specific for the mutated segment of various oncogenic peptides can be elicited by immunization in vivo with synthetic peptides corresponding to the mutated segments (Peace et al., 1993; Cheever et al., 1993). In chronic myelogenous leukemia the human c-abl proto-oncogene from chromosome 9 is translocated to specific breakpoint cluster (bcr) region on chromosome 22. This results in the formation of a bcr-abl fusion gene that encodes a 210 kDa chimeric protein. The joining region segment of chimeric bcr-abl protein is composed of a unique combination c-abl and bcr aminoacids and is expressed only by malignant cells. Peptides corresponding to this region can elicit both CD4+ as well as CTL responses (Cheever et al., 1993). Wreschner et al., (1990) analyzed human epithelial tumor antigen cDNA sequences and concluded that differential splicing of these genes may generate multiple protein forms. These tumor antigens are unique to a tumor and are not shared by other tumors, even of the same tissue origin. Basonbrioso (1970) reported that out of 25 sarcomas induced by methylcholanthrene, no cross reactivity was found.
1.3. Antigens unique to a class of tumor

Oncofetal antigens or developmental antigens are the primary examples in this group. Alphafetoprotein (AFP), which is expressed only during embryonic stages in the liver has been found to be expressed in many hepatocarcinomas. The tissue specificity of AFP has been useful in the diagnosis and classification of liver tumors (Abelev, et al., 1979).

Carcinoembryonic antigen (CEA) is also developmentally regulated and is another example of this class of tumor antigens. It is expressed in embryonic stages in the colonic mucosa and in carcinomas of the gastrointestinal tract. It is a 180 kDa glycoprotein and is a member of a family of isoantigens (Shimada et al., 1994). PE4, a 66 kDa glycoprotein antigen, defined by mAb E4 is expressed on the surface of rat colon and mammary carcinomas (Chadeneau et al., 1994). CA-19-9 is another example of this class of antigens expressed in colonic carcinomas and has potential for use in immunotherapy (Garcia de Palazzo et al., 1992). The secreted tumor associated antigen 90K, defined by mAb SP-2 is a potent immune stimulator and is expressed in breast cancer cells (Ullrich et al., 1994). It has stimulatory effects on NK cells, LAK activity and its stimulatory effects may be due to the induction of IL-2 and other cytokines. A monoclonal antibody, L3, against human lung adenocarcinoma derived cell line, A549, defines an antigen of 40-50 kDa size (L3p40-50) which is expressed by some adenocarcinomas of the lung and is weakly expressed in normal tissues (Brezicka, 1994).

Melanomas are highly immunogenic and various antigens from these tumors have been identified using either CTLs or sera from melanoma patients. These antigens are well characterized and they are expressed in most of the melanomas of different origin. The gp75 is a shared melanoma antigen and is defined by the HLA-A31 restricted tumor infiltrating lymphocyte line TIL 586. It was originally identified by using sera from melanoma patients and was found to be a tyrosinase related protein. The tyrosinase gene defined by autologous HLA-A2 restricted CTLs from melanoma patients is expressed by most melanomas, and among normal tissues, is expressed only by melanocytes (Brichard et al., 1993). Gp75, a tyrosinase related protein, is found to be expressed in melanomas, melanocyte cell lines and retina but not in any other normal tissue or tumors (Wang et al., 1995). Another melanoma
antigen, MART-1, cloned using tumor infiltrating lymphocytes is a transmembrane protein whose expression is limited to melanomas and melanoma cell lines and retina (Kawakami et al., 1994a). The melanoma cell line SK-29-MEL expresses 3 distinct antigens A, B, C, defined by HLA-A2 restricted CTLs. All these antigens are restricted to melanomas and melanocyte cell lines (Wolfel et al., 1993).

1.4. Antigens unique to the transforming agent

In case of viral carcinogenesis, the origin of the tumor antigens and their crucial role in transformation or tumorigenic process is well known. Proteins of viral origin like SV40 large T, middle T, and small T antigens are not only required for the transformation process, but also act as tumor antigens. The immune response evoked by these antigens is usually strong. Mouse embryo fibroblasts (Hb), transformed with recombinant plasmid containing the SV40 large T antigen coding sequence, express the antigen on the cell surface. CTLs specific for SV40 distinguish at least 2 antigens on these cells (Tevethia et al., 1984). Mice immunized with recombinant SV40 T antigen produce both humoral as well as cellular responses against SV40 T antigen and are immune to tumor challenge. The carboxy terminal of the T antigen was found to contain the epitopes for both humoral and cellular responses (Bright et al., 1994). Immune lymphocytes from these mice were found to produce IL-2, and IFN-γ but not either IL-4 or IL-5 when cultured with recombinant SV40 T antigen suggesting a Th1 type of response (Bright et al., 1995). Cellular immunity was also seen when mice were immunized with pSV3-neo, a plasmid containing the large T antigen.

The adenoviral E1A gene product is a strong tumor antigen and protects the animals from adenovirus induced tumors. Sawada et al., (1994) have shown that there is a group specificity exhibited by the E1A antigen of group A and group C adenoviruses. Mapping the domains of the E1A gene responsible for protection in these groups revealed that the antigenic activities of the adenovirus A and C are controlled by different domains of their respective E1A genes.
Bispecific antibodies with specificities for CD3 and the gp52 antigen of MMTV can redirect specific CTLs to kill tumor cells. Dendritic cells pulsed with the human papilloma virus type 16 (HPV16) E7 peptides can protect mice from HPV transformed cells (C3). Protective tumor immunity was mediated by CTLs that recognized HPV E7 (49-57) peptide pulsed target cells as well as C3 cells in vitro (Ossevoort et al., 1995).

1.5. Antigens shared by more than one type of tumor

This group comprises of proteins which have been detected in various classes of tumor cells. The tumor associated antigen L6 is a transmembrane protein and its expression has been shown in lung, breast, colon and ovarian carcinomas. Two other members of this family of transmembrane proteins (shown by DNA sequence homology), CD63(ME491) and CO029 are also highly expressed by various tumor cells (Marken et al., 1992). The murine counterpart of this antigen has been cloned and experiments suggest L6 as a promising target molecule for immunotherapy (Edwards et al., 1995).

The peptides of the HER2/neu proto-oncogene are shared antigens among human non-small cell lung cancer and ovarian cancer (Yoshino et al., 1994). Another mouse tumor antigen, 791Tgp72 is expressed on colorectal, gastric and ovarian human tumors. Murine monoclonal anti-idiotypic antibodies (mab2) representing the internal image of this antigen induce cellular responses (Durant et al., 1995).

Mucins have been implicated as tumor-associated antigens of adenocarcinoma from a variety of organ and tissue sites. One member of this family was isolated from a human pancreatic cell line HPAF. Rabbit monospecific polyclonal antibody against pancreatic apomucins reacted with a 200 kDa species. This antibody also cross-reacted with a breast mucin (Lan et al., 1990).

The carcinoembryonic antigen (CEA) family of proteins which exhibit a high degree of cross-reactivity among themselves are highly expressed by both embryonic colonic mucosa and carcinoma of the gastro-intestinal tract. These comprise a family of isoantigens (Herlyn et al.,
Another carcinoma associated antigen CA-195, is also a shared antigen among carcinomas of various tissue origins. The melanoma antigen MZ2-D encoded by the MAGE3 gene recognized by the autologous CTLs is expressed in melanomas and head and neck squamous cell carcinomas and breast carcinomas but not in normal tissues. These antigens represent attractive targets for developing broad range immuno-therapies, but the antigenicity of these proteins vary depending upon the tissue type of the tumor.

In addition to the above mentioned antigens, there are other types of tumor antigens which merit some detailed description. The primary ones among them are - 1) antigens resulting from post-translational modifications and 2) stress induced proteins.

1.6. Antigens resulting from post-translational modifications

Changes in the levels and patterns of glycosylation upon transformation lead to altered pattern of glycoproteins/glycolipids and increased levels of proteases/glycosidases lead to exposure of new antigenic determinants. Carbohydrate antigens are the most potent immunogenic agents among tumor antigens. Mostly humoral responses are evoked against this kind of antigens. Monoclonal antibodies to tumor-associated carbohydrate antigens may serve not only as classic immunological reagents but also have therapeutic value (Hakomari et al., 1991). Ganglioside GM2, a major ganglioside expressed by tumors of the neuroectodermal origin, is a T cell independent tumor antigen and evokes only antibody responses in rodents and humans. KM696 and KM697 are mabs against GM2, both of which are of the IgM class. Chimeric antibodies of these with mouse/human IgG1 not only retain strong reactivity with GM2 but mAb K966 mediates ADCC with PBL and complement dependent cytotoxicity with complement from human serum (Nakamura et al., 1994). Viral transformation of cells can also alter the glycolipid patterns. Glycolipid extracts of SV40 transformed Syrian golden hamster cell lines were found to be immunogenic (Ansel et al., 1984).

Melanoma patients undergoing vaccinia melanoma oncolysate (VMO) therapy developed IgG antibodies to a 31 kDa heavily glycosylated protein, which most likely represents a new melanoma antigen. Periodate treatment of proteins transferred to nitrocellulose membranes
showed that the relevant epitope could be a carbohydrate moiety (Berthier-Vergnes et al., 1994).

Two monoclonal antibodies against the fibrosarcoma of rats, KMT-17 define carbohydrate antigenic determinants. mAb K.-1 recognizes a globotriglycosyl ceramide and mAb KH-2 recognizes a lactocele ceramide and lactoneotetraglycyl ceramide. An MHC unrestricted cell line WD from a pancreatic cancer patient recognizes a large and heavily glycosylated mucin molecule, expressed on pancreatic and breast tumor cell lines (Barnd et al., 1989).

1.7. Stress induced proteins

Old and coworkers isolated a p84/86 tumor specific antigen from cytosolic fractions of Meth A cells which could provide immunity to Meth A cells. N terminal sequencing revealed that p84 and p86 were identical except for short segments which were unique to each isoform (Ullrich et al., 1986). Immunolocalization studies revealed that a substantial amount of p84/86 was localized to the cytosol, though a small proportion was seen on the cell surface.

cDNA cloning and sequencing of p84/86 antigens revealed that they are murine counterparts of the hsp90 family of proteins that are common to organisms from E. coli to man. (Moore et al., 1987). The gp96 antigen was purified from Meth A cells and was found to be a surface glycoprotein. A similar protein was isolated from CMS5 cells. Tumor immunity elicited by isolated Meth A and CMS5 gp96 protein show the same specificity as elicited by intact tumor cells. Using rabbit antisera to gp96 molecules, the distribution of gp96 was found in a wide range of normal tissues. The gp96 molecules were shown to be related to the hsp100 family of heat shock proteins. In addition the gp84/86 and the gp96 proteins were shown to be inducible by heat shock. Recently the human homolog of the gp96 protein has been cloned (Maki et al., 1990).

Because hsp90 are known for their ability to bind to a diverse array of molecules (Lindquist et al., 1988), it has been suggested that gp96 and p84/86 are not antigenic per se but act as carriers of immunogenic peptides and the specificity of immunogenicity resides in the peptides
rather than in the carrier (Srivastava and Maki, 1991). A large proportion of the gp96 molecules resides in the endoplasmic reticulum- the presumed site of peptide charging of MHC class I - and a role for gp96 in presentation of peptides to MHC class I has been proposed (Srivastava and Maki, 1991). In addition gp96 contains ATP binding cassettes, binds ATP, and possesses Mg** dependent ATPase activity (Li et al., 1993). Gp96 preparations from normal tissues did not elicit any tumor specific immunity. Two other hsps from Meth A sarcoma, hsp90 and hsp70 were isolated and their immunogenicity tested. Hsp70 was found to be highly immunogenic but the immunogenicity of hsp90 was 10% of that of gp96. Hsp90 also lacked measurable ATPase activity. The ATPase activity has been implicated in the ability of hsps to transfer peptides to acceptor molecules in the endoplasmic reticulum (Udono and Srivastava, 1994).

Srivastava et al., (1994), also proposed that hsps carrying endogenous antigenic peptides from viral infected cells or tumor cells can be released by lysis of these cells by the action of antibodies or other non specific mechanisms and taken up by professional antigen presenting cells, presumably by receptor mediated mechanisms. The hsp borne peptide is then routed to the endogenous antigen presenting pathway of the antigen presenting cell. Udono et al., (1994) showed that gp96 molecules can prime CD8\(^+\) cells in vivo and elicit a CTL response against Meth A sarcoma. Hsp based vaccines do not depend on the availability of cell lines or CTLs nor do they require definition of the antigenic epitopes of cancer cells. These advantages among others, make hsps attractive and novel immunogens against cancer.

1.8. Immune responses to tumors

For the host to mount a rejection response against a tumor, the immune system needs to be sufficiently activated. The different components of the immune system, the T cells (both CD8\(^+\) and CD4\(^+\)), antibody responses, non-antigen specific effector cells such as macrophages and NK cells, and production of cytokines have to be optimally activated. All the above mentioned responses to tumor cells and their antigens are known, though different tumor cells activate these components to different levels. In the following pages, each of these responses to tumor antigens will be dealt with in detail.
1.9. CD8+ T cell responses

The dazzling discoveries of the last 15 years on specific antigen recognition by T cells have demonstrated in detail how superbly designed this recognition system is to defend the body against intracellular pathogens such as viruses. The same features also make T cells markedly effective against virus induced tumors and other immunogenic tumors. The first breakthrough in this area was the realization that T cells recognize an antigen only in the context of Major Histocompatibility Complex molecules at the cell surface (Zinkernagel and Doherty, 1974; Bevan, 1975). The second landmark discovery concerns the fact that T cells do not recognize large proteins, but instead recognize small peptides presented in the context of the MHC. Such peptides are derived from the endogenous processing of proteins by fragmentation into peptides that have the ability to associate with MHC (Townsend et al., 1985). The third milestone in this field is the elucidation of the 3D crystal structure of the MHC class I molecule HLA-A2 (Bjorkman et al., 1987a; 1987b).

Activation of the CD8+ T cell requires the engagement of the T cell receptor complex by the appropriate MHC class I molecule associated with the antigenic peptide specific to the TCR on the target cell. Following activation, the death signal is delivered to the target cell by degranulation of the cytoplasmic granules which contain granzymes, perforins and TNF, causing the target cell lesion and apoptosis.

Unlike the antibody defined tumor antigens, CTL defined tumor antigens were not easy to study because of the difficulty involved in their isolation. Boon and colleagues made use of a direct genetic approach to identify tumor rejection antigens encoded by the cellular genome. They mutagenised clonal mouse tumors with the mutagen N-methyl-N'-nitrosoguanidine (MNNG) and obtained variants of these tumors which were unable to form tumors in syngeneic mice. These variants were called tum' variants (Van Pel et al., 1979; Boon, 1983). They subsequently showed that the failure of the tum' variants to form tumors is the consequence of an immune rejection response. This immune memory could be transferred adoptively by T lymphocytes (Boon and Van Pel, 1978). Several viral antigens that induce tumor rejection have been identified. The *gag* and *env* encoded components of Friend's
Leukaemia virus are recognized by class II restricted lymphocytes (Klarnet et al., 1989). The product of the adenovirus E1A is recognized by CTLs (Kast et al., 1989) and so is the nuclear antigen protein EBNA of the Epstein-Barr virus (Burrows et al., 1990).

Antigens of human tumors recognized by autologous T lymphocytes have been identified. The antigen MZ2-E, encoded by the MAGE-1 gene was identified on a melanoma with autologous HLA-A1 restricted, CTLs obtained by in vitro stimulation with tumor cells (Van der Bruggen et al., 1991). A second human tumor rejection antigen that has been identified is recognized on HLA-A2 melanomas by autologous CTLs. It is encoded by the tyrosinase gene (Brichard et al., 1993). Other antigens also recognized by HLA-A2 restricted CTLs and isolated from melanomas are the A, B, C antigens of the melanoma cell line SK-29-MEL (Wolfel et al., 1993) and the MART-1 melanoma antigen (Kawakami et al., 1994b).

Minev et al., (1994) showed that addition of an endoplasmic reticulum insertion signal to a naturally occurring tumor antigen expressed by the murine mastocytoma P815 (P1A aminoacids 35-43) significantly enhanced priming of specific CD8+ T cells in vivo. The signal sequence was shown not to merely enhance antigenicity nor did it act as a helper epitope. Using elegant biochemical techniques Cox et al., (1994) purified a nine residue peptide epitope presented on the HLA-A2.1 molecule of a melanoma cell line that is recognized by CTLs from five different melanoma patients. The high affinity of the CTL lines for this peptide suggests it to be a promising candidate for a peptide based melanoma vaccine.

Tumor reactive CTLs from patients with breast adenocarcinoma have been isolated which kill the tumor cells in an MHC restricted fashion (Jerome et al., 1991). The antigen that is recognized by these cells is a ductal epithelial mucin produced by breast and pancreatic adenocarcinomas. The tumor specific but MHC unrestricted lytic activity of these cells is mediated by the α/β T cell receptor. The protein core of the mucin consists of tandem repeats of a 20 aminoacid sequence. It appears that this part of the mucin crosslinks the T cell
receptor on mucin specific T cells and therefore accounts for the lack of MHC restriction seen in this system. This mucin core epitope is not expressed in normal cells and therefore represents truly a tumor rejection antigen.

These tumor antigens described above represent a few of the large number of tumor antigens that stimulate T cells. However, there is evidence that many human tumors can evade the immune system by either losing or down regulating the antigens or the MHC molecules on their surface (Doherty et al., 1984). In such cases, tumor specific CTL, though present may not be effective in eliminating the tumor. Redirected lytic activity of polyclonally activated or cloned CTLs by bispecific antibodies binding to the TCR/CD3 complex on effector cells and to the antigens on the target cells was reported (Staerz et al., 1985; Perez et al., 1985; Liu et al., 1985). The mechanism of action seems to involve IFNγ, TNFα and possibly other mediators like cytolysin and fragmentin (Shi et al., 1992).

1.10. CD4+ T cell responses
CD4+ T cells initiate and maintain antigen specific immunity. They do so by providing help, in the form of secreted cytokines, for both B-cell responses and CD8+ T cell responses, as well as by sustaining immunological memory in the competent host. They may also perform antigen specific effector function directly via cytokine secretion, or less commonly via cytolysis. Given their central role in the complex immune network that leads to antigen specific reactivity, it seems incongruous that relatively little is known about the participation of CD4+ T cells in the anti-tumor immune response. Even so, a thorough inspection of the extensive literature concerning specific humoral and CD8+ cellular responses against a variety of murine and human tumors suggests that tumor reactive CD4+ T cells are now beginning to be defined, opening new possibilities for the design of more effective immunological approaches to cancer immunotherapy. The rapidly growing literature about cytokine secreting tumor cell vaccines has provided definitive evidence for the participation of T helper (Th) cells in generating and maintaining anti-tumor immunity.
That the secretion of IL-2 by antigen activated CD4+ T cells is important for recruiting CD8+ T cells to the immune response was shown by Fearon et al., (1990). CT26 colon carcinoma cells, which are poorly immunogenic by themselves were genetically engineered to secrete IL-2. Mice could reject large doses of the genetically modified tumor cells and in vivo depletion of T cell subsets demonstrated that CD4+ T cells were not critical for primary tumor rejection. However, these mice were unable to establish long-term immunity and failed to reject secondary tumor challenges, indicating that CD4+ T cells are required for establishment of immunological memory. Other studies (Pulaski et al., 1993; Karp et al., 1993) confirmed these findings that CD4+ T cell responses though not essential for the primary response, were required for establishing long lasting systemic immunity.

Tumor cells have the potential to act as presenters of their own antigens via the MHC class II pathway for CD4+ T cell recognition. To perform this function efficiently, tumor cells may need to be modified to express sufficient quantities of MHC class II antigens and/or the appropriate costimulatory molecules (Ostrand-Rosenberg, 1994). It has recently been shown that professional APCs, such as macrophages, langerhans cells and dendritic cells can act as third party cells for Th cell recruitment to the anti-tumor response. The B16 melanoma was genetically modified to secrete large quantities of GM-CSF and used as a prophylactic tumor vaccine. Vaccinated mice were immune to secondary challenges of parental tumor, but not to an unrelated tumor demonstrating specific and long lasting tumor immunity. In vivo depletion studies demonstrated the roles of CD4+ and CD8+ T cells in the primary and recall phases of anti-tumor response. Extensive infiltration of the primary vaccination site by mononuclear cells was observed, implicating these cells in tumor antigen presentation (Dranoff et al., 1993). Using a murine system, Cohen et al., (1994) showed that professional APCs pulsed with tumor digests can be used to detect and expand tumor reactive CD4+ T cells in vitro. These CD4+ T cells were shown to be highly specific for the immunizing tumor. EBV transformed B cells have been shown to process and present human melanoma antigens to melanoma specific CD4+ T lymphocytes (Topalian et al., 1994).
That the role of CD4⁺ T cells in tumor response is independent of CD8⁺ T cells has been demonstrated by Greenberg et al., (1985). They showed that either purified CD4⁺ or CD8⁺ anti-FBL immune cells could induce regression of established FBL tumor upon adoptive transfer to immunocompromised mice. The authors hypothesized a role for professional APCs in processing tumor antigens for CD4⁺ T cell recognition. The antigen recognized by CD4⁺ T cells in this system is encoded by the env gene of the Friend's leukaemia virus. They generated transgenic mice for the env gene which are tolerized for the env antigen, and these mice were not able to mount a response to the FBL tumor. In contrast, non transgenic mice could be immunized successfully. Thus CD4⁺ T cells were shown to be a critical component of the tumor regression mechanism.

1.1. Role of NK cells
Since the early 1970s natural killer cells have been recognized as a functionally distinct subset of lymphocytes (Gorelik et al., 1982; Herberman and Holden, 1978). In rodents, as well as in man, these cells have a morphology of large granular lymphocytes (LGLs) and are endowed with the ability to kill a variety of cell types, including a wide range of tumor cells and virus infected cells. NK cells isolated from human peripheral blood, or from rodent blood and spleen, kill these targets spontaneously without the need for prior sensitization and without class I MHC restriction. It is because of their unique and spontaneous ability to eliminate tumor cells, but not normal tissue cells, in in vitro cytotoxicity assays that this subset of lymphocytes has been named natural killer cells and is presumed to play an important role in surveillance of cancer in vivo (Herberman, 1983).

NK cells also have another property that is essential for their surveillance function: they are responsive to a wide variety of biological, and other agents by the up regulation of cytolytic, proliferative and other functions and by the secretion of a variety of other cytokines (Kasahara et al., 1983). The mechanism of NK mediated oncolysis involves recognition and conjugation of effectors to targets and delivery of the death signals and disintegration and the death of target cells (Herberman et al., 1986; Young, 1989). The nature of the target antigens and the receptors on the NK cells that recognize these antigens have not yet been conclusively
established. Cross-linking of the low affinity Fc receptor on NK cells to its ligand, the Fc portion on the target cell surface, initiates a cascade of intracellular biochemical signals in the NK cells that ultimately induces antibody dependent cell-mediated cytotoxicity (ADCC).

On the basis of the data derived from studies of animal models of tumor growth and metastasis, it is generally proposed that NK cells are able to recognize and eliminate tumor cells in the peripheral circulation. In a series of classic experiments Gorelik and Herberman (1986) showed that NK depleted mice with B16 melanoma had uncontrolled metastases. Conversely, Barlozzari et al., (1983) showed that adoptive transfer of purified LGLs could restore the resistance to metastases in immunocompromised rats. In humans, accumulating evidence indicates that patients with advanced metastatic disease often have abnormalities NK cell function and/or NK cell numbers (Whiteside and Herberman, 1994).

The earliest events in cancer development occur not in the circulation but in tissues, when a transformed cell begins its multi step process of forming a primary tumor. Recent insights into the biology of NK cells indicates that these cells are capable of interfering with this process. The NK cells appear to be able to discriminate between abnormal and normal cells by at least two interactive receptor systems: the NK receptor (NKR-P1), which recognizes oligosaccharide moieties on target cells and may trigger killing of abnormal cells (Bezouska et al., 1994); and another receptor family, which recognizes autologous MHC class I molecules expressed on all normal nucleated cells and turns off cytotoxic activity triggered by the first receptor. Moreover, it appears that in humans, NK cells are resident lymphocyte populations in a variety of tissues. In normal human liver, NK cells represent up to 50% of liver associated lymphocytes (LALs) (Hata et al., 1991). All these evidences indicate that NK cells may be the key immune effector lymphocytes capable of mediating the first line of defense against malignant and virus infected cells.

On the basis of their ability to respond to 2-22 nM of IL-2 within minutes by rapidly adhering to solid surfaces, Vujanovic et al., (1993) have defined two phenotypically and functionally distinct subsets of NK cells. This property of IL-2 induced adherence distinguishes activated,
adherent (A-NK) cells from activated non adherent (NA-NK) cells. Only A-NK cells are able to efficiently enter tumor tissues, eliminate established tumor metastases, or prolong survival following adoptive transfer of these cells together with IL-2 in animal models of tumor growth or metastasis (Yasamura et al., 1994).

As mentioned earlier, the exact mechanism of NK mediated oncolysis remains ill defined but involves a complex array of events such as (i) recognition and conjugation of effector to target, (ii) NK cell activation and delivery of the lethal hit and (iii) target cell disintegration and death. Antibody dependent cell mediated cytotoxicity (ADCC) of target cells by NK cells is another mechanism by which NK cells mediate oncolysis. Kausalya et al., (1994) have shown that binding and conjugate formation of NK cells isolated from tumor bearing rats with target tumor cells requires the participation of antitumor antibody. NK cells from normal animals when activated by IL-2 are able to form conjugates with the target cells and mediate ADCC.

1.12. Humoral responses

Antibodies against autologous tumor cell surface antigens were first reported by Carey et al., (1976; 1979) and Shiku et al., (1976; 1977) who used sensitive microsurgical assays and cultured tumor cells. These authors demonstrated antibodies against autologous tumor cell surface antigens in a third of melanoma patients studied. However, due to the low incidence and weak titer of autologous antibody to melanoma, questions were raised regarding the relevance of humoral immunity in melanoma. The presence of circulating antigen in patients and the resulting formation of immune complexes may explain this. In native serum from patients, the levels of these antibodies are usually low with decreased titre. Dissociation of immune complexes by acidification and ultrafiltration of sera augments autologous antibody reactivity in the majority of cases studied (Kirkwood et al., 1984). Using acid dissociated and ultrapurified autologous antiserum S150 against the melanoma cell line Y-Mel 84:420, a 66 kDa antigen was purified by Vlock et al., (1988) and shown to be an acidic glycoprotein (Vlock et al., 1991).
In addition to their diagnostic value, autologous polyclonal and monoclonal antibodies to tumor antigens have uses in the immunotherapy of cancer. Using different adjuvants, IgG monoclonal antibodies to the well defined tumor associated antigen CA-195 were produced which have a high diagnostic value (Kuus-Reichel et al., 1994). Monoclonal antibodies, either radiolabeled or coupled to anti-tumor drugs have been demonstrated to destroy tumor cells specifically and safely (Jurcic et al., 1994).

Winter et al., (1993) demonstrated that antibodies against autologous tumor cell proteins in patients with small cell lung cancer are associated with improved survival. A murine monoclonal antibody BR96 against a human carcinoma can inhibit the growth of human tumors in nude mice. BR96 is a murine IgG3 that internalizes and is cytotoxic to cells expressing the antigen in vitro and also elicits a strong ADCC and complement dependent cytotoxicity. A mouse human chimeric form and an IgG1 class switched variant of BR96 was also used. The chimeric form had the strongest effects indicating that Fc dependent host effector functions were primarily responsible for its in vivo activity.

Another murine mAb of the IgG2a type was developed against endocrine pancreatic carcinoma associated liver and peritoneal metastases as well as therapy resistant Verner-Morrison's syndrome. When passive immunotherapy with this antibody was given, disappearance of metastases and alleviation of other symptoms was observed. A full length cDNA for CEA under the cytomegalovirus early promoter/enhancer (pCEA) was used as a polynucleotide vaccine and was shown to induce a CEA specific response. This vaccine protects 100% of the mice from CEA expressing colonic cancer cells. Both cellular and humoral responses against CEA are seen in the protected mice (Conry et al., 1995).

An anti-CEA antibody PR1A3, used successfully for imaging colorectal cancers in vivo, recognizes a conformational epitope at the site of membrane attachment which involves parts of the glycosyl-phosphatidyl inositol anchor and the B3 domain of CEA. Access of the mAb to this epitope is possible only when the antigen is attached to the cell membrane (Durbin et al., 1994). Surface immunoglobulin idiotype expressed by B cell tumors induce anti-idiotypic
responses and these responses have been shown to be involved in suppression of certain B cell lymphomas. Immunization of Balb/C mice with idiotypic IgM from the syngeneic B cell lymphoma BCL1 protects the immunized mice against challenge with tumor cells. Cytotoxic anti-idiotypic antibody in sera of immunized mice and splenic T cells that proliferated specifically in response to the idiotypic antibody were observed. Passive transfer studies with the anti-idiotypic antibody demonstrated a major role for antibody in the protection against the tumor (George et al., 1987).

Patients with B-cell lymphomas were immunized with the Ig expressed by the tumors along with the adjuvant. Seven out of nine patients showed strong humoral and cell mediated responses against the tumor. In two of the patients the tumor regressed completely indicating that both humoral and cellular responses are required for effective elimination of tumor (Kwak et al., 1992).

Many tumor antigens though capable of inducing immune responses and being localized to tumor tissues only, do not afford complete protection to the immunized host. In such cases, anti-idiotypic antibodies, i.e., antibodies to the monoclonal antibodies recognizing the tumor antigen have been found to be better than the tumor antigen itself. Anti-idiotypic antibody (Ab2) provides specific immunity in two ways; (i) presents the critical epitope of the antigen in a different way and (ii) induces the production of Ab3 which can bind to the tumor antigen. In addition, the idiotypic network is activated which leads to an efficient response and elimination of the tumor through mechanisms which are yet to be defined. Immunization with such an idiotypic antibody, OC125, mimicking the TAA CA125, leads to prolongation of survival rate even for extended stages of the tumor (Wagner et al., 1992). Immunization of a patient with an anti-idiotypic antibody to mAb 17-1A raised in goats recognizes the tumor antigen 17-1A of colorectal carcinoma and resulted in an enhanced humoral and cellular response against the tumor. 791Tgp72 is a tumor associated antigen expressed on colorectal, gastric and ovarian human tumors. Anti-idiotypic antibodies against a mAb 105AD7 recognizing this antigen was used to induce strong cellular responses against the tumor (Durrant et al., 1995).
Recent developments in recombinant DNA technology have led to the development of genetically engineered antibodies which have specificities to both the antigen expressed by the tumor cell and to a receptor on the host T cells. Hypothetically, these antibodies can bring the T cells of the host in contact with the tumor cell and thus lead to its elimination by the CTLs. Such molecules have been prepared as chemically linked antibody heteroconjugates (Karpovsky et al., 1984) or as bispecific monoclonal antibodies derived by somatic cell hybridization of two existing hybridomas (Staerz et al., 1986). These bispecific antibodies have been quite successful in experimental tumor systems and show promise in development of future immunotherapies. A bispecific antibody, CL158, recognizing the CA19-9 antigen and the CD16 molecule expressed by LGLs, macrophages and some T cells, significantly reduced the growth of implanted tumors in mice (García de Pallazzo et al., 1992).

Mechanistically, bispecific antibodies seem to act through the activation of both the T cell receptor and costimulatory molecules. Bispecific antibodies that bind to a Hodgkin’s tumor associated antigen CD30 on the tumor cell and to either CD3 or CD28 on the T cells were produced. Immunodeficient mice were cured of established human tumors when mice were treated with both the CD3-CD30 and CD28-CD30 bispecific antibodies and then given human peripheral blood lymphocytes previously incubated with the CD3-CD30 bispecific antibodies and cells expressing CD30 (Kroesen et al., 1995). Enrichment of human T cells within the tumor and the fact that established tumors can be cured may indicate in situ activation of both T cell receptor and the costimulatory molecule CD28 (Renner et al., 1994).

Chimeric murine monoclonal antibodies carrying human Fc portions have been found to be more effective in mediating cytotoxicity against tumor cells presumably through their ability to mediate ADCC with human NK and T cells and complement dependent cytotoxicity (George et al., 1994). A chimeric anti-pancarcinoma monoclonal antibody (323/A3), recognizing the 17-1A antigen has the ability to mediate ADCC and complement dependent cytotoxicity with human peripheral blood lymphocytes against LS180 cells derived from human colon carcinoma (Velders et al., 1994).
1.13. The AK-5 tumor model

The system used during the course of this study is a rat transplantable macrophage like tumor cell line, the AK-5, which arose spontaneously in one of the animals in an inbred Wistar rat colony of our laboratory and is passaged intraperitoneally as ascites. The lineage of AK-5 has been established by a careful analysis comprising histochemical, biochemical, histological and physiological studies (Khar 1986; Khar et al., 1990). AK-5 tested positive for the presence of Fc receptor, C3d receptor, MHC class II antigen, leukocyte common antigen (CD45), and MO1 antigen. The tumor cell extracts showed lysozyme, non specific esterase, acid phosphatase and peroxidase activities. Electron micrography revealed a kidney shaped nucleus typical of macrophages. Most convincingly of all, AK-5 demonstrated phagocytosis. All these criteria indicate that AK-5 belongs to the macrophage class.

In addition to these features which establish it to be a macrophage cell line, AK-5 is highly immunogenic. When passaged intraperitoneally, AK-5 is highly malignant, killing 100% of the animals within 8 days. However, subcutaneous injections of AK-5 into syngeneic animals result in solid tumors which grow relatively slowly. By day 14 post-transplantation, the solid tumor starts regressing spontaneously, accompanied by necrosis in about 80% of the animals and disappears completely by day 25 in these animals. Animals that reject the tumor do not accept further challenges of AK-5, either intraperitoneally or subcutaneously (Khar, 1986). This property of AK-5 is extremely interesting, since spontaneous tumors are not known to be highly immunogenic. Investigating the mechanisms of the spontaneous regression of AK-5 has been the main focus of our laboratory for the past few years.

Earlier work in our laboratory had shown that the mechanism of rejection of AK-5 involves activation of multiple factors and effectors of the immune system. Animals that reject the tumor have high titres of circulating anti-tumor antibody in their serum, which reach peak titres around day 10 post-transplantation. The anti-AK-5 antibody obtained from tumor regressed animals can lyse the tumor cells in the presence of rabbit complement in a complement mediated lysis assay (Khar, 1993). The anti-AK-5 serum was specific in that it did not lyse other tumor cells like ZAH, YAS or Meth-A. Cell membrane or extracts of AK-
5 inhibit the complement mediated lysis of AK-5 cells by the anti-AK-5 antibody. It was also shown that NK cells from tumor bearing animals mediate killing of AK-5 cells *in vitro* through ADCC, anti-AK-5 being the specific mediator in this reaction (Khar, 1993).

The aim of the present study was to identify and characterize the antigenic molecule(s) responsible for such a strong humoral response in the tumor bearing animals. To achieve this end, we decided to employ a molecular cloning strategy using the anti-tumor antibody as probe. This approach has two advantages. First, there is the possibility that there may be more than one antigen responsible for the antibody response, and this strategy would most likely pick up the protein with the strongest reactivity to the anti-AK-5 serum. Second, the antigen thus isolated would be amenable to *in vitro* manipulation and expression and allow the characterization of its antigenic epitopes and its utility as a tumor vaccine. What follows is a description of the isolation and characterization of one such antigen obtained by immunoscreening a cDNA expression library of AK-5 using the anti-AK-5 serum from tumor bearing animals.