Apoptotic cell death plays an important role during development of an organism in the morphogenesis as well as in the regulation of tissue homeostasis. Morphologically, this type of cell death is characterized by cellular blebbing, nuclear shrinkage, nuclear fragmentation into apoptotic bodies, and DNA fragmentation into nucleosomal fragments, which can be resolved as a ladder in agarose gels. This type of DNA breakage is considered to be the hallmark of apoptotic cell death.

Very early evidences describe the genetic basis of cell death which was termed as 'programmed cell death'. The mutations in the ced-3, ced-4 genes of Caenorhabditis elegans, switch the programme of some of the cells, which are programmed to die during development, towards continued survival. The homologues of these genes are involved in the apoptotic cell death pathway of higher organisms. The mammalian homologue of another gene ced-9, which is involved in the cell survival pathway is bcl-2, which is upregulated in many of the human lymphomas; bcl-2 encodes for a mitochondrial membrane protein involved in the regulation of the mitochondrial membrane potential. The mammalian homologues of ced-3 are the interleukin-1-β-converting enzymes (ICE/caspase) family of proteins. The caspases are a family of cysteine proteases which are implicated in the apoptotic pathway.

Oncogenesis can be thought of as the result of disruption of the cell death pathway. It is known that DNA damage of cells can commit them to undergo apoptosis. If such a pathway is disrupted, the cell would be directed towards uncontrolled growth, and therefore, cancer. Inducing apoptosis in tumor cells specifically, would then be the target of any anti-cancer therapy.
The aim of this project was therefore, directed to study the mechanism of apoptosis in the AK-5 histiocytoma which was used as the model system.

AK-5 tumor is a histiocytoma associated with the property of spontaneous regression, when injected subcutaneously into syngeneic Wistar rats. It is, therefore, a good system to study host-tumor interactions, and the mechanisms adopted by the host against the tumor, which could provide clues for an effective anti-tumor therapy. Mechanisms like ADCC (Antibody Dependent Cellular Cytotoxicity) are known to be operative in the regression of this tumor. Further, it was observed that the anti AK-5 antiserum itself, without any intervention of either complement factors or Natural Killer cells (NK cells), was effective in killing AK-5 tumor cells \textit{in vitro}. It is also known that AK-5 tumor undergoes apoptotic cell death \textit{in vivo}, when injected intraperitoneally in the animals which have rejected the sub-cutaneous tumor. It was therefore postulated that the anti AK-5 antiserum contained 'factor(s)' responsible for the tumor cell death \textit{in vitro}. The focus of this study was to isolate, identify and characterize such a factor specific and responsible for inducing apoptotic cell death in AK-5 cells.

The strategy used to study AK-5 apoptosis were: the standardization of apoptosis detection protocols, the specificity of apoptotic cell death, purification and characterization of the apoptotic factor from the anti AK-5 antiserum and its downstream signalling mechanisms.

BC-8 cells which are the clonal population of AK-5 and adapted to tissue culture conditions, were used for the \textit{in vitro} assays of cell death. Detection of cell death was done by microscopic observations, propidium iodide staining of the nuclei and fluorescence microscopy observations, confocal microscopy and FACS (Fluorescence Activated Cell Sorter) analysis.

Purification of the apoptotic factor involved ammonium sulphate precipitation(s), DEAE ion exchange chromatography, Protein G affinity chromatography etc. The anti AK-5 anti-serum was collected, heat inactivated and subjected to ammonium sulphate precipitations, ion-
exchange chromatography, affinity chromatography, successively, wherein, the apoptotic activity was tested in all the fractions at the end of each fractionation step, and only the active fraction was used in the succeeding step of fractionation.

The detection of the purified protein, was by SDS-polyacrylamide gel electrophoresis and subsequent staining by Coomassie Brilliant Blue or ammonical silver nitrate. The binding of the purified apoptotic factor to AK-5 cells was studied by immunofluorescence, western analysis and immunoprecipitation.

The signalling mechanisms studied were phosphorylation of AK-5 proteins and calcium flux. These were detected by \textit{in vitro} phosphorylation assays. The cells were incubated with the purified apoptotic factor (anti AK-5 antibody) for different times, after which the cells were lysed and incubated with \( \gamma \text{P}^{32} \)-ATP for 15 min at room temperature and the reaction was stopped by adding the SDS-PAGE sample buffer and boiling for 5 min. The proteins were resolved on a 10% polyacrylamide gel. The phosphorylated proteins were detected by autoradiography after drying the gel.

Calcium ion dynamics were studied using the dual wavelength dye INDO-I-AM. The dye was loaded into BC-8 cells and relative emissions of fluorescence at two different wavelengths, 485nm for free INDO-I and 405nm for INDO-I bound to calcium were monitored. The ratio of fluorescence intensities between the two wavelengths, of control and treated BC-8 cells were plotted as a function of time.

The other aspect looked into was the involvement of oncogenes in the apoptotic pathway. Northern analysis provided the tool for detecting the involvement of various genes during the AK-5 cell death. RNA was isolated by phenol-chloroform extraction and ethanol precipitations; RNA was quantitated and resolved on 1% agarose gel containing formaldehyde and transferred on to nylon membranes by vacuum transfer method. The blots were probed with labelled cDNA under stringent conditions, washed and autoradiographed.
The probe was prepared by either Random Priming Method or by Nick Translation method, using dATP-[P³²] as the label.

The results obtained from this study indicate that the purified apoptotic activity in the antibody fraction of the anti AK-5 antiserum, increased to 200-fold. This antibody had the capacity to bind to AK-5 cell surface, as seen by immunofluorescence and confocal microscopic techniques. Though, this antibody would be of a polyspecific polyclonal nature, attempts were made to identify the protein(s) to which the antibody could bind, through immunoprecipitation and western blotting techniques.

As stated above, the binding of the anti AK-5 antibody to AK-5 cell surface was the causal event leading to AK-5 cell death. This indicated a downstream cellular signalling cascade. It is well known that the cell death through the apoptotic pathway is a well controlled cascade involving protein phosphorylation, Ras pathway, G-proteins etc., all converging at the cysteine proteases, which are considered to be the cell death effectors.

An attempt at understanding signalling mechanism namely, phosphorylation of proteins as a consequence of the interaction of the anti AK-5 antibody and AK-5 cells yielded the following results: there are at least three proteins of AK-5 cells, with the relative molecular weights: 94, 78, and 73kDa, which are phosphorylated within a few seconds of addition of the anti AK-5 antibody. An instantaneous phosphorylation of proteins is known to be of importance in many signalling pathways. The 78 and the 73kDa proteins are phosphorylated at the serine/threonine amino acid residues, while the 94kDa protein is phosphorylated at the tyrosine residue, as shown by the alkali hydrolysis pattern. The phosphorylation of the 78 and the 73kDa proteins as well as AK-5 cell death is inhibited by the protein kinase inhibitor staurosporine. These results clearly implicate the necessity for protein phosphorylation in AK-5 cell death pathway. The next step was to investigate the molecules involved further downstream to this cascade and the nature of the signal transmitted to the nucleus. In this
context, the relevance of the proto-oncogenes which act as transcription factors, becomes an important aspect of the study.

It is known that the regulation of the proto-oncogene proteins is necessary for the maintenance of cellular homeostasis as well as for cell survival and growth. Some of these proto-oncogene families are \textit{myc} and \textit{jun}, the proteins of which act as transcription regulators. In the anti AK-5 antibody induced AK-5 apoptosis there is marked decrease in the mRNA levels of the above mentioned proto-oncogenes, as detected by the northern blot analysis. Further, this depression in the mRNA levels is observed within a period of 3h of treatment of AK-5 cells with anti AK-5 antiserum or purified antibody. This process is reversed by the addition of specific peptide caspase inhibitors such as DEVD and YVAD, transfection of the AK-5 cells with the anti-sense of caspase-2, overexpression of \textit{bcl-2} gene, indicating the upstream action of the caspases and \textit{bcl-2} to proto-oncogenes \textit{myc} and \textit{jun}.

There is evidence to indicate that the anti AK-5 antibody induced AK-5 apoptosis is a mechanism completely different from the well known anti Fas antibody and Fas receptor interaction. The northern blot analysis indicates the absence of the Fas mRNA. Also, the possibility of AK-5 cell death due to serum TNF\textalpha is ruled out.

The antibody present in the anti AK-5 anti-serum is clearly the factor responsible for AK-5 cell death and proceeds as a cascade of events outlined below:

Anti AK-5 antibody binds to the AK-5 cell surface protein(s), which leads to the phosphorylation of two proteins of relative molecular weights 78 and 73kDa. This signaling is instantaneous and sustained and is necessary for AK-5 cell death. The blocking of phosphorylation by protein kinase inhibitor, staurosporine, inhibits AK-5 cell death. Further, the down regulation of the mRNA of proto-oncogenes \textit{c-myc} and \textit{c-jun} occurs as a downstream event. Interestingly, it is blocked by the caspase inhibitors DEVD and YVAD and caspase-2 antisense, implying the role of caspases upstream to the \textit{c-myc} and \textit{c-jun}.