MATERIALS AND METHODS

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ANIMALS

Inbred Swiss (albino) mice were obtained from Indian Institute of Chemical Biology, Calcutta and maintained in our animal Centre with pellet food and water *ad libitum*. Six to ten weeks old mice were used in all experiments because spleen NK cell activity in mice is highest at one to three months of age and there after falls considerably.(Herberman *et al.*, 1975; Kiessling *et al.*, 1975; Roder,1980; Weindruch *et al.*, 1983).

ANAESTHETIZING THE ANIMALS

For surgery, animals were anaesthetized with intraperitoneal injections of thiopentone sodium (May and Baker, Bombay) at a dose of 0.07 mg/gm of body weight.

TUMOUR INDUCTION

(a) **Maintenance of Tumour cell line :** Ascitic fibrosarcoma cell line (Ehrlich ascitic cell line) obtained from Chittaranjan National Cancer Research Institute, Calcutta is maintained in our Laboratory by serial passage in inbred Swiss mice by intraperitoneal injections of $1x10^6$ ascitic fibrosarcoma cells per animal every fifteen days.

(b) **Induction of solid tumours :** $2\ x10^6$ fibrosarcoma cells were injected subcutaneously at right hand side of the abdomen near the base of the hind leg of each animal for induction of solid tumour. Rate of growth of tumours was noted as change in mean diameter (cms) of the tumour with time. Size

of the tumour of individual mouse was measured every seven days by a slide calliper.

CELL SUSPENSION

Spleens were aseptically collected and the cells were dissociated in phosphate buffered saline (PBS) (pH7.2) with the help of sterilized stainless steel wire mesh. Erythrocytes were lysed by exposure to tris buffered 0.84% ammonium chloride solution (pH 7.2) for ten minutes. The cells were washed thoroughly twice with PBS and finally suspended in complete medium.

MEDIUM

For *in vitro* works, lymphocytes were cultured in RPMI 1640 (HI- Media, Bombay) supplemented with 25 mM HEPES buffer, 200 mg $NaHCO_3$ /100 ml, 50µl /ml of penicillin-streptomycin, 50µl /ml nystatin and 10% heat inactivated sterile goat serum. Henceforth, if not otherwise indicated, complete medium will mean the medium with all the supplementations.

SERUM

In our Laboratory, goat serum was found to be equally effective as Fetal Calf Serum (FCS) for *in vitro* culture of lymphocytes (Chaudhuri and Chakravarty 1983).

Goat blood was collected aseptically from jugular vein and was allowed to stand at room temperature for 45 mins for clotting and then at 4°C for the next three hours. Serum was gently collected and then centrifuged at 1000 rpm for ten minutes. The clear serum was preserved in aliquotes at

-20 °C until use. When required, the serum was thawed, heat inactivated at 56 °C for 30 minutes and sterilized by passing through 0.45 millipore filters (Sartorius).

COLLECTION OF COMPLEMENT

Guineapig serum was used as a source of complement obtained by direct heart puncture of healthy guineapigs as per the method of Herbert (1978). The blood samples were kept at room temperature for a few hours and the serum separated from the blood clot was centrifuged at 1000 rpm for 10 minutes. Sera from different guineapigs were mixed together for maximun efficiency. Small aliquotes of the serum were preserved at -20° C until use. The complement was used in a 1:10 dilution of total working volume.

SEPARATION OF NK CELLS

To get a pure population of NK^N cells, a stepwise separation of macrophages and the lymphocytes was followed.

(a) Separation of Macrophages

Spleen cell suspension free of erythrocytes were resuspended in fresh medium and incubated in sterilized plastic petridishes (LAXBRO) for 45 minutes in humidified atmosphere at 37 ° C containing 5% CO_2 in air. The plastic nonadherent cells were separated from the plastic adherent macrophages by gentle shaking and collecting the supernatant containing the plastic nonadherent cells.. The plastic non adherent cells were then centrifuged and resuspended in fresh medium.

(b) **Separation of B cells by nylon wool column technique**

The B cells were separated from the remaining lymphocyte suspension by nylon wool fibre column technique as outlined by Julius and co-workers (1973).This technique was selected for its simplicity and rapidity and also because the cells are not exposed to any harsh treatment during this procedure.

About100 mg of nylon wool (Fenwal lab; Deerfield, Illinois) was teased into loose fibres devoid of knots. The wool was then boiled in 1(N) HCl for 10 minutes to remove any toxicity. Then the nylon wool was washed three to four times with triple distilled water to remove the acidity. The fibres were then washed first with 1(N) NaOH to neutralize the acidity and then again two to three times with triple distilled water. The nylon wool was then soaked overnight in a solution containing 0.2% EDTA and 0.2% $NaHCO_3$, washed in distilled water, dried and autoclaved.

The sterilized nylon wool was loosened by soaking in medium and packed into a 2 ml syringe. About 1 ml of cell suspension containing upto $5x10^6$ cells were loaded carefully in the nylon wool column and incubated for 1 hr at 37 °C in humidified atmosphere containing 5% CO_2 in air. Then the nylon wool non adherent cells were eluted out with an excess amount of warm medium (37 °C) and resuspended in fresh medium. The non adherent cell suspension mainly consisted of a mixture of T and NK cells after separation of B cells. The cell number was determined with the help of a haemocytometer in presence of trypan blue.

c) **Removal of T cells**

The T cells were removed by lysis with monoclonal antibody directed against specific cell surface markers on T cells. 10^7 cells were spun down to 0.2 ml volume to which was added 0.2 ml anti Thy 1.2 (J.I.J.10) monoclonal antibody. The volume was made upto 4.5 ml with medium to which 0.5 ml guineapig complement was added, mixed gently and incubated for 1 hr at 37° C in humidified atmosphere containing 5% CO_2 .After lysis of T cells, the NK cells were collected, washed thrice with fresh medium to remove any trace of antibody and complement and then counted with the help of a haemocytometer in presence of trypan blue.

GENERATION OF IL-2

Procedure described by Gillis *et al.,* (1978) was followed with some modifications for generation of IL-2. Spleen cells were suspended at a concentration of $5x10^6$ cells /ml in medium containing Con A as a stimulator (10 µg /ml) (Type IV Chem Co. U.S.A.). The cells in aliquotes of 2 ml were incubated at 37 °C in humidified atmosphere containing 5% CO_2 in air. After 6 hours of incubation, the cells were centrifuged and washed twice with fresh prewarmed medium to remove the Con A. The cells were again resuspended in complete medium to its original volume of 2 ml per tube and subjected to 48 hours incubation. After incubation, the cell suspension was centrifuged for 10 minutes at 5000 rpm. The IL-2 rich fraction of supernatant was collected, doubly concentrated by vacuum dialysis, filtered and stored in aliquotes at -20°C. This IL-2 is referred as generated IL-2 (gIL-2) throughout the investigation. 50 µl of this IL-2 was used to

stimulate $0.5x10^6$ NK cells /ml for 24 hrs. Henceforth if not otherwise indicated activation with 50 µl of gIL-2 written in the running text will mean activation with 50µl of gIL-2 for 24 hrs.

RAISING OF ANTI TUMOUR ASSOCIATED ANTIGEN (TAA) SERUM

Anti TAA serum was raised in a rabbit. O.5 ml PBS containing $5x10^6$ washed ascitic fibrosarcoma cells were mixed thoroughly with 0.5 ml Freunds' Complete Adjuvant (Difco Laboratories U.S.A.). The emulsion was injected intramuscularly in the thigh of a healthy rabbit from which normal serum had already been collected for control experiment. The injection was repeated every week. After 72 hrs of the seventh injection blood was collected by ear vein puncture of the rabbit. The blood was allowed to stand for three hours at room temperature and the serum was collected. The serum was heat inactivated at 56 ° C for half an hour and stored at -20° C for further use.

AMMONIUM SULPHATE PRECIPITATION OF ANTI TAA SERUM RAISED IN RABBIT

Ammonium sulphate precipitation of anti TAA serum was carried out according to the method outlined by Nowotny (1979). 10 ml of anti TAA serum was precipitated by gentle addition of 6ml of saturated Ammonium sulphate solution under constant stirring at room temperature. The pH was adjusted by 2(N) Sodium hydroxide to 7.4. After half an hour of constant stirring by a magnetic stirrer, the precipitate was separated by centrifugation at 10,000 rpm for 10 minutes and reconstituted to 10 ml volume by triple

distilled water. To this volume, 5 ml of saturated ammonium sulphate solution was slowly added under constant stirring in cold. The precipitate formed was centrifuged, the supernatant thrown and dissolved in 10 ml triple distilled water. The solution was then finally dialysed in cold against phosphate buffered saline until the outer fluid was sulphate negative.This was tested by adding a few drops of the outer fluid in Barium chloride solution until precipitation stopped completely. Dialysed volume containing anti TAA globulin fraction was stored in aliquotes at -20 ° C.

DETECTION OF ANTI TAA ANTIBODY BY OUCHTERLONY METHOD

1% agarose in 0.89% sodium chloride solution was poured hot on glass slides to form a layer of about 2.3 mm thickness. Two holes were punched on the gel surrounding a central hole of 5 mm diameter by a gel cutter and the distance employed between the adjacent margins of the two holes was 7mm. The bottom of the holes were sealed with a small amount of hot agarose (0.3%) in order to avoid any leakage. Anti TAA antibody solution was diluted 2 fold and 20µl was poured in the central well. Cell membrane antigen of ascitic tumour cell was poured in one side well marked '1'. and ascitic fluid in another side well marked '2'. Diffusion was allowed to proceed overnight at room temperature. Distinct reaction lines could be seen between the central well and the two surrounding wells indicating that antigenic determinants on tumour cells can be detected with antisera raised by injecting tumour cells in rabbit.(Plate no.3)

BLASTOGENESIS

The transformation of NK^N cells into blasts after *in vitro* activation by IL-2 was studied. Cell suspension containing $0.5x10^6$ NK cells per ml in complete medium were stimulated with varying doses of recombinant IL-2(rIL-2) (Sigma Co. St. Louis, U.S.A.) such as 10U, 50U, 100U and 200U and 50µl of generated IL-2 for 24 and 48 hrs. Samples were removed from culture and blast cells counted by haemocytometer in presence of trypan blue under microscope fitted with an occulometer. Cells with diameter greater than 7 µm were scored as medium sized blasts and cells with greater than 10 -11µm in diameter were scored as large blasts. The proportion of transformed cells was expressed as percent blast cells by counting the viable medium plus large lymphocytes out of every 100 of total viable lymphocytes on haemocytometer in presence of trypan blue (Chakravarty and Clark 1977).

DNA SYNTHESIS

Activation of NK cells lead to blast transformation and is usually accompanied by DNA synthesis and proliferation. The rate of DNA synthesis is indicative of the degree of activation and was measured by the incorporation of tritiated thymidine in DNA at different hours. Cells after isolation were suspended at a concentration of $5x10^4$ cells/ml and separately treated with 10U,50U, 100U and 200U of rIL-2 and 50µl of gIL-2 per ml at the onset of culture. 200µl of cell suspension was poured in each well of a 96 well tissue culture plate (LAXBRO). The samples were incubated for 24 and 48 hrs in humidified atmosphere containing 5% CO_2 in air at 37°C. The samples were labelled with 1µCi ^{3}H-TdR per well for 10 hours before

termination of culture. At the end of respective culture periods, cells were harvested with PHD cell harvester onto glass fibre filters, washed with methanol, dried and kept in scintillation vials.

Prior to radioactive count, 5 ml of scintillation fluid containing 6 gms of PPO and 0.05 gms of POPOP in 1 litre of toluene was added to each vial. The radioactivity incorporated by the cells was measured in Beckman LS-1800 liquid Scintillation Counter. All assays were done in triplicates and the level of ^{3}H-TdR released was expressed as counts per minute.

CYTOTOXICITY ASSAY

The cytotoxic capability of NK cells and IL-2 activated NK cells from both normal and tumour bearing mice were measured in ^{51} Cr release assay. 10^{7} ascitic fibrosarcoma tumour cells which were used as target cells were taken in 1 ml complete medium and incubated at 37° C for 1.5 hours with 200 µCi of $Na_2{}^{51}CrO_4$ (Sp.Act 130-139 mCi/mg, Bhabha Atomic Research Centre, Bombay) with intermittent shaking to facilitate proper labelling. The cells were then washed thrice with PBS.

An aliquot of 10^{4} radiolabelled tumour target cells in 0.25 ml medium was mixed at different ratios (1:10 -1:100) with NK^{N} or NK^{T} cells in 1 ml of medium in small glass culture tubes and further incubated in humidified atmosphere of 5% CO_2 in air at 37°C for 4 hours.

At the end of incubation, the tubes were centrifuged and the radioactivity released in the supernatant from the tumour target cells was assessed by using a Gamma Ray Spectrometer (Electronic Corporation

of India Limited, Hyderabad). The percentage of cytotoxicity of ^{51} Cr released by the action of effector cells was calculated as follows:

$$\frac{\text{Experimental release - Spontaneous release}}{\text{Maximum release - Spontaneous release}} \times 100$$

The index of cytotoxicity was calculated as the mean values of triplicates for each point.

ANTIBODY DEPENDENT CELL MEDIATED CYTOTOXICITY (ADCC) ASSAY

ADCC of NK cells was demonstrated by incubating NK^N and NK^T cells before and after activation with IL-2 for various hours (16, 24, 48 hrs) with radiolabelled tumour target cells preincubated with heat inactivated anti TAA serum for 1 hour at various target : effector ratios (1: 25 to 1:100) in a 4 hr ^{51} Cr release assay. Radioactivity released in the supernatant of each tube was measured by a gamma ray spectrometer. The percentage of ADCC was calculated by the procedure described for cytotoxicity assay.

HOMING OF RADIOLABELLED IL-2 ACTIVATED NK^N CELLS AFTER ADOPTIVE TRANSFER IN TUMOUR BEARING MICE

$2x10^6$ NK^N cells activated with IL-2 for 24 hours were radiolabelled with 1 μCi ^{3}H-TdR (BARC, Bombay) for eight hours. The cells were washed thoroughly with PBS and injected intravenously in mice containing tumours of average size (1 to 1.1 cms in diameter). At 12, 24 and 48 hrs of adoptive transfer, the animals were sacrificed and small tissue pieces ($0.3mm^3$ approx.) from peripheral and central zones of the tumour, liver, spleen, skin, kidney and mesentric lymph nodes were collected in PBS following the

technique defined by Chakravarty and Jha (1997). Three pieces of each kind of tissue was collected from each animal. The pieces of tissues were soaked on filter papers and transferred into standard scintillation vials. The tissues were solubilized with 50µl Methylbenzothonium hydroxide (Sigma Co. St. Louis U.S.A.) and kept in dark till counting. The radioactivity incorporated by each kind of tissue was measured in Beckman LS-1800 Liquid Scintillation Counter after adding regular Scintillation cocktail in each vial as indicated in connection with DNA synthesis. Four mice were used for an experiment and the experiment was repeated three times.

REPEATED INJECTIONS OF LOW DOSE gIL-2 IN TUMOUR BEARING MICE

Repeated injections of low dose IL-2 (50µl gIL-2 /animal) were given to tumour bearing mice where tumours had been induced seven days earlier. In one group of animals, the injections were made by intravenous route of tail vein after necessary warming up of the animals. In a second group of animals, IL-2 injections were given at the tumour site. In all the experiments repeated IL-2 injections were made at 7 days intervals. Control animals were given intravenous injections of same amount of normal saline.

ADOPTIVE CELL TRANSFER

a) Adoptive transfer of NK^N cells to the tumour bearing hosts

Seven days after induction of the solid tumour, 0.5×10^6 NK^N cells in 0.1 ml were adoptively transferred once at the tumour site. In control animals, same volume of normal saline was injected. The growth of the tumours in different weeks and survivality of these animals were noted.

b) Adoptive transfer of IL-2 activated NK^N cells to the tumour bearing hosts.

Three groups of animals were taken for tumour induction. Seven days after induction of solid tumours, $0.5x10^6$ cells NK^N cells activated with 50µl gIL-2 were injected at the tumour site in one set of the animals and intravenously in the other set. In both sets of animals, weekly IL-2 injections were given through respective routes for five times.The third set was control animals in which only saline was injected.

In a separate experiment, the number of gIL-2 activated NK^N cells was increased to 10^6 cells and injected at the tumour site. This protocol was followed by weekly 50 µl IL-2 injections.

The weekly growth of tumours and survivality of the animals were noted.

c) Adoptive transfer of IL-2 activated NK^T cells to the tumour bearing hosts

$0.5x10^6$ NK^T cells were activated with 50 µl gIL-2 and adoptively transferred at the tumour site in one set of animals and intravenously in another set of animals, 7 days after induction of solid tumours in mice. IL-2 were injected through respective routes 5 times at weekly intervals.

In all cases, the growth of the tumours in different weeks were noted on the basis of mean diameter (cms) of tumours. At the time of plotting the data, the least square fit method was employed. The survivality of these experimental and control animals were also noted separately.

ADOPTIVE IMMUNOTHERAPY OF NK CELLS^N AFTER SURGICAL REMOVAL OF TUMOUR MASS

Mice bearing tumours (1 to 1.1 cms in diameter) were selected. The tumours were removed surgically under asceptic condition and care was taken to see that every trace of tumour was removed. Then the incision in the skin was stitched with asceptic nylon thread and the area was treated with Nebasulf powder.The mice were ready for adoptive immunotherapy 24 hours after surgery.

In one set of these animals, $0.5x10^6$ NK^N cells activated with 50µl of gIL-2 were adoptively transferred via intravenous route. This was followed by repeated intravenous 50µl of gIL-2 injections for five times every week. In another set of animals, after surgery, only 50µl gIL-2 was administered intravenously for five times at weekly intervals. The control animals were injected with similar amount of normal saline every week for the same period after surgical removal of tumour load.

The mice were under observation every week to check the recurrence of tumours. The survivality of the mice were also noted regardless of recurrence or nonrecurrence of tumours.