III. METHODS

1. Induction of tumors:

S-180 ascites tumor maintained by serial passage in Swiss mice, at the Tumor Immunobiology Laboratory of Chittaranjan National Cancer Research Centre, Calcutta, was used as parent cell line for induction of tumor. The tumor cells were collected from mice bearing 6 to 7 days old tumor by injecting 5 ml sterile PBS, into the peritoneal cavity of the mice and then withdrawing the fluid. The cells were separated from the fluid and washed twice with PBS by centrifugation at 1000 rpm for 10 mins. The tumor cells suspended in PBS were tested for viability by staining with Trypan Blue and were kept on ice till use. Approximately, 95% of the freshly drawn cells were found viable. A suspension of $2 \times 10^6$ cells/ml PBS was prepared after counting the cells in a Neubauer Chamber and 0.5 ml aliquots were inoculated intraperitoneally (i.p.) into the groups of mice which were used for experiments on different days of tumor growth.

2. Collection of Sera from tumor bearing mice:

A group of mice were inoculated i.p. with S-180 cells ($1 \times 10^6$ cells/mouse). Six to seven days
after tumor inoculation, the tumor bearing mice were bled by slitting the jugular vein. The blood samples were allowed to clot at 4°C for 3-4 hrs. and the sera were pooled and separated by centrifugation. Decomplementation of the pooled serum was done by incubating in a water bath at 56°C for 30 mins. This was stored at -20°C till use.

3. Raising of antiSRBC serum in rabbit:

A rabbit was immunized against SRBC by 7 intravenous injections of 1ml of 10% SRBC in PBS given on alternate days. The SRBC were separated from freshly drawn sheep blood and washed prior to immunisation. The immune rabbit was bled by cardiac puncture on day 15 and the serum was separated and stored at -20°C till use.


1. S-180 Agglutination test:

S-180 tumor cells (10⁶) were mixed with 0.1ml of decomplemented tumor bearing mice serum collected from 6-7 days old tumor bearing mice and incubated at 37°C for 30 mins. On microscopic observation of the treated cells spreaded on slides a number of cells forming aggregates an clumps were seen which indicated presence of antitumor antibodies in the serum.
2. Passive Hemagglutination (HA) assay:

Passive hemagglutination tests for detection of tumor specific antibody in the tumor bearing mouse serum were carried out according to the method of Boyden (290).

The serum obtained from mice bearing 6-7 days old S-180 tumor was decomplemented at 56°C for 1 hr. One ml of this decomplemented sera was absorbed twice on a 0.5 ml pellet of S-180 tumor cells for 48 hrs. The tumor cell absorbed serum and one aliquot of the unabsorbed serum were then absorbed twice with SRBC for another 48 hrs.

A 5% suspension of SRBC was incubated with equal volume of 10 mg% Tannic Acid solution at 37°C for 15 mins. in a water bath. The excess tannic acid was removed by centrifugation and washing with PBS. The tanned SRBC in PBS was incubated with equal volume of tumor antigen containing 2mg/ml protein.

For preparation of antigen, S-180 tumor cells, free of TAM, were suspended in PBS and subjected to four cycles of freeze thawing, the freezing temperature being -40°C. The suspension was then successively centrifuged for 1 hr. at 12,000g and at 40,000g. The supernatant
containing tumor antigens was collected and the protein content of the supernatant was determined by the method of Lowry et al (291).

Following incubation at 37°C for 30 mins. in a water bath, the excess antigens were removed by centrifugation. The SRBC coated with tumor antigen were then resuspended in PBS to attain a cell suspension of 1%. The HA assay was carried out in microtitre plates (Laxbro Manufacturing Co., India). The antigen coated SRBC in the wells of the microtitre plate were allowed to react with an increasing dilution of the SRBC absorbed tumor bearing mice serum and incubated at 37°C for 30 mins. The positive passive hemagglutination reaction was characterized by even carpeting of SRBC.

The controls included in the assay were:

(a) The unabsorbed serum with tanned SRBC not coated with antigen (b) tumor cell absorbed serum with tanned SRBC not coated with antigen (c) the antigen coated tanned SRBC in PBS and (d) tumor cell absorbed sera with antigen coated tanned SRBC. Positive reaction in 1:2 dilutions of the tumor bearing mice serum, and abrogation of the reaction when the serum was absorbed with S-180 tumor cells, along with the negative findings in
the controls indicated presence of tumor antibodies in the serum.

5. **Determination of sub-agglutinating titres of sera:**

1. **Sub-agglutinating titre of tumor bearing mouse serum:**

A serial dilution of the heat inactivated tumor bearing mice serum was prepared to get 1:5, 1:10, 1:25, 1:50, 1:100 and 1:150 dilutions in PBS. \(1 \times 10^6\) S-180 tumor cells were treated separately with each dilution of the serum for 30 mins, at \(37^\circ C\). The percent agglutinated cells were enumerated microscopically and number of cells aggregated in clump were determined. At 1:100 dilution of the sera, a high percentage of 2 cell clump was obtained, but at 1:150 dilution occasional aggregate of two tumor cells were observed. Therefore, 100 was considered as the sub-agglutinating titre of the serum.

2. **Sub-agglutinating titre of rabbit antiserum for SRBC:**

Rabbit antiserum for SRBC was serially diluted in PBS to 1:100, 1:250, 1:500, 1:750, 1:1000, 1:1,500, 1:2,000, 1:2,500. With each of the above dilutions \(1 \times 10^7\) SRBC was incubated at \(37^\circ C\) for 30 mins, and the agglutination of SRBC was studied under the microscope. Two to
three cell clumps were noted with 1:2,000 dilution of the serum and with higher dilution no cell aggregate was observed. The subagglutinating titer of the rabbit antiserum was therefore determined as 2000.

6. Elicitation of peritoneal cells in mice.

The peritoneal cells (PC) of 8 to 10 weeks old male Swiss mice were elicited by a non-specific stimulant TG broth. One ml of TG broth (0.0295gm/ml) was injected i.p. to a group of 6-8 normal mice. The PC were harvested 5 days after TG broth inoculation.

7. Preparation of PC of mice:

1. Harvesting of the PC:

Normal, tumor bearing and TG treated mice were sacrificed by cervical dislocation. A small incision was made in the abdomen and through the incision, the peritoneal cavity was rinsed several times with cold PBS. The washings containing PC were pooled and collected in a glass Petridish kept on ice.

2. Isolation of cells of the monocyte-macrophage lineage as a monolayer.

The peritoneal washings of mice were collected in glass Petridishes and kept at 37°C for 45 mins.
This would allow the cells of the monocyte-macrophage lineage to adhere on to the glass surface to form a confluent cell monolayer. The non-adherent cells were removed with several vigorous washings of the plates with cold PBS.

3. Recovery of the adherent cells from the monolayer.

To recover cells from the monolayer, the adhered cells in Petridishes were treated by overlaying the cell surface with 0.02% EDTA in PBS for 1 hr. at 4°C. The cells were then detached by jets of chilled PBS and the cell suspension collected in centrifuge tubes were kept on ice. EDTA was washed off by centrifuging at 400g for 10 mins. The cell pellets obtained were suspended in cold RPMI-1640 or PBS as needed by the experiment to be followed.

8. Characterization of the adherent PC:

1. Morphological characterization:

To study the morphological characters of the adherent PC of normal, tumor bearing and TG-treated mice, the PC were adhered on cover glasses, kept in Petridishes, at 37°C for 45 mins. The non-adhered cells were
washed off carefully. The adhered cells on cover glasses were blotted dry and fixed at room temperature by air drying, which were then characterised by staining with Giemsa, Wright's stain and Leishman's stain.

A. Staining with Giemsa:

The stock solution of Giemsa stain (Appendix ) was diluted in 50 volumes of distilled water buffered to pH 6.5 with 0.01M Phosphate Buffer. The adhered cells on cover glasses were treated with the stain(292) for 20 mins. at room temperature and was then rinsed gently in Phosphate Buffer. The stained cover glasses dried in air was then mounted in Canada Balsam and examined under a light microscope.

B. Staining with Wright's Stain:

The adhered cells on cover glass were treated with two or three drops of the undiluted Wright's stain for 2 mins. at room temperature. The cells were then treated for 10 mins. with the stain diluted two to three times with Phosphate Buffer 0.01M, pH 6.5. The cover glasses were then washed gently with distilled water until the cells appeared pink. This was then allowed to dry at room temperature, was mounted in Canada Balsam, and examined under microscope.
C. **Staining with Leishman's Stain:**

The air dried adhered cells on cover glass were treated with four to six drops of the Leishman's stain for 1 min. The staining solution was then diluted with double the volume of distilled water buffered with Phosphate Buffer, 0.01M, pH 6.5 and staining was done for another 15 mins. The stained cover glass was rinsed with water, air dried in room temperature and examined under microscope after mounting in Canada Balsam.

2. **Staining of the adherent PC for non-specific esterase:**

The air dried adhered cells on cover glasses were fixed in cold buffered Fixative Solution for 30 seconds and rinsed in distilled water. This was then dried at room temperature for 30 mins. The dried cells were covered with a solution containing 1% L-Naphthyl Acetate in Acetone, 0.1M Phosphate Buffer and Fast Blue B salt (Appendix .) for 10 mins. at room temperature. This was rinsed again in distilled water and air dried (293). The stained adhered cells on cover glass were mounted in Canada Balsam and examined under microscope.
3. *Fc*-receptor assay:

The adherent PC of normal and tumor bearing mice were suspended in PBS and mixed with hemolysin coated SRBC (Section III, 8.3) at a ratio of 1:50 and spun at 200g for 5 mins. The cell pellet was carefully dispersed and the cells having 4 or more bound SRBC were enumerated microscopically as rosette forming cells and the percentage of such rosette forming cells was recorded.

9. Preparation of tumor cells free of adherent PC:

The S-180 ascites tumor cells grown in the peritoneal cavity of experimental mice were made free of the tumor associated macrophages (TAM). The PC of the tumor bearing mice were collected at different days of tumor growth by extensive lavage of peritoneal cavity. The cells were made to adhere on glass Petridishes for 45 mins. at 37°C. The tumor cells along with other non-adherent cells were collected in centrifuge tubes by washing the Petridishes. The cells were then washed two to three times either with RPMI-1640 or PBS, by centrifuging at 400g for 10 mins. & suspended in RPMI-1640. The viability of the cells were determined by trypan blue dye exclusion test.
10. **Labelling of S-180 tumor cells and SRBC with $Na_2^{51}CrO_4$:**

1. **Labelling of S-180 tumor cells:**

The S-180 tumor cells, depleted of associated adherent macrophages were incubated with $Na_2^{51}CrO_4$ (500 $\mu$Ci/ml), taken at a concentration of 50 $\mu$Ci/10$^6$ cells at 37°C for 30 mins. The excess isotope was removed by 3 to 4 washings with chilled RPMI-1640. The labelled tumor cells were then allowed to stand on ice for 60 mins, before use.

2. **Labelling of SRBC:**

An aliquot of SRBC kept in Alsever's solution was washed thrice with cold PBS. The washed cells were suspended in RPMI-1640, counted in a Neubauer Chamber and kept in cold.

$10^7$ SRBC suspended in RPMI-1640 were then incubated with 100 $\mu$Ci of $Na_2^{51}CrO_4$ at 37°C for 30 mins. Following three washes with cold RPMI-1640 the cell pellet was suspended in fresh cold medium and allowed to stand on ice for 30 mins, before use.

11. **Coating of target cells with specific antibody:**
1. Coating of S-180 tumor cells with tumor bearing mouse serum:

The S-180 tumor bearing mouse serum containing antibodies against the S-180 cells were used to coat the tumor cells. The tumor cells depleted of TAM and in some cases labelled with radioactive chromium were mixed with the mouse serum suitably diluted to contain subagglutinating titre of antibodies, and incubated at 37°C for 30 mins. Following incubation the excess serum was removed by centrifugation. The serum coated tumor cells were suspended in cold RPMI-1640 and kept on ice till use.

For specificity control experiments, the S-180 tumor cells were coated with serum from non-tumor bearing normal Swiss mice following the method described above.

2. Coating of SRBC with rabbit antiSRBC serum:

An aliquot of SRBC stored in Alsever's solution was washed thrice with PBS. 10^7 cells as such or labelled with Na_2^{51}CrO_4 were then incubated at 37°C for 30 mins. with rabbit antiSRBC serum diluted to 1:2000 so that a subagglutinating titre of antiSRBC antibodies was achieved. The excess antiserum was removed by centrifugation and coated SRBC suspended in RPMI-1640 were kept in cold till use.
12. **Phagocytosis:**

Phagocytosis was assayed following a method slightly modified from Shaw and Griffin (294). The cells of the monocyte macrophage lineage from the peritoneal cavity of normal and S-180 tumor bearing mice were prepared as effector cells. Antibody coated tumor cells and SRBC were used as targets.

1. **Phagocytosis of tumor cells:**

S-180 ascites tumor cells were prepared as target cells free of TAM as described earlier. These were labelled with Na$_2$$^5$CrO$_4$ and sensitised with iso- logous serum suitably diluted to contain subagglutinating titre of antibodies against the S-180 cells (Section III, 5.1).

The adherent PC of normal mice, or resident macrophages (RM), suspended in RPMI-1640 supplemented with 10% FCS (RPMI-FCS), were plated in the wells of a flat-bottomed multicavity culture tray at a concentration of $2\times10^5$ cells/well and allowed to adhere for 40 mins. at 37°C. Tumor target cells suspended in RPMI- FCS were added to the effector cells at different effec- tor: target ratio (E:T) in a total volume of 1.5 ml of assay medium and allowed to react for varying lengths
of time, ranging from 2 to 5 hrs, at 37°C. At the end of incubation, the wells were washed off the excess tumor cells and medium. The adhered cells were lysed by treating over night with 0.3% SDS in PBS at 37°C. A total of 3ml lysate was quantitated for radioactivity in a gamma counter (Model: 125I Gamma Counter IC4702, Electronics Corporation of India Limited).

Similarly, antibody coated, radiolabelled autologous tumor cells (ATC) were added to the adherent cells separated from the ascites tumor i.e. TAM plated in the wells (2x10^5 cells/well) of a culture tray at an E:T of 1:1 and incubated for 5 hrs, at 37°C. After removing the non-engulfed tumor cells, the adhered TAM were lysed with 0.3% SDS and the radioactivity in the lysate was quantitated.

The results were expressed as the percent engulfment and was calculated as:

\[
\text{% Engulfment} = \frac{\text{Lysate count/100 sec.}}{\text{Total incorporated count/100 sec.}} \times 100
\]

The total amount of radioactivity incorporated in the tumor target cells was obtained by subjecting the cells to three cycles of freeze thawing.
2. Phagocytosis of SRBC:

SRBC were coated with rabbit anti-SRBC serum and labelled with Na$_2^{51}$CrO$_4$ (Section III, 11.2 and 10.2) and used as targets for phagocytosis by RM and TAM. The TAM isolated on different days of tumor growth or RM were suspended in RPMI-FCS and plated in the wells of the multieavity culture tray at a concentration of 2x10$^5$ cells/well and allowed to adhere for 40 mins. at 37°C. The $^{51}$Cr-labelled, hemolysin coated SRBC suspended in RPMI-FCS were added to the effector cells at an effective E:T of 1:5 and the total volume of the assay medium was maintained at 1.5 ml. The reactants were incubated for 4 hrs. at 37°C. Following incubation, the assay medium was discarded and the wells were washed off excess SRBC with Tris Ammonia Chloride (Appendix-) and PBS. The adhered cells were then lysed with 0.3% SDS and the lysate was quantitated for radioactivity as described above.

The results were expressed as percent engulfment and calculated as above.

The total amount of radioactivity incorporated in the SRBC was obtained by lysis of the cells
subjected to hypotonic stress.

13. Cytolysis:

1. Lysis of antibody coated tumor cells mediated by macrophages:

To assess cytotoxicity mediated by the TAM, RM and TG elicited macrophages to the S-180 tumor cells the effector cells were prepared by the method described in Section III.7. The TG elicited inflammatory macrophages were harvested from normal Swiss mice 3 days after the i.p. injection of 0.5 ml of TG broth.

S-180 ascites tumor cells were prepared as target cells free of TAM (Section III.9). These were labelled with $\text{Na}_2^{51}\text{CrO}_4$ and sensitized with serum from tumor bearing mice suitably diluted to contain subagglutinating titre of antibodies against the tumor cells (Section III.10 and 11.1). The resultant lysis of the targets was considered as ADCC.

To determine the effective ratio of effector to antibody coated tumor target cells, TAM prepared at day 3 after tumor inoculation was suspended in RPMI-FCS and plated in the wells of a flat bottom multicavity culture tray at a concentration of $2 \times 10^5$ cells/well
and allowed to adhere for 40 mins. at 37°C. The antibody coated tumor cells suspended in RPMI-FCS were added to the effector cells at different E:T and the total volume of the assay medium was made to 1.5 ml. The reactants were incubated for 4 hrs. at 37°C. At the end of incubation the supernatants with washings were collected from each well and the released radioactivity in the supernatants were measured in a gamma counter, the results were expressed as percent specific cytolysis (% SCL), which were calculated as:

\[
\% \text{SCL} = \frac{\text{Experimental Count} - \text{Spontaneous Release Count}}{\text{Total Releasable Count}} \times 100
\]

Spontaneous release of radioactivity and the total releasable radioactivity were obtained by incubating the target cells alone in the medium and by three cycles of freeze thawing of the target, respectively. This method was essentially the same as described by Garagiola et al (295) with slight modifications.

Subsequent to the determination of the effective E:T the antibody coated ⁵¹Cr-labelled ATC were added to the TAM prepared at different days of tumor growth at an E:T of 50:1 and the cytotoxicity
assay was carried out as described earlier. To study the cytotoxicity of RM and TG elicited macrophages, the antibody coated $^{51}$Cr-labelled 6 day old S-180 tumor cells was added to the macrophages at an E:T of 50:1 and incubated for 4 hrs. at 37°C. The released radioactivity in the supernatant was quantitated and the results were expressed as %SCL as described above. All the assays were done in triplicate.

2. Direct cytolysis of tumor cells mediated by macrophages:

Peritoneal cells of the monocyte-macrophage lineage from S-180 tumor bearing mice on days 3 and 10 of tumor growth were collected and prepared as effector cells as described in Section III.7. RM prepared from normal mice were the effector cells used as control. S-180 cells free of adherent TAM at days 6, 3, and 10 of tumor growth were labelled with Na$_2$$^{51}$CrO$_4$ and prepared as target cells for RM and TAM respectively as described earlier.

The TAM or RM were plated in the wells of the multicavity culture trays ($2 \times 10^5$ cells/well), to which was added the tumor target cells at an E:T of 50:1. The reactants were incubated at 37°C for 4 hrs.
The radioactivity released in the supernatants after 4 hrs. of incubation was measured and results were expressed as percent specific cytolysis (Section III. 13.1).

3. Lectin-dependent cellular cytotoxicity (LDCC) to tumor cells mediated by macrophages.

To study LDCC of the ATC the TAM were prepared as effector cells at days 3 and 10 following tumor inoculation. The RM was used as effector cells for LDCC to 6 day old S-180 tumor cells.

The tumor cells, free of adherent PC and labelled with Na$_2^{51}$CrO$_4$ (Section III.10), were used as target cells. The effectors and targets were mixed in a ratio of 50:1 in the wells of a multicavity culture tray. The lectins, WGA and Con.A were added individually at different concentrations, ranging from 3.3 ug-66.6 ug/ml to the effector target reactants and the reactants were incubated at 37°C for 4 hrs. The supernatant with washings was collected from each well and the radioactivity was measured in gamma counter. The results were expressed as %SCL (III.13.1) by measuring the radioactivity released in the supernatants, that released spontaneously from an equal number of
target cells incubated in presence of the respective concentrations of lectins and total releasable activity.

14. Binding of target cells by the macrophages:

Binding of target by TAM and RM was carried out by the method described by Garagiola et al (295).

1. Binding of antibody coated tumor cells by TAM and RM.

TAM collected at different days of growth of S-180 ascites tumor in Swiss mice and RM from non-tumor bearing mice were prepared by the process described in Section III.7. The tumor cells were depleted of adherent PC as described earlier and were used as target cells for binding by the TAM and RM after being coated with subagglutinating titre of antibodies.

The antibody coated ATC, suspended in RPMI-1640, were mixed with the TAM taken in suspension in the centrifuge tubes at an E:T of 1:50 to ensure availability of enough number of target cells for binding. Six days old antibody coated tumor cells were added to RM at the same E:T. The reactant cells were then incubated at 4°C for 15 mins. followed by centrifugation for 5 mins. at 200g at room temperature. The cells were
resuspended gently with Pasteur pipettes and the percent macrophages were enumerated microscopically having 2-5 or more than 5 bound tumor target cell.

2. Direct binding of the tumor cells by Macrophages:

The S-180 tumor cells were depleted of TAM as described earlier in Section III.9. The cells were added to the autologous TAM prepared as effectors at day 3 and 10 of tumor growth at a E:T of 1:50. The tumor cells prepared at day 6 of tumor growth were added to RM at the same E:T of 1:50. The binding of the tumor cells by RM or TAM was assessed by the method described in Section III.14.1. Enumeration of effector cells having 2-5 and more than 5 bound tumor target cells was done microscopically in duplicate.

3. Binding of the hemolysin coated SRBC by Macrophages:

SRBC, kept in Alsever's Solution was washed with PBS and coated with subagglutinating titre of rabbit antiserum to SRBC (Section III.11.2). The hemolysin coated SRBC were then added to the TAM prepared at different days of tumor growth and to RM at an E:T of 1:50 and were allowed to react for 15 mins. at 4°C. Following
centrifugation of the reactants at 200g for 5 mins, the cell pellet was resuspended. The % effector cells forming conjugates and having 2-8 or more than 8 bound SRBC were enumerated microscopically. The conjugates with 2-8 and 8 SRBC were designated as small and super rosettes, respectively. Each binding assay was done in duplicate.

15. Separation of the PC of mice into sub-populations by density gradient centrifugation:

The PC of normal mice and the TAM isolated from tumor bearing mice were subjected to density gradient centrifugation in Percoll following the method described by Brook et al (296) and Hester et al (297). A stock solution of the gradient medium was prepared by adding 1 part of 90% saline to 9 parts of Percoll Solution. This is kept in sterile condition at 4°C. Using stock solution a number of dilutions of Percoll were prepared with PBS. A discontinuous gradient was formed by layering in centrifuge tubes from down to upward 1 ml each of 60%, 55%, 50%, 45%, 40% and 20% Percoll.

The PC and the TAM prepared as described earlier were washed thoroughly with PBS and suspended in 2 ml of RPMI-1640. The total number of cells in the suspension
was counted and layered on top of the uppermost layer of 20% Percoll. This was then set to centrifuge at 2000rpm for 20 mins. at 20°C. The cells layered as a band at each interface of the Percoll gradients were carefully recovered and washed with PBS and the number of cells at each band were counted.

The cells of each band were morphologically characterized by staining with Leishman Stain or Giemsa Stain (Section III.8.1). The Fc receptor containing cells in each band were assessed by enumeration of rosettes formed by the cells with antibody coated SRBC (Section III.8.3).

16. **Statistical analysis:**

The statistical significance of the data were assessed by students 't' test.