MATERIALS AND METHODS
MATERIALS AND METHODS

2.1.0 Animals:
Rats of Wistar strain, maintained as an inbred colony established in our laboratory, were used for this study.

2.2.0 Tumor and cell lines:
This study was conducted on AK-5 tumor, which had arisen spontaneously in one of the animals of the inbred Wistar rats (Khar, 1986), and is maintained by passaging serially in the peritoneal cavity of a syngeneic line of Wistar rats.

2.3.0 Serum collection for apoptotic assays:
The animals were injected with AK-5 tumor cells ($5 \times 10^5$) subcutaneously which form a tumor which regresses after a period of about 30 days. Subsequently the animals are challenged with the tumor intra-peritoneally. The animals were ready for the blood collection about 8 days later. The blood was allowed to clot and the clear supernatant was collected as serum, which was used for apoptotic tests.

2.4.0 Cell Culture:
A single cell clone of the AK-5 tumor, named as BC-8, was adapted to and grown in culture conditions. These cells were grown in the Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum at 37°C in a CO$_2$ incubator. All the experiments were conducted on BC-8 cells in \textit{in vitro} conditions.

The other cell lines, such as ZAH (Zajdela ascitic hepatoma), Meth A, used for the study were maintained in conditions similar to the aforesaid.
2.5.0 Methods used to detect apoptosis:

2.5.1 Phase contrast microscopy:

The cells were observed under the Nikon phase contrast light microscope using phase rings, using 10X eye piece and 10X or 20X objectives.

2.5.2 Fluorescence Microscopy:

The cells were fixed in 80% methanol for 1 hour or overnight. The fixative was removed by washing cells with PBS. The cells were treated with Propidium Iodide reagent, for 1h at room temperature. The composition of the reagent is as follows: Propidium Iodide (Boehringer Mannheim Inc) 50 μg/ml, Sodium citrate 0.1% and Triton X-100 0.1%. About 10 μl of the cell suspension was placed on a clean glass slide and covered with a coverslip. The cells were observed at either 40X or at 100X oil immersion lens using Xenon lamp as the UV light source and a red filter of 488nm.

2.5.3 Confocal Microscopy:

BC-8 cells were treated with the anti AK-5 antiserum or Apoptotic Factor, purified from anti AK-5 antiserum, for various times, after which the cells were fixed in 2% paraformaldehyde. The proteins attached to the cells would thus, get fixed to wherever the localization or binding occurs. The cells were then treated with FITC conjugated anti rat secondary antibody (Amersham, USA). The cells were simultaneously stained with Propidium Iodide, and observed with the confocal microscope.

2.5.4 DNA Ladder Assay:

BC-8 cells (2 x 10^6) were treated with anti AK-5 antiserum or Apoptotic Factor, purified from anti AK-5 antiserum, overnight. The cells were fixed in 70% ethanol overnight. The
procedure of DNA extraction used was of Gong et al, 1994. The cells were centrifuged at 1000 rpm for 1 min in a table top Remi centrifuge. The ethanol was carefully and thoroughly aspirated out and the cell pellet was resuspended in 40μl Phosphate–citrate (PC) buffer of pH 7.8, consisting of 192 parts of 0.2M Disodium hydrogen phosphate and 8 parts of 0.1M citric acid. The cells were left at room temperature for 30-40 min. The cells were centrifuged again at 1000 rpm for 5 min and the supernatant was transferred to fresh tubes. The supernatant was treated with 0.25 % Nonidet NP-40 (Sigma) and 3 μl of 1mg /ml RNase A (Sigma) and incubated at 37°C for 30 min. The contents of the tubes were mixed with appropriate amount of DNA loading dye and loaded in the wells of a 1% agarose gel. The electrophoresis was carried out at 2V /cm for 16 h. The DNA was visualised by staining the gel with ethidium bromide, in an UV trans-illuminator.

2.5.5 FACS:

Apoptosis was also detected and quantitated by FACS using the Beckman Dickinson FACStar machine, with excitation wavelength at 488nm and emission at 630nm. The data analysis was done using the software Cellquest.

2.6.0 Methods used for protein isolation and purification:

2.6.1 Serum isolation:

The blood was collected through the retro-orbital plexus and was allowed to clot. The serum was prepared from each animal, and the complement was heat inactivated at 56°C for 30 min. The sera were then tested for the apoptotic activity on cultured BC-8 cells and the positive samples were pooled for further fractionation.
2.6.2 Ammonium sulphate precipitation:

For 45% saturation of ammonium sulphate, 2.77g of solid ammonium sulphate was added to 10ml of anti AK-5 antiserum, slowly with constant stirring at 4°C and was left for 6 hours for optimal precipitation of the proteins. The solution was centrifuged at 6000 rpm in SS-34 rotor, in Sorvall centrifuge at 4°C, for 20 min. The supernatant was aspirated out and the precipitate was dissolved in 3-4ml of sterile PBS (Phosphate Buffered Saline). The supernatant and the precipitate were subjected to extensive dialysis against 1L of PBS with three changes for 16-20 h. The supernatant and the precipitate were individually tested for apoptotic activity as described in section 2.5. The positive fraction was subjected to ion exchange chromatography.

2.6.3 Ion Exchange Chromatography:

A column of 2.5cm diameter and 50cm height was packed with DEAE (diethyl amino ethyl) Sephadex (Pharmacia, USA) under gravity and allowed to equilibrate overnight with a 10mM Tris buffer, pH 8.3. 10ml of the active fraction with protein concentration of 600-800mg/ml was applied to the column and was allowed to bind for 30 min. The column was washed with the 10mM Tris buffer (pH 8.3) and 3ml fractions were collected until the absorbance at 280nm (A280) was close to background absorbance. The column was developed with 300ml of 0-0.5M linear sodium chloride gradient and 3-4ml fractions were collected. The eluate fractions were monitored for protein by measuring the absorbance at 280nm. The fractions containing protein were pooled and concentrated by ultrafiltration, using 30kDa cutoff pore size membrane, PM30 (Amicon Corp., Ireland). The ultrafiltration apparatus (Amicon Corp., Ireland), was fixed with PM30 membrane, and protein solution was concentrated with constant slow stirring, at a pressure of 2-5 pascals, generated with the help of nitrogen cylinder. The concentrated protein solutions were dialysed against 200-400
volumes of PBS for 22-24 h with 3 changes. The samples were then tested for apoptosis as described in section 2.5

2.6.4 **Affinity Chromatography:**
The partially pure fraction from the previous step of purification, capable of inducing apoptotic cell death in BC-8 cells, was applied on to a 3ml Protein G Sepharose-4 fast flow (Amersham Pharmacia, Sweden) column which had been equilibrated with 10mM phosphate buffer pH 7.0. The protein was allowed to bind for 30 min after which the column was washed with 30 volumes of 10mM phosphate buffer pH 7.0. The bound protein was eluted with 100mM glycine-HCl buffer pH 2.7. The wash and the eluate flow-through were collected as 1ml fractions. The protein content was detected by measuring the absorbance at 280nm and the fractions with protein were pooled, concentrated and dialysed as stated above, in section 2.6.2. As before, the fractionated proteins were tested for apoptotic activity of BC-8 cells.

2.6.5 **Purification of apoptotic factor using Capryllic acid:**
The serum was clarified by centrifuging at 8000-10000 rpm in a Sorvall Centrifuge. The serum was transferred to a beaker with a magnetic bead, and diluted with 4 volumes of 60mM Acetate buffer pH 4.2, and the pH was adjusted to 4.5 with 1 N NaOH. Caprylic acid, at a concentration of 28µl per ml of the diluted serum solution, was added with constant stirring. The solution was allowed to be stirred for another 30 min., at room temperature, after which, it was transferred into centrifuge tubes and centrifuged at 10000 rpm, 4°C, for 20 min. The supernatant was aspirated out in another beaker. 1M Hepes buffer was added to a final concentration of 20mM, and the pH was immediately adjusted to 7.4 using 1 N NaOH. The supernatant was then cooled to 4°C, and solid ammonium sulphate was added slowly, with
constant stirring, such that it reached a saturation of 45%. The stirring was continued for another 30 min and, as before, the solution was transferred to centrifuge tubes and centrifuged at 10000 rpm, 4°C for 10 min. The supernatant was discarded, the pellet was suspended in minimum volume of PBS, and dialysed against PBS as described in section 2.6.2.

2.6.6 Purification of apoptotic factor by Sodium Sulphate method:

Solid sodium sulphate was added slowly with constant stirring, on a magnetic stirrer to anti AK-5 antiserum, such that the concentration was 18%. The solution was stirred for another 30 min after which it was transferred to centrifuge tubes and centrifuged at 10000 rpm, 4°C for 20 min. The supernatant was discarded, and the pellet was suspended in minimum volume of PBS. The suspended pellet was transferred to a beaker and additional solid sodium sulphate to a concentration of 12% was added, with constant stirring. The solution was centrifuged as before, and the pellet was suspended in minimum volume of PBS and dialysed against PBS.

2.7.0 Quantitation of Proteins:

2.7.1 Bradford's Method:

Bradford's method was used for protein quantitation. A 400 μg/ml stock solution of BSA (Bovine Serum Albumin) was made for constructing the standard curve. Various concentrations of BSA were taken in the wells of a microtitre plate. 50 μl of Bradford's Reagent (Pharmacia, USA) was added to each of the wells. The final volume was made up to 250 μl with deionised water and the absorbance was read at 490nm. A standard curve was constructed with the absorbance as a function of protein concentration. The absorbance of
unknown protein samples were similarly monitored and the protein concentration was read from the standard curve.

2.8.0 Methods used for detection of proteins:

2.8.1 SDS-Poly Acrylamide gel electrophoresis:

The SDS-PAGE of proteins in the fractions was done by the discontinuous buffer system as described by Laemmli et al, 1970. A stock solution of 30% acrylamide was prepared by dissolving 29.2g of acrylamide (Life Technologies, USA) and 0.8g of N, N' methylene bis-acrylamide (Serva, Sweden) in distilled water, and the volume was made up to 100ml. The solution was then filtered and stored at 4°C.

The resolving gel was polymerised as either 8% or 10% acrylamide in 0.375M Tris-HCl, pH 8.8, while the stacking gel was 4.5% acrylamide constituted in 0.125M Tris.HCl, pH 6.8, each with 0.1% SDS. The gels were polymerised using ammonium persulphate (200 µl of 10% solution) and TEMED (10µl for 30ml gel mixture), in a vertical gel apparatus (Hoeffer, USA). The protein samples were prepared by boiling the protein solution in Sample Buffer (50mM Tris.Cl pH 7.5, 3% SDS, 10% glycerol, 4mM EDTA, 0.01% bromophenol blue and 5% β-mercaptoethanol). Proteins were loaded in the wells of the stacking gel and electrophoresis was done in constant current mode, at 20 mA, until the proteins reached the resolving gel, after which the current was increased to 40mA.

2.8.2 Staining the gels with Coomassie Blue:

The gels were stained with 0.25% Coomassie Brilliant Blue R250 in methanol: acetic acid: water (45:10:45, v/v/v) for 30 min to 1 h, and destained with destaining solution of methanol: acetic acid: water (20:10:70, v/v/v) for 6-8 h, with 2-3 changes of destaining solution on a rocking platform.
2.8.3 *Ammonical Silver Staining:*  
The gels were left in 50% methanol for 2-4 h for fixing. The gels were then incubated in ammonical silver nitrate for 10 min with constant shaking. The gel was washed with 3 changes of deionised water for 10 min each with constant agitation. The gel was then placed in a container with the Developer (1% Citric Acid and 0.0002% formaldehyde), with gentle shaking, for 10-15', till the bands developed. The gel was fixed in the Fixer (40% methanol and 10% acetic acid).

The ammonical silver nitrate solution was prepared as follows:

0.6 g of Silver Nitrate was dissolved in 3ml of deionised water.

1.05ml of liquid Ammonia was added to 16ml of 0.36% sodium hydroxide and the volume made up to 75ml with deionised water. The silver nitrate solution was added drop wise to the ammonical sodium hydroxide with constant shaking till the brown precipitate dissolved completely. This solution was labelled as ‘ammonical silver nitrate’.

2.8.4 *Western Blot:*

The gel was incubated in Transfer Buffer (39mM glycine, 48mM Tris base, 0.0375% SDS and 20% methanol) for 5-10' and transferred on to nitrocellulose membrane (Hybond C, Amersham) or on Immobilon-P (Millipore).

The procedure was as follows:

The nitrocellulose paper and the gel were wetted with Transfer buffer and sandwiched between three strips of Whatman-3 or six of Whatman-1 paper, cut according to the size of the gel. The sandwich was placed between two electrode plates of an electro-blot apparatus (Nova blot Apparatus, Pharmacia; Millipore Transfer Apparatus) with the membrane facing the anode. The transfer was done at constant current of 0.8mA/ cm².
Subsequent to the transfer, the membrane was incubated with Blocking Buffer (3% BSA in PBS) for either 4 h or overnight.

The membranes were then treated with first antibody at a dilution of 1-10 μg/ml at a volume of 0.1ml/cm² of the membrane, for 1 h. The membranes were washed with TNT buffer (10mM Tris-HCl, pH 8.0, 150mM sodium chloride, 0.05% Tween-20). Three changes of the buffer for 20 min each were given. The membrane was then incubated with either peroxidase or alkaline phosphatase conjugated second antibody, for 45 min and washed again as above. The proteins bound to the antibodies were detected by the enzyme reaction by Electro Chemiluminescence (ECL) kit (Amersham,U.K.) for peroxidase conjugated second antibody. The reagents A and B provided by the manufacturer were mixed in the ratio of 100:1 and layered on the blot at a concentration of 0.1ml/cm² for one minute in the dark. The blot was exposed to X-ray film for 1-10 min and the film was developed.

The enzyme reaction for alkaline phosphatase was as follows:

The blot was incubated with 100mM Tris-HCl, pH 9.5 containing 10mM MgCl₂, 0.005% BCIP, 0.01% NBT and the alkaline phosphatase enzyme reaction was monitored by the color development was monitored.

2.8.5 Immunoprecipitation:

About 8 X 10⁶ cells were taken, washed with PBS and pelleted. All the subsequent steps were done on ice or at 4°C. The cells were lysed in 0.8-1.0ml of Extraction Buffer (Tris-HCL, pH 7.4 50mM, Triton X-100 0.5%, DTT 1mM, PMSF 0.5mM, Aprotinin-leupeptin-pepstatin 5 μg/ml, EDTA 1mM, NaCl 500mM, SDS 0.1%, Sodium deoxycholate 0.5%) and passed through 18 and 24 gauge needles 10 times each. The unbroken nuclei and DNA were pelleted by centrifugation at 14000 rpm at 4°C, after which, upper layer of clear solution was aspirated and used for immunoprecipitation. The solution was incubated with ~20μl of
Protein G Sepharose beads (Amersham Pharmacia, Sweden) for pre-clearing. One set of the cells was used as control and was treated with normal rat antibody, while the other was treated with equal amount of Apoptotic Factor, and was incubated at 4°C for 90 min. Protein G Sepharose beads were added to each at this point and the cell extracts were further incubated for 30 min. The beads were then precipitated by low speed centrifugation (~500 rpm). The beads were washed twice with Lysis Buffer, boiled with Sample Buffer and loaded on a 10% SDS-Polyacrylamide gel. The electrophoresis was done at 20mA constant current, until bromophenol blue reaches the resolving gel, after which the proteins were resolved at 40mA constant current. The gel was then transferred on to nitrocellulose/PVDF (Amersham) membrane and western analysis was done, as explained above, with apoptotic factor as primary probe and anti rat antibody tagged to alkaline phosphatase as the detection antibody. The enzyme reaction was developed as stated in section 2.8.4.

2.8.6 Immunofluorescence:

The cells were either live or fixed in 2% paraformaldehyde, washed with PBS and stained with first antibody for 1 h on ice. The cells were then washed with cold PBS 4-5 times and treated with FITC conjugated anti-rat second antibody (Amersham, U.K.) for 45 min. The cells were washed again as described above. A drop of the cell suspension with antifade was placed on a slide and viewed under a fluorescence microscope (Nikon) with the blue filter.

2.9.0 Detection of phosphorylation of cellular proteins:

Cells (7 X 10^4 – 8 X 10^4) were treated with the apoptotic factor for different periods of time at 37°C. The cells were then lysed in Lysis Buffer (20mM HEPES buffer pH 7.5, 1mM EDTA, 10mM MgCl2, 1mM DTT, 0.5% NP-40/ TritonX-100, 1mM PMSF, 5μg/ml aprotinin+leupeptin, 1mM vanadate) and treated with a mixture of γp32 ATP and cold ATP,
for 15 min at room temperature. The reaction was stopped by the addition of Sample Buffer (50mM Tris.HCL pH 7.5, 3% SDS, 10% glycerol, 4mM EDTA, 0.01% bromophenol blue and 5% 2-mercaptoethanol) and the samples were boiled for 5 min in boiling water bath. The proteins were resolved by SDS-PAGE. The gel was stained with Coomassie Blue as described above, dried under vacuum and exposed to X-ray film at room temperature. The phosphorylated proteins were detected by autoradiography.

2.9.1 Densitometric quantitation of phosphorylation:

The developed X-ray films with imprints of radioactive proteins were scanned and the intensities of each imprint was calculated using Image-quant software.

2.9.2 Detection of phosphorylated aminoacid in the protein:

The proteins, after the phosphorylation reaction were resolved by SDS-PAGE as described section 2.8.1. The gel was then treated with 1N sodium hydroxide at 50°C for 1h. The gel was carefully removed from the alkali solution and treated with the de-stainer (40% methanol and 10% acetic acid). The gel was dried in a vacuum gel drier and autoradiographed. The rationale of this procedure is that the phosphate attached to tyrosine is not hydrolysed by alkali but the bond attached to an aliphatic chain such as that of serine or threonine is readily hydrolysed. Therefore the autoradiogram detects tyrosine bound phosphate.

2.9.3 Inhibition of phosphorylation by kinase inhibitors:

The experiment was done as described above, and some of the samples were treated with protein kinase inhibitor, Herbimycin A and Staurosporine. Herbimycin A is a specific tyrosine kinase inhibitor while Staurosporine is a wide range kinase inhibitor with different specificities for different kinases- Staurosporine, at a concentration of 2nM inhibits protein
kinase C, at 5nM inhibits serine/threonine kinases while at 10nM inhibits protein tyrosine kinases. The cells were pre-incubated with the inhibitors for 15 min. These were then incubated with anti AK-5 antibody for 20 min. The phosphorylation assay was done as described above. The proteins were resolved on a 10% SDS polyacrylamide gel. The gel was dried and the proteins detected by autoradiography.

2.9.4 Inhibition of cell death by the protein kinase inhibitor:

The inhibition of cell death was assessed microscopically and quantitated by FACS analysis. About 2 million cells were incubated with staurosporine 30 min and further incubated with the anti AK-5 antibody/anti AK-5 antiserum for 20 h. The cells were observed under the phase contrast microscope and stained with propidium iodide. The percentage of apoptosis was assessed by Fluorescence Activated Cell Sorter and the pre-G1 peak was taken to be the apoptotic cells.

2.10.0 Northern Analysis:

2.10.1 Preparation of Total cell RNA:

Acid-guanidium thiocynate-phenol-chloroform procedure as described by Chomczynski and Sacchi (1987) was used to isolate total RNA from cells. Cells (10 x 10^6) were suspended in 3ml of Guanidium thiocynate solution (4M guanidium thiocynate, 25mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1M 2-mercaptoethanol), and were sonicated for 1 min. The extract was incubated with 1/10 the volume of 2M sodium acetate, 1/5 volume of chloroform: isoamyl alcohol (24:1) and equal volume of water saturated phenol. The mixture was allowed to stand on ice for 10 min., and was centrifuged at 10000g for 20 min, at 4°C. The aqueous phase was transferred to a fresh tube and RNA was precipitated by the addition of equal volume of isopropanol, after incubation at -20°C for 1h. The centrifugation was repeated as above and
the pellet was resuspended in minimum volume of guanidium thiocynate solution. RNA was precipitated as above, the pellet was washed with 70% ethanol and with absolute alcohol and air dried. RNA pellet was, next, dissolved in small volume of autoclaved deionised water. RNA was quantitated spectrophotometrically, by reading the optical density at 260nm, and calculating the concentration using the equation 40ng of RNA = 1A<sub>260</sub>.

2.10.2 Isolation of mRNA:
Poly A<sup>+</sup> RNA was isolated from the total RNA by using oligo (dT) coupled cellulose beads. The total RNA (about 100μg) was incubated with the bead suspension, washed and the RNA was eluted in deionised water heated to 65°C for 5min.

2.10.3 Northern transfer and hybridisation:
The RNA was denatured in Denaturation solution (100μl of MOPS, 500μl of formamide, 177μl of formaldehyde and make up the volume with water to 1ml) and heated at 65°C for 5min. The samples were mixed with gel loading buffer (50% glycerol, 1mM EDTA, 0.25% Bromophenol blue and 0.25% xylene cyanol) and loaded on a 1% agarose gel containing 20mM MOPS, 8mM sodium acetate, 3% formaldehyde and 1mM EDTA and electrophoresed. The RNA was visualised by ethidium bromide (the stain was added in the gel) and vacuum-transferred on a Hybond N<sup>+</sup> membrane (Amersham, USA) using 50mM sodium hydroxide for 1h and 30min. The blots were washed twice with 2X SSC or SSPE buffer and incubated with the radioactively labelled probe overnight. The blots were washed with concentrations upto 0.5X SSPE at 65°C (high stringency), in steps and were exposed to either phosphor screen and detected with phosphor-imager instrument, or to X-ray film in cassettes provided with radioactivity sensitive screen. The cassettes were incubated at −70°C for a maximum of 1 week and the X-ray film was developed.
2.10.4 Nick translation:

Plasmid DNA was radiolabelled, as described using nick translation kit (BRIT). The reaction was carried 16°C for 2 h, in a 25μl volume containing 200-300ng of DNA, 200μM of each unlabelled dNTPs other the one either which is radioactive, either 50 μCi [α-P³²] dATP, or dCTP, 50mM of Tris-HCl pH 7.2, 10mM MgCl₂, 0.1mM DTT, 50pg of DNAse I and 5 units of *E.Coli* DNA polymerase I. The reaction was stopped by adding EDTA to a final concentration of 10mM. The unincorporated dNTPs were separated from the incorporated ones by column chromatography using Sephadex G-50, swollen in TE (Tris-HCL pH 8, EDTA) as follows: The slurry was poured in a 2ml syringe which was plugged with sterile glass wool. The nick translation reaction mixture was diluted to 100μl with TE and was applied to the column. The label-incorporated probe was collected by spinning the column at 100 rpm in a Remi table top centrifuge.

2.10.5 Random priming:

Radiolabelled probe was also prepared by random priming of the plasmid or insert DNA by using Random Priming Kit supplied by BRIT. 50μg of DNA was taken in an 1.5ml Eppendorf tube, and was denatured in a boiling water bath for 5 min and immediately placed on ice. For a 50μl reaction, typically, 4μl of all the nucleotides other than the radiolabelled nucleotide were added along with 5μl of the random primer, 5μl of the buffer solution and 1μl of Klenow enzyme. The mixture was mixed gently and the reaction was allowed to proceed for 45 min at 37°C. The unincorporated nucleotides were separated by column chromatography as detailed in section 2.10.4.
2.11.0 Monitoring calcium flux using Indo-1-AM and confocal microscopy:

AK-5 cells (2x10^6) were incubated with Indo-1-AM (Molecular Probes, USA), at a concentration of 1μM for 30 min, washed with phenol red free DMEM (Sigma) and suspended in 1-2ml of the same medium. The cells were loaded in a shallow cavity slide, and excited with the UV (350-364 nm) channel using an Argon LASER, and the emission was monitored at 485nm/45 band pass filter for the unbound INDO-1 which is represented as green in pseudocolor, and 405nm/45 band pass filter, for INDO-1 bound to calcium, which is represented as red in pseudocolor. The dichroic was 445LP. The image was scanned using an Olympus IMT2 inverted microscope, with 100X oil immersion lens. The image analysis was done using Meridian Ultima Master programV4.19. The test solution (purified apoptotic factor or dexamethasone) was introduced carefully (without disturbing the cells), and the image was scanned immediately and after a time lag of 5 min. The relative change in emission ~485 nm for the dye unbound with calcium and ~405 for dye bound to calcium was calculated.