Materials and Methods

Animals: 8-12 weeks old inbred C57Bl/6 and Balb/c and Swiss outbred mice were used in this study. New Zealand white rabbits bred in J.N.U animal house were used for generation of antisera. All animals were maintained at the Animal House Facility in J.N.U., New Delhi.

Human donors: Normal human blood samples were obtained from Red Cross Society of India, New Delhi as well as from student volunteers from the School of Life Sciences, J.N.U. Leukemia samples were obtained from patients diagnosed at the Institute Rotary Cancer Hospital, All India Institute of Medical Sciences, New Delhi.

Culture Medium: Cell cultures were maintained in RPMI-1640 supplemented with 5% Fetal calf serum (FCS), 2 x 10^{-5} M 2-mercaptoethanol, 300 μg/ml glutamine and 50 μg/ml gentamycin (complete medium). The medium was prepared in deionized (milli-Q) water and filtered through a 0.22 micron millipore membrane by positive pressure. Sterility checks were performed by incubating medium with nutrient broth, at a 1:1 ratio at 37°C for 48 hours in glass tubes. For toxicity checks, 0.1-0.2 x 10^6 mouse spleen cells were cultured in 200 μl of the freshly prepared medium in 96 well flat bottomed microtiter plates at 37°C for 48 hours.

Cytokines: Human recombinant interleukin-2 (IL-2), obtained as a gift from Cetus Corporation, Emeryville, California, was used for generation of lymphokine activated killer cells. Human recombinant gamma interferon preparations were obtained from Biogene. Murine IFN-g was a gift from Pittsburg Cancer Institute (courtesy R.H Goldfarb). Potency of IL-2 preparation was monitored in standard bioassays using an IL-2 dependant CTLL cell line (27). Potencies of IFN-g preparations were checked by using WISH/VSV system (human IFN-g) and L929/VSV system (mouse IFN-g) in virus protection

assays (111).

Monoclonal antibodies: Supernatants of ATCC hybridomas HB102 (anti H-2D^d), HB95 (anti HLA-A,B and C), TIB 99 (anti Thy-1), TIB 122 (anti mouse common leukocyte antigen), B468 (anti H-2^k) and B896 (anti H-2^b) were used as sources of the respective monoclonal antibodies.

A comparison of the concentration of monoclonal antibodies in different hybridoma supernatants was done by the technique of capture ELISA. The wells were coated with 0.2 μg/well of affinity purified rabbit anti mouse IgG polyclonal antibodies. 3% BSA was used to block the wells. The hybridoma supernatants were used as source of monoclonal antibodies to be captured, followed by rabbit anti mouse IgG-HRPO conjugate. OPD was used as the substrate and color development was monitored at 492 nm.

Rabbit antisera: For generation of rabbit anti mouse IgG serum, rabbits were immunized with affinity purified mouse IgG (generated as described below). Primary immunization of 100 μg protein was given in CFA followed by four weekly boosters of 50 μg protein each.Five days after the last booster, rabbits were bled from ear vein and about 30 ml of blood was collected in clean glass tubes. Blood was allowed to coagulate at 37°C for 1-2 hours. After detaching the clot from the walls, the tubes were further incubated at 4°C overnight in order to allow the the clots to retract. The samples were centrifuged at 2000 rpm for 20 minutes at 10°C. The clear serum was carefully collected without disturbing the pelleted clot. In case of RBC contamination, the sera was centrifuged once again. The serum was decomplemented at 56°C for 30 minutes.

Affinity purification of mouse IgG and preparation of mouse IgG-sepharose columns: Swiss mice were bled through eyes. Each mouse yielded approximately 1-1.5 ml of blood. Sera was collected from blood samples as described above. For the purification of mouse

IgG, antibody from the serum was precipitated with ammonium sulphate (50% saturation). The precipitate was dissolved in minimum volume of phosphate buffer (pH 7.0). This preparation was loaded on a Protein-A Sepharose-CL-4B affinity column at a flow rate of 10 ml per hour. After washing the column thoroughly with phosphate buffer, the bound antibody was eluted using citrate buffer (0.1M, pH 3.5) and collected in 2 ml fractions. pH was neutralized with 0.1M tris-HCl. Protein concentration was estimated at 280 nm and fractions containing the protein peak were pooled. This was dialysed against PBS and stored at -70°C in aliquots. Antisera against this preparation (first antibody) were raised in rabbits as described above to obtain rabbit anti mouse IgG antisera.

Mouse IgG affinity column was prepared by tagging mouse IgG to cyanogen bromide activated sepharose beads as described elsewhere (1). Rabbit anti mouse IgG, antiserum was diluted wih an equal volume of PBS and loaded on to the column packed with mouse IgG sepharose beads, at a rate of 20 ml per hour. After washing the column with about 5 bed volumes of PBS, the bound antibody was eluted with glycine-HCl buffer (0.1M, pH 2.8).

Conjugation of rabbit anti mouse IgG with Horse-radish peroxidase: Rabbit anti mouse IgG was coupled to HRPO by the method of Nakane et.al (75). 10 mg of HRPO (grade-1, sp. activity 250 U/mg, RZ value 3.0, Boehringer) was dissolved in 10 ml of freshly prepared 0.3M $NaHCO_3$. 20 μl of 10% fluoro dinitro benzene (FDNB, 10% solution freshly made in absolute ethanol) was added to the HRPO solution. Contents of the tube were mixed and incubated for one hour at room temperature. 1 ml of 100 mM periodate solution (sodium-meta periodate, Sigma) was added to the tube and incubation continued at room temperature for another 30 minutes. 0.1 ml of ethylene glycol solution (3.2M in distilled water) was added and incubated at room temperature for one hour. The sample mixture was dialysed against two changes of 0.5 litres of carbonate buffer (0.1M, pH 9.5).

10 mg of the affinity purified rabbit anti mouse IgG in 2 ml of carbonate buffer and 10 mg of activated HRPO in an equal volume of the same buffer were mixed and held in a water bath at 37°C for 3 hours with constant agitation. 10 mg of sodium borohydride was added to the tube, mixed and incubated for 3 hours. Conjugated antibody-HRPO complex was purified on a G-100 sephadex column. For storage, BSA was added (3% final concentration) to the purified antibody-HRPO complex and the aliquots were stored at -70°C.

Effector Cells: Spleen from mice were aseptically removed and teased in phosphate buffered saline (PBS) to obtain a single cell suspension. Tissue debris was allowed to settle and the floating cells were collected in sterile centrifuge tubes. After giving three washes in PBS, the cells were suspended at the desired concentrations in complete medium.

Heparinized blood from normal human donors was diluted with an equal volume of PBS and layered on Histopaque (density 1.077 gm/ml) in sterile tubes and centrifuged at 1400 rpm for 30 minutes at room temperature. At the end of the centrifugation, peripheral blood mononuclear cells (PBMC) were harvested from the interface using a pasteur pipette. The cells were washed thrice in PBS at 1000 rpm to remove the platelets. The final cell suspension was made in complete medium.

For the generation of LAK effector cells, murine spleen cells and human peripheral blood lymphocytes (HPBLs) were cultured at 5 million cells/ml in complete medium with 200 U/ml of Human recombinant Interleukin 2 (HRIL-2). After 2 days, cultures were split into two and replenished with 50% v/v fresh medium and 200 U/ml HRIL-2. LAK cells harvested on day 5 of the culture were used as effectors in different assays.

Target Cells:

(a) Murine tumor cell lines: YAC, a T-cell lymphoma (ATCC TIB160), P815, a mastocytoma (ATCC TIB64), SP2O, a B cell myeloma

(ATCC CRL1581), EL4, a T-cell lymphoma (ATCC TIB39) and L929, a fibroblast cell line (ATCC CCL1) were originally obtained from NIA, NIH (courtesy Dr. W.H Adler).

(b) Human tumor cell lines: K562, a chronic myelogenous leukemia (ATCC-CCL 243), MOLT-4, a T cell leukemia (ATCC-CRL 1582), Raji (ATCC-CCL 86) and Daudi (ATCC-CCL 213), B cell leukemia cell lines were originally obtained from NIA, NIH (courtesy Dr. W.H Adler). HR7, a gastric carcinoma cell line (117) was originally obtained from Pittsburg Cancer Institute (courtesy Dr. T.L Whiteside). Both murine and human cell lines were propagated by serial transfers in RPMI based complete medium. All cell lines except murine L929 and human HR7 were maintained as suspension cultures. L929 and HR7 cell lines had to be detached from plastic surface by using trypsin-EDTA (25 μl of 10 mg/ml trypsin and 4-5 ml of 1mM EDTA per 25cm^2 flask for 3-4 minutes). Trypsinized tumor cells were washed before reseeding into fresh complete medium.

Freezing and thawing of tumor cell lines: When not in use, tumor cell lines were stored frozen in liquid nitrogen. Exponentially growing tumor cells (5-10 x 10^6) were pelleted by centrifugation and resuspended in 1 ml of ice cold freezing medium (10% DMSO in complete medium containing 10% FCS). Cell suspensions were transferred to freezing vials and kept at -70°C for 24-48 hours and in liquid nitrogen vapour phase thereafter. Frozen cells were revived by rapidly thawing the frozen cell suspension at 37°C. Thawed cell suspensions were washed and cultured in complete medium. Viability of the cell lines was assessed by trypan blue exclusion.

(c) Leukemia cells: Lymphocytes from the peripheral blood of untreated patients of acute lymphocytic (ALL) and chronic myeloid leukemia (CML) diagnosed at the Institute Rotary Cancer Hospital (All India Institute of Medical Sciences, New Delhi), were isolated by centrifugation over Ficoll histopaque as described

above.

IFN Treatment of tumor cells: Mouse and human tumor cells were cultured at 0.2 x 10^6 cells/ml with 200 U/ml of murine or human recombinant gamma interferon (IFN-g) respectively for 48 hours at 37°C. At the end of the incubation, cells were harvested and washed twice in PBS and used for further assays.

Exposure of tumor cells to pH 3.0: Selective depletion of class I MHC antigens on tumor cells by briefly exposing them to pH 3.0, was done as described by Suguwara et.al (128). Cell pellets (1-10 x 10^6) were suspended in 0.5 ml of cold 0.2 M citric acid-sodium phosphate, buffer of pH 3.0, containing 1% w/v of BSA. After a 2 minutes incubation at 4°C cold complete medium was added in excess to neutralize the pH and cells were washed twice in fresh medium. The viability of these cells was monitored using the trypan blue exclusion method. For regeneration of MHC I antigens, these cells were resuspended in 1 ml of complete medium and incubated at 37°C.

Estimation of relative levels of class I MHC antigens on tumor cells: Expression of class I MHC antigens on tumor cells was assayed by a cell ELISA technique described by Sarin and Saxena (104). Briefly, 1-10 x 10^6 pelleted tumor cells per tube were suspended in 0.1 ml of hybridoma supernatant (HB102 for YAC, P815 and SP2O cells, B468 for L929 cells, B869 for EL4 cells, HB95 for K562, MOLT-4, Raji, Daudi, HR7 cells and human PBLs) and incubated at 4°C for 30 minutes. At the end of the incubation, cells were washed thrice with PBS and suspended in 0.1 ml of 1:200 diluted rabbit anti mouse IgG-HRPO conjugate and incubated at 4°C for 45 minutes. At the end of the incubation, cells were washed thrice in PBS. 0.5 ml of substrate solution containing 0.5% ortho-phenylene diamine (OPD), citrate phosphate buffer (0.1M, pH 5.2) was added to each tube and incubated at 37°C for 20 minutes. The reaction was quenched by adding 0.1 ml of 5N sulphuric acid to each tube. 0.2 ml of the reaction mixture was transferred in duplicates to

wells of 96 well microtiter plates and absorbance was read at 492 nm on an ELISA reader.

Estimation of cell size: 1-2 x 10^6 untreated tumor cells were washed in PBS and fixed in 500 μl of 0.1% paraformaldehyde. The forward angle light scatter was measured on a flowcytometer (EPICS-Coulter).

Chromium labelling of target cells: To target cell pellets of 1-2 million cells, 0.1 mCi of sodium chromate (Cr^{51}) solution in normal saline (sp. activity 0.1 Ci/mg chromium, from Bhabha Atomic Research Centre, Trombay) and 50 μl of fetal calf serum were added and the cell suspension incubated for one hour at 37°C in a water bath. At the end of the incubation, labelled cells were washed thrice in PBS and suspended in complete medium at a concentration of 10^5 cells/ml.

Chromium release assay of cytotoxicity: Chromium release assay was performed as described previously (110). 100 μl of the target cell suspension (10^4 cells) was added to different dilutions of effector cells (E/T ratios ranging from 6/1 to 100/1) and were deposited in round bottomed wells of 96 well microtiter plates (0.2 ml/well). Assay plates were centrifuged at 500 rpm for 5 minutes and incubated at 37°C in a humified atmosphere of 5% CO_2 and 95% air for 4 hours. The amount of radioactive chromium released in each assay well (ER) was estimated by counting 0.1 ml of culture supernatant from the assay wells in an LKB gamma counter. Spontaneous release (SR) was determined by incubating target cells in the absence of effector cells. Maximum release (MR) was determined by incubating target cells with 20 μl of 1% Triton X-100 and 80 μl of water. Percent target lysis was calculated using the formula:

$$\% \text{ Target lysis} = (ER - SR) / (MR - SR) \times 100.$$

The percent target lysis values at different E/T ratios were

converted into lytic units/10^7 cells using a computer program developed by David Coggin at National Institutes of Health, Bethesda, MD.

Cold target competition assays: For competition assays, an E/T of 25/1 was used and non-labelled tumor cells (competitors) were included into the cytotoxicity assay at competitor/target (C/T) ratios of 1, 2, 4 and 8. Control lysis in the absence of competitor cells was also determined and the percent inhibition caused by the addition of competitor cells was calculated.

Target binding assay: To a pellet of 1 x 10^6 target cells added 25 μl of a 0.5 mg/ml preparation of flourescein di acetate (FDA) in PBS and incubated at 4°C for 10 minutes. The cells were washed and further incubated with 10^5 effector cells in 100 μl of PBS (E/T ratio of 1/10). The cell suspension was spun at 500 rpm for 3 minutes and then incubated on ice for 20-30 minutes. At the end of the incubation, 10 μl of the cell suspension was loaded on a glass slide and counted under a fluorescence microscope. A minimum of 200 flourescing cells were counted and percent target binding was calculated as follows:

$$\% \text{ bound cells} = \frac{\text{(Number of bound fluorescent cells/Total number of fluorescent cells)}}{} \times 100$$

Activation of human PBLs and proliferation assay: Normal human PBLs were cocultured with gamma-irradiated Raji tumor cells for 5 days at a PBL:Raji ratio of 10:1. Proliferation activity of PBLs was assessed by giving a ^{3}H-thymidine pulse (0.5 μCi/well for 18 hours). Cells were harvested onto glass fibre discs and deposited in scintillation vials using an automated cell harvestor (PHD cell harvester, Cambridge Technology). Scintillation cocktail (1 ml) was added to the vials which were stored for 12 hours in the dark before counting in a LKB beta scintillation counter.

Statistical analysis: Paired t-test was employed to determine the levels of significance in different experiments.