Normal cell has multiple independent mechanisms that regulate its growth and differentiation potential and several separate events are needed to override these control mechanisms as well as to induce the other aspects of the transformed phenotype (119). Among the various changes that occur in a normal cell upon transformation, alterations in the cell surface profile are crucial. A large number of antigens, which are expressed as a consequence of transformation are components of cell surface (120-124). The antigens detected on the malignant cells are not strictly tumor specific (39,40). Based on the molecular nature and the pattern of the antigen expression during the life cycle of a cell, tumor specific antigens are further divided into various classes (21,26) (see Introduction). Of these several classes, two, which have attracted considerable attention are tumor specific transplantation antigens (125) and normal cellular proteins which are either over-expressed or have altered conformation upon transformation (126). Besides their tumor specific nature, the precise biological function of these antigens is unknown (127,128). Many of these antigens are expressed as a result of the aberrant expression of the proteins upon transformation, which is well documented (129).

4.1. Observations of the present study:

An albumin like antigen of molecular weight 67 kd has been purified to homogeneity from the 3M KCl extracts of AK-5 cells. The antigen can protect the tumor cells from lysis by anti-tumor antibody and complement in in vitro cytotoxicity assay.
The protein resembles albumin, when analysed by different biochemical criteria such as the molecular weight, pattern of glycosylation, isoelectric point, and single dimensional peptide mapping. The immunological crossreactivity of the protein with rat serum albumin has been tested using the antialbumin antiserum.

The organisation of the gene coding for albumin has been studied using the normal liver as a control and the gene specific for albumin appears to be normal in AK-5 with respect to the copy number and the arrangement. The size of the albumin specific transcript in AK-5 is same as that of the liver.

The purified antigen is tumor specific as indicated by the cytotoxicity assay which utilises the tumor specific antiserum raised in syngeneic animals. It can be purified from the membranes of the tumor (AK-5) cells using the same protocol of purification. The antibodies against the tumor recognised the antigen in a Western blot as well as in the immunoprecipitations.

The protein can be distinguished from albumin using the antibody against the tumor which is unable to recognise rat serum albumin though the antialbumin antibody can recognise the antigen and AK-5 cells in turn.

Based on the above mentioned observations underlying questions which need to be addressed pertain to issues, such as the ectopic expression of genes upon malignant transformation, secretion of the proteins across the plasma membrane and the context dependent changes in the patterns of immunogenicity of the proteins.
4.2. Protein purification:

A scheme of protein purification usually exploits the variation in physical or chemical properties among different molecules. 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 scientific principles.

4.2.1. Purification of tumor antigens:

The purification of a tumor specific antigen which is cell surface associated involves three major steps, dissociation of the antigen from plasma membrane, assay of the antigen and purification of the antigen(s) (130). The only location consistent with the role of tumor antigens as targets for the destruction of the tumor cell by immune lymphocytes or by antibodies and complement is plasma membrane (8).

It is relatively simpler to purify a protein whose biological activity can be assayed. Before designing the scheme for the purification of the tumor specific antigen, a suitable assay was needed to be standardised for the detection of its activity. In the past, tumor specific antigens have been assayed either by using transplantation procedures or by cytotoxicity assays (131). Tumor specific antigens have the ability to induce immune response both at cellular as well as humoral level. Also, as a tumor specific antigen(s) is the major determinant of immune response purified or semipurified preparations of these antigens can be
used to prime a syngeneic host against a subsequent tumor challenge by immunisation. Both the characteristics of tumor specific antigens mentioned above have been exploited while designing suitable assay procedures.

Transplantation test involves challenging immunised and control animals with viable syngeneic tumor cells and monitoring the subsequent growth of these cells by tumor incidence, tumor size or death from tumor. The test is made more viable by challenging with several doses of tumor cells \((10^3, 10^4, \text{and } 10^5 \text{ viable cells per animal})\). On certain occasions irradiated tumor cells have been utilised for immunisation in order to avoid the multiplication of tumor cells although X-irradiation may result in some loss of immunogenicity.

Though useful, transplantation tests are expensive and slow, since they require large number of animals and several weeks of observation time. Initially cytotoxicity tests utilised colony inhibition assay in which decreased ability of tumor cells to form colonies in presence of immune lymphocytes was assayed. Colony inhibition assay is largely replaced by microcytotoxicity and radioisotope release assay. Assays of cellular immunity \textit{in vitro} allow the effector cells to be characterised but the greatest disadvantage with these tests is that \textit{in vitro} assays often do not correlate well with transplantation tests. The complement mediated antibody dependent cytotoxicity assay which was utilised during the course of this study is based on the principle of competition between the soluble antigen and the cell bound antigen for the cytotoxic antibody and hence the complement fixation. The assay was expected to work only, if the cytotoxic antibodies were directed primarily against a single epitope. The specificity of the assay was ensured by using the antiserum which was purified using various
other cell types. The assay was preferred over the rest of its kind for its specificity, speed and operational simplicity.

4.2.2. Solubilisation of cell surface antigen:

Between the two kinds of membrane proteins, peripheral and integral, the former can be dissociated from the membrane by mild treatment such as changing the ionic strength or adding chelating agents while a complete disruption by a detergent is required for the latter. Salt extraction usually with hypertonic (3M) KCl has been extensively used to solubilise major histocompatibility complex antigens from spleen and tumor specific antigens from the tumors. Also mild protease treatment can be used to release the antigen from the membrane.

In spite of the possibility of denaturing the structure of the antigen, detergents are frequently used for this purpose. Nonionic detergents such as Triton X-100 or Nonidet P-40 are widely used since they do not denature the antigens and have little effect on antigen antibody interaction. In addition, non-ionic detergents do not dissolve the nuclear membrane and hence nuclear contamination can be avoided.

4.2.3. Use of high salt extractions to obtain the crude antigen for purification:

Use of detergents to solubilise the membrane proteins is limited primarily due to the possibility of loss of the function of the protein which is demonstrable when the protein is in the membrane associated form. Moreover, no single detergent has emerged as the best choice. Though most of the detergents solubilise peripheral as well as integral proteins, a milder method like 3M KCl...
extraction was preferred over the use of a detergent. Salt extraction procedures have been utilised to isolate and purify tumor-specific antigens (78). The peripheral localisation of the antigen was indicated by its removal using salt extraction method. Digestion of the extract with nonspecific proteases abolished the activity suggesting the proteinaceous nature of the antigen.

4.2.4. Purification of cell surface antigens:

Two different methods can be employed for the purification of tumor antigens. One is to use biochemical techniques to fractionate the antigen preparation while the other is to use specific antibody to purify tumor antigens immunochemically. Both methods are often combined.

In addition to the techniques such as gel filtration, ion exchange chromatography and isoelectric focusing, affinity chromatography on columns of lectins coupled to sepharose are being increasingly used as a rapid mild method to purify cell surface antigens. This exploits the glycoprotein nature of the antigen which is detected by the lectins.

As mentioned earlier (see Introduction), a small number of tumor specific antigens are biochemically defined. Notable among these are Gp96 antigens which were identified by separating fractions of cytosol and plasma membranes of 3 different MCA-induced Balb/c mouse sarcomas (Meth A, CMS5, CMS13). Another antigen, p84 was isolated from Meth A sarcoma by column chromatography of cytosol fractions. In recent past, a tumor specific antigen has been characterized from a melanoma which is shown to resemble albumin using immunological techniques.
4.2.5. Purification of the antigen using chromatographic procedures:

Naturally occurring polysaccharide and synthetic polymers that can be formed into beads with varying pore sizes depending on the extent of interchain polymer crosslinking are used to resolve the mixture of the proteins based on their ability to penetrate the beads.

Use of Sephadex G-150 column allowed successful fractionation of proteins from KCl extract into various peaks while the activity of the protein as an antigen as judged by the assay was confined to the peak collected just after the void volume.

Specific and reversible adsorption to a ligand which is immobilised on an insoluble matrix forms the basis of the affinity chromatography. It has been utilised to purify substances from the complex biological mixtures in a concentrated form in a single step procedure. The purification protocol devised for the tumor specific antigen of AK-5 involves two affinity chromatography procedures out of which blue sepharose CL-6B contains the dye, cibacron blue F3G-A coupled to sepharose and is used for the purification of a wide variety of NAD/NADP containing enzymes, albumin, interferon, etc. Cibacron Blue is covalently attached to the crosslinked agarose gel to generate the final form of the column material.

Purification to near homogeneity was achieved by the use of blue sepharose column chromatography as is seen from the figure 4. Antialbumin antibody affinity column was utilised to purify the antigen, essentially to exploit the possibility of immunological crossreactivity of the protein with rat serum albumin.
4.2.6. Determination of the molecular weight and resemblance of the antigen with rat serum albumin:

The specific antibodies raised against rat serum albumin were coupled to CNBr activated sepharose 4B to make an affinity column. In addition, to achieve further purification, this step was used to test the ability of the antigen to bind to the column based on the epitope profile. Immunological crossreactivity of the antigen with rat serum albumin was clearly indicated by the elution profile. Purified fractions obtained from both purification procedures performed sequentially, were run on an SDS polyacrylamide gel. The molecular weight of the protein (antigen) was 67 kd which is the same as that of rat serum albumin.

Co-migration of the protein with rat serum albumin and its ability to bind to antialbumin antibody column and blue sepharose indicated the similarity between the two proteins, albumin like antigen and albumin.

4.3. Comparative analysis of albumin like antigen and albumin:

Chemically induced tumors of rodents generally express individually distinct immunogenic antigens, although occasional instances of cross reactivity have been recorded. In case of tumors which are induced by DNA viruses, similar antigens are shown to be involved in tumor rejection (135). The molecular nature of non-viral cross-reactive antigens is not known. Identity of tumor antigens which elicit individually distinct immunity has been the subject of much speculation and little structural analysis (24).

In the light of these observations, the similarity between the antigen and albumin is noteworthy. What follows is a detailed discussion of comparative analysis of albumin like antigen and albumin. Amino acid composition and
sequence are the two properties which impart unique characteristics to each protein. Depending on these two factors, the physical and chemical properties of a protein vary. The criteria which are used for the characterisation of the protein are more or less similar to those used during the design of the purification protocol. In order to compare two proteins, techniques which are utilised are primarily based on the molecular weight, charge, amino acid sequence and composition. The post translational modifications and epitope profile based on the antibody recognition are two other parameters which have been exploited for the same purpose. Albumin and albumin-like antigen appeared to be similar when compared using several of these techniques.

4.3.1. Immunological crossreactivity of the antigen with albumin:

The antigenic determinants or epitopes are defined as the structurally defined sites of three dimensional composition on an antigen where the specific antibodies bind by their complementary binding sites. The complex antigens possess a mosaic of different antigenic determinants to which a multiplicity of the antibody specificities may be present as a result of polyclonal stimulation. Each antibody molecule is specific to the antigenic determinant to which it binds while the specificity of an antiserum is constituted by the specificities of individual antibodies it contains (136).

When the antigenic determinants are shared between the molecules, antibodies raised in response to one antigen may bind to another antigen and are said to be crossreactive. In other words, a crossreactive antigen is one that binds the antibodies induced in response to a different molecule by virtue of shared antigenic determinant (136).
The pattern obtained using the Western blot and the subsequent immunostaining using the fluorescence labelled antibodies reveals the shared epitopes between the two molecules and also reflects upon the extent of relatedness in terms of the antigenicity.

Both the proteins under consideration were found to be immunologically crossreactive using the antialbumin antibody raised in rabbit in a Western blot. This result also explained the ability of the antigen to bind to the antialbumin antibody affinity column.

4.3.2. Analysis of the albumin specific transcript in AK-5:

Inability to detect any difference between the two proteins on the basis of techniques such as isoelectric focusing, peptide mapping, glycosylation pattern/PAS staining and amino acid analysis led to the analysis of the message using hybridisation procedures.

Experiments done to investigate the possibility of the presence of a message specific for the albumin gene indicated ectopic expression of this liver specific gene in AK-5 which is a macrophage line. Conclusions derived using the northern hybridisations are noteworthy i.e., the size of the albumin specific transcript in AK-5 is comparable to that from normal liver and the hepatoma although the amount of the message was found to be less.

Initiation of the messenger RNA (mRNA) synthesis is the primary control point in the regulation of differential gene expression (137). The regulatory elements for transcription by RNA polymerase II are scattered, both downstream and upstream of the RNA start site for a gene. The cis regulatory elements are the binding sites for the transcription factors which have the ability to promote or
repress the transcription and they are arranged in a position dependent manner (138).

The cis acting regulatory sequences involved in the activation of transcription are usually distinguished in promoters and enhancers. The former is made up of a cluster of cis acting elements located immediately upstream of the initiation site and is generally defined as the minimal DNA segment capable of directing transcription. The enhancers are defined as the DNA sequences able to activate transcription over and above that observed with the promoter alone and can exert their effect in a position- and orientation-independent manner (139). Recently, however the distinction between the promoters and enhancers has become less sharp. Both the modular units, containing common and/or distinct short DNA motifs, are the binding sites for the trans-acting factors and have a specific role in the tissue specificity or in the general enhancement of transcription (140,141).

In brief, in the case of eukaryotes, the regulatory sequences contain variety of sequence motifs which have the ability to respond to different stimuli. As mentioned earlier, the establishment of regulatory network involves, in addition to the cis elements, the transfactors which bind at the cis regulatory elements and modulate the transcription.

During mammalian development, the albumin gene is expressed in a tissue specific and temporally regulated manner (100). Regulation of the albumin gene expression has been analyzed utilizing various hepatoma cell lines as well as the normal liver (142). The control of albumin synthesis has been shown to be modulated at the transcriptional level (110,112). A transient expression
system has been developed to analyze the role of the trans-acting factors which are crucial with respect to the tissue-specific expression (106). A recent report indicated the presence of four trans-acting factors involved in the tissue-specific expression which bind to the regulatory site 150 bp upstream of the transcriptional initiation site (103).

In light of the facts regarding the regulation of the albumin gene expression and the mechanism of its transcription control, our results obtained using AK-5 cell line as a model system can provide significant clues. Important observations with respect to the expression of albumin indicate similar transcriptional and translational regulation of the albumin gene in AK-5 and the liver. This fact is reflected in the biochemical behaviour of both the proteins. The claim is further supported by the analysis of the albumin specific message in AK-5. The organization of the albumin gene is same as that of the normal liver and hepatoma, which is consistent with the observations of the other workers (100).

4.4. Is the aberrant expression of the gene in AK-5, a consequence of presence of liver specific trans-factors?

Deviation from the normal pattern of expression of the albumin gene can be explained using one or many mutations in either the cis control elements or the trans controls i.e., trans factors or both. The possible experiments that could be designed can be listed as follows:

1) Cloning of the cis control elements from genomic library of AK-5 using the available rat specific clones which code for 5' promoter region as well as enhancer region which is upstream (10 kb).
2) Purification of trans factors from the cell or nuclear extracts of AK-5 which are aberrantly expressed in a macrophage like cell line.

3) To check for the presence of other liver specific genes which would indirectly indicate the activity of liver specific trans factors in a histiocyтомa.

   It has been suggested that the production of trans-factors is carefully regulated in non-transformed cells, but their expression becomes deregulated during malignant development. Thus, AK-5 is a useful model system for studying liver specific transcription factors.

4.5. Tumor specific nature of the antigen:

   Tumors elicit immune response in the syngeneic host of origin due to the presence of tumor specific antigens. These antigens may serve as useful markers for diagnosis or immunotherapy (143-145). The tumor specific antigens were first detected during a series of studies with chemically induced tumors of inbred rats and mice (146). The purified preparations of such antigens were shown to induce specific immunity (147). The specificity has been demonstrated in a wide variety of sarcomas and carcinomas induced by a number of carcinogens including UV irradiation (148-150). Unlike the individually distinct antigens of chemically transformed tumors, the viral antigens display remarkable similarity. Also the immunity induced by these has been found to be cross reactive (151). Interestingly, antigens unrelated to that of viral origin show homology with stress induced proteins.

   The second interesting aspect of this work relates to the peculiar behaviour of the protein as a tumor-specific antigen i.e., its capacity to neutralize the anti-tumor antibody. While antigen can complex with the antialbumin
antibody, serum albumin does not act as an antigen in the lysis assay. Also, it is not recognized by the anti-tumor antibody in immunoprecipitations or Western blots. Under normal circumstances, albumin is the largest component of the serum proteins and is secreted by liver constitutively. Though several variants of serum albumin exist, none of them have been reported to be immunogenic within the same species. Amino acid analyses of both the antigen and rat serum albumin are remarkably similar, although rat serum albumin has lesser cystine (0.19%) as compared to the antigen (2.35%). There are certain other minor differences which cannot be interpreted easily at this stage (glycine and alanine contents of rat serum albumin appear to be marginally more than that of the antigen). Out of the 24 amino acids that are sequenced, excluding the three at the N-terminus, rest are identical.

An explanation which can account for most, if not all the observations mentioned above, is based on the fact that both the proteins i.e., the antigen and serum albumin are largely similar (as indicated by immunoblotting with the anti-albumin, peptide mapping and expression studies), but unidentical (as indicated by the immunoblotting and immunolysis assays with the anti-AK-5 antiserum). The common antigenic determinants, which are generated due to similarity at the primary structure level, are recognized by antialbumin antibody and explains the ability of the antialbumin antibody to recognize the AK-5 cells, and in turn lyse them in presence of complement while the ability of the antigen to evoke an immune response within a syngeneic host may be due to an epitope unique to it. This antigenic determinant is recognized by the anti-AK-5 antibody and hence the protein can protect the cells from the lysis by anti-AK-5 antibody, while
albumin cannot do so due to the absence of the epitope, which is involved in binding with the anti-AK-5 antibody. The ability of the antigen to neutralize the anti-albumin antibody, and in turn, its capacity to protect the cells from lysis in the presence of the antialbumin antibody, supports the fact that both the proteins share antigenic determinants which is, probably, a result of commonality at the primary structure level.

Several non-viral tumor antigens have turned out to bear homology with the stress induced proteins. Each of these proteins is shown to be present not only in the tumors, but also in normal tissues (39,40). In recent past, a mouse melanoma is shown to express an albumin related antigen (B700), which elicits crossreactive immunity (152). The immunity induced by the administration of B700 protein provided the first indication of its similarity to albumin, though the mechanism of antigenicity of the protein is yet unclear. The possibilities which can be outlined to explain our observations are: first, a crucial difference at the level of primary structure, significant with respect to the epitope profile, which is not detected as yet. Second, the association of the protein with cell surface might give rise to a conformation which is immunodominant in nature. Third, the fact that the protein is placed in an entirely different millieu allows it to be immunogenic without any alteration. At this juncture, all the three possibilities are essentially speculative and experiments are under progress to establish the exact mechanism.

4.6. Possible Involvement of AK-5 antigen in rejection:

Tumor rejection is intimately associated with the presence of highly immunogenic protein(s) on the cell surface (147). Viral antigens which act as
TSTAs have provided important insights into mechanisms of transplantation rejection (153). A tumor rejection antigen evokes a response which includes activation of T cells as well as production of antibodies (154). Ability of tumor rejection antigens to stimulate the host immune system is variable (155) and is dependent on several factors (156). The humoral response is of great value technically and antisera can be used for serological analysis as well as for their purification while cellular response is important because of its role in tumor rejection (157, 158).

Although spontaneous rejection of tumor is a rare phenomenon, a few highly immunogenic tumors such as UV-induced fibrosarcomas and virally induced tumors are studied extensively (159). Immunisation of syngeneic hosts with tumor specific transplantation antigens from the immunogenic tumor cells elicits cell mediated immunity against the respective tumors but not towards other transformed cells (160, 161).

The role of lymphocytes in the recognition of tumor surface antigens is known to be essential for the rejection of the tumor (18). The cytotoxic T cells co-recognise tumor antigens on the cells with the membrane products of the major histocompatibility complex and thereby kill them (162). Another subset of lymphocytes, NK cells are shown to act on various tumor cells but their role in tumor rejection is still inconclusive.

Intraperitoneal injection of AK-5 tumor cells kills all the animals whereas on subcutaneous injection, all the animals develop the tumor but about half of them reject it subsequently. Tumor rejecting animals produce antitumor antibodies which are cytotoxic to AK-5 cells in the presence of complement. The
T-cell fraction from the spleens of these animals possesses cytotoxic activity against the tumor cells in the presence of anti-AK-5 antibody. The cytotoxicity of the immune spleen cells is totally lost on treating the effector cell fraction with anti-thy.1 and complement indicating the involvement of thy.1 positive cells in the rejection of AK-5 cells. OX-8* (CD8*) subset of natural killer cells is cytotoxic to AK-5 cells in vitro in the presence of anti-AK-5 antibody. Immune cells when treated with either OX8 monoclonal antibody and complement or anti NK monoclonal and complement loses cytotoxicity against AK-5 cells. The mechanism of cytotoxicity mediated by NK cells is still unclear. Several modes of action involving ADCC, release of cytolytics, release of NK cytotoxic factor have been described. In AK-5, action of NK cells seems to be mediated with the help of anti-tumor antibody i.e., the anti-tumor antibody which is produced in response to the immunogenic antigen binds to tumor cells. The antigen-antibody complex on the tumor cell surface facilitates the recognition of these cells by NK cells involved in lysis. Therefore the antigen which is purified and characterised from AK-5 in this scheme may be involved in the recognition process.

4.7. Expression of an albumin like antigen In a rat histiocytoma - can one speculate based on such an event regarding the origin?

The transforming principle and mechanism of its origin are not yet clear. This tumor arose in the peritoneal cavity of one of the rats when cell free ascitic fluid from a hepatoma (Zajdela ascitic hepatoma) was injected intraperitoneally into 35 inbred animals. This observation has been irreproducible. The macrophage nature of the tumor has been established beyond any doubt as illustrated previously. Also the possibility of the tumor being a hepatoma variant
has been ruled out based on presence of the various markers which are macrophage specific and are absent in the liver or in the hepatoma. The similarity in the pattern of expression of the albumin gene observed in the case of ZAH and AK-5 is rather intriguing. Another observation which needs to be noted here is the complete absence of the transcript specific for alpha-fetoprotein which has been shown to be present in various hepatoma cell lines. Both these observations suggest that a factor(s) present in the cell free ascitic fluid from ZAH was involved in the origin of the macrophage like tumor.

The possibilities which can be outlined to explain the hybrid behaviour include in vivo fusion of two cells of different types i.e., peritoneal macrophage fused with a ZAH cell which lead to the origin of AK-5. The evidence in support of this possibility would be, close to the normal karyotype of the tumor cells (AK-5), which is a characteristic feature of the fusion products between a normal and malignant cell. Of course such a possibility is based on an assumption that the fluid which was considered to be cell free to start with actually did contain certain number of cells. In vivo transfection of ZAH DNA into peritoneal macrophages is another possible explanation which can lead to the origin of the cell line. Spontaneous occurrence of such a tumor though seems unlikely cannot be ruled out. At this juncture, each scenario mentioned above seems possible. The correlation which could logically bridge the gap between the 'chimeric' behaviour of the tumor and origin of the transformed phenotype remains obscure.

Molecular characterisation of a tumor associated antigen has been explored to provide clues regarding the transforming principle. In other words, the initial event which transforms a normal cell into a malignant one leaves its imprint
in the form of a protein which by virtue of being foreign acts as an antigen. Obviously using such a protein, it is possible to trace back the nature of the carcinogenic insult which would have played a decisive role. At the same time, it should be kept in mind that from a wide variety of the neoantigens that are expressed upon transformation, only a few are relevant to the origin and the maintenance of the malignant phenotype, while the rest of the moieties are mere consequences of transformation and hence trivial. Obviously the molecules which belong to the second category cannot provide any information regarding the origin of the tumor though the first category of the antigens can be exploited for the same.