CHAPTER II
MATERIALS AND METHODS

1. MATERIALS
2. METHODS (General)
3. PREPARATION OF EXPERIMENTAL ANIMALS
1. MATERIALS

Reagents

(1) Alsever's reagents:
- Dextrose: 20.50 gms
- Na-Citrate: 8.00 gms
- Citric Acid: 0.55 gm
- NaCl: 4.20 gms
- Streptomycin: 0.10 gm
- Penicillin: 0.063 gm
Distilled water made upto 1 litre.

(2) 0.85% NaCl

(3) Phosphate buffer saline (PBS)
- \( \text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O} \): 8.90 gms
- \( \text{KH}_2\text{PO}_4 \): 1.36 gms
- NaCl: 5.84 gms
Distilled water made upto 1 litre.
pH: 7.0 to 7.4
(7.2 for cytotoxicity test)

(4) Trypan blue solution
- 0.2% trypan blue (in Normal Saline)
- 0.4% trypan blue (in Normal Saline)

(5) Hank's Balance Salt Solution (HBSS)
- NaCl: 8.000 gms
- KCl: 0.400 gm
- MgSO_4 \cdot 7H_2O: 0.200 gm
- \( \text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O} \): 0.060 gm
- Glucose: 1.000 gm
- \( \text{KH}_2\text{PO}_4 \): 0.060 gm
- Phenol Red: 0.020 gm
CaCl₂, H₂O
(if fused)
Distilled water made up to 1 litre
pH
(6.9 - 7.4)
(pH adjusted with 0.8% Na₂CO₃)

(6) Giemsa Stain
- Methylene blue chloride 2 gms
- Azure A eosinate 1 gm
- Azure B eosinate 5 gms
- Methelene blue eosine 4 gms

(7) 10% Buffered formaline
- Formal 100 ml
- Sodium acetate 20 gms
- Distilled water 900 ml

(8) EDTA Solution (10 mM)
- EDTA 3.722 gms
- Distilled water made up to 1 litre.

(9) Turk's solution
- 0.01% Gention violet in 3% acetic acid.

(10) Gluteraldehyde (2.5% with phosphate buffer)

(11) Acetone (Pure)

(12) Graded alcohol (50% onwards up to 100%)
(100% alcohol prepared by applying dried copper sulphate which absorbed the last drop of water from alcohol. Dried copper sulphate prepared by heating the blue copper sulphate to white copper sulphate)

(13) Hydrochloric acid

(14) Nitroblue tetrazolium - Sigma Chemical Co., U.S.A.

(15) Pyridine

(16) Sodium Iodide (NaI)
(17) Sodium thiosulphate

(18) Trichloroacetic acid


(20) Iodine $^{131}$ (NaI$^{131}$) — Carrier free (Bhabha Atomic Research Centre, Trombay, Bombay, India.)

(21) Chromium $^{51}$ (Na$_2$Cr$^{51}$O$_4$) — Carrier free (Bhabha Atomic Research Centre, Trombay, Bombay, India).

(22) Percoll:

Separation of lymphocytes from spleen cell suspension has been carried out with a newly developed density gradient material, Percoll (Pharmacia Fine Chemicals AB, Uppsala, Sweden). Percoll is a new gradient material based on colloidal silica. The particles are rendered non-toxic by coating with polyvinyl pyrrolidone (PVP) and have the following properties: (a) low osmolality (b) low viscosity (c) non-toxic to cells (d) impermeable to biological membrane and (e) spontaneous gradient formation during centrifugation.

In the present study attempts to separate splenic lymphocytes with different percentages of percoll, namely, 40%, 45%, 50%, 60%, 70% and 75% have been made. A gradient of 60% was found to be most suitable.

Physiologically iso-osmolar percoll was obtained by mixing 9 parts of original material with one part of 10x (8.5%) saline. A 60% gradient is then made by using 0.85% NaCl. Separation of lymphocyte was achieved by gently layering 3-4 ml of single cell suspension on to 5-6 ml of 60% gradient and centrifuged at 400 'g' for 20 minutes at room temperature. After centrifugation lymphocytes were aspirated out from the interphase of percoll and media. Further details will appear in Chapter IV, Section 1.
Tissue Culture Media:

In the immunological procedure, different media are required to provide the cells with ample nutrition for the maintenance of their metabolic functions and also to facilitate their growth with care to avoid the growth of unwanted micro-organisms. The range of media vary extensively with the particular cells or tissue types. The tissue culture media generally include different salts and buffers in balanced proportions, various vitamins, protein hydrolysates, often different proportions of essential amino acids, carbohydrates and fat constituents. Phenol red is generally used as an indicator and antibiotics and antifungal agents are added in definite concentrations to guard bacterial and fungal growths. Animals sera like fetal calf serum or human AB sera are added in complete media and used according to their requirement. The following media were used according to our need.

(a) Tissue Culture Medium - TC-199
(Difco Laboratories, Detroit, Michigan, U.S.A; Gibco Laboratories, Grand Island, New York, U.S.A.)

(b) Hank's Eagle's Medium (Difco Laboratories, Detroit, Michigan, U.S.A.)

(c) RPMI - 1640 (Gibco Laboratories, Grand Island, New York, U.S.A.)

Complete Media

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI</td>
<td>10.40 gms</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>2.00 gms</td>
</tr>
<tr>
<td>HEPES</td>
<td>5.95 gms</td>
</tr>
<tr>
<td>Streptomycine</td>
<td>0.10 gm</td>
</tr>
<tr>
<td>Penicillin</td>
<td>0.063 gm</td>
</tr>
<tr>
<td>FBS</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

Distilled water made upto 1 litre. pH 7.4 to 7.5

Fetal Bovine Serum(FBS); Heat inactivated:

Purchased from Gibco Chemicals, U.S.A.
Instrumentations:

(1) Laminar flow - 1154-ST-SM
(Klenzaids Contamination Control Pvt. Ltd., Bombay-400 016)

Klenzaids class 100 Vertical Gradvel Laminar Down flow
Clean Air Work station straddle, designed to provide a bioclean
work space measuring 4'W x 2'D x 2'6''H, and comprising, as
standard features, internationally patent GRADVEL HEPA filter;
Micro-Klenz prefilter.

(2) CO₂ incubator Mod Cl. CO-21 New Brunswick Scientific, N.J., USA

(3) U-V-lamps - Philips, Holland

(4) Gamma Scintillation Counter - Electronic Corporation of India,
Hyderabad, India.


(6) Scanning Electron Microscope (PSEM-500), Philips, Holland.

(7) Edward's Sputter Coater, Philips, Holland.

(8) Spectronic-20, Bosch & Lomb, U.S.A.

(9) Autoclave.

(10) Hot air oven.

(11) Table top centrifuge - Remi, India.

(12) Cold Centrifuge - K24 - Janetzki.

(13) Light microscope, Binocular type, Olympus, Japan.

(14) Fridge and Deep Fridge.

(15) U.V. hood for Isotopic administration.

(16) Haemocytometer (Neubauer, W. Germany).

(17) Membrane filter (Schleicher and Schull, GmbH, Postfach-4
D-3354 Dassel, W. Germany).
Choice of Animals

Most of the materials for studies in cancer biology are usually formed by experimental animals. Human subject for the purpose for obvious reasons is extremely limited. However, human material became available and used only under special circumstances.

Although various types of animals have been used for studying cancer biology, mouse is by far the most commonly used animal. It owes its unique position to its (1) capacity to grow spontaneous tumors (Haaland, 1911; Woglow, 1913) (2) strong genetic basis for easy tumor transplantation and maintenance (Little, 1941) (3) resemblance of neoplastic nature with that in human (Haaland, 1911; Woglow, 1913; Cloudman, 1941) (4) best known immune system in the animal kingdom and (5) some other facts involving high fecundity resemblance with man in being largely unspecialised, easy domestication and adaptation to cagelife, low cost and availability (Lane-Petter, 1976).

The genetic basis of tumor transplantation in mice has been discussed as early as 1901-1902 by Loeb and later by Little (1941). The transplantability of a particular tumor line to a strain (of mice) of its own origin (syngeneic) is thus explained on this genetic basis and related to the "histocompatibility gene" (H_{2} locus) (Snell, 1948). Transplantation of the particular tumor line to an allogeneic group of mice (a strain other than that of the tumor origin) thus becomes a failure as being different in their "histocompatibility."

In the present investigation, Swiss mice was found to be syngeneic to the APS tumor line and was the strain of choice. Pure inbred swiss mice were obtained from Tata Memorial Cancer Research Institute, Parel, Bombay and were maintained by brother-sister mating in our laboratory in cages at 26-28°C. They were kept on a balanced diet (Hindlever pellet). Drinking water ad libitum was supplied containing antibiotic neomycin (1 gm/10 litre) with polymixin (100 mg/10 litre).
Tumor Line

Ascites fibrosarcoma tumor line (Dimethyl cholanthrene induced) obtained from Tata Memorial Cancer Institute, Bombay, India, was maintained by serial transplantation ($5 \times 10^5$ cells). The tumor is syngeneic to the Swiss mice.

Tissue Components

(1) Spleen cell suspension: Single cell preparation:

Animals were killed by dislocation at the neck and then the spleen was taken out aseptically and kept in 3–4 ml of chilled PBS containing kanamycin sulphate (100 µg/ml). The spleen was then minced with iris forceps and finally passed through 80 mesh steel wire gauge to get single cell suspension of spleen. The details have been discussed in Chapter IV, Section 1.

(2) Isolation of Macrophages from Peritoneal Exudate:

Method followed for the isolation of macrophages from peritoneal exudate is similar to that followed by William and Chase (1976). Briefly, mice from all groups are killed by dislocation and pinned to a board; the abdominal skin is flushed with 70% ethanol. The skin is carefully reflected, leaving the peritoneal lining intact. Peritoneal lining is tented with forceps and 2.5 ml balanced salt solution (PBS) is injected forcefully with no. 20 needle to ensure adequate mixing. The fluid is then harvested by means of a fine-tipped Pasteur pipette and rubber bulb. The whole of the peritoneal exudate has been layered on surface of a petri dish or a microscopic slide containing a small amount of culture media (RPMI-1640) and incubated at 37°C in CO₂ atmosphere. After 30 mins incubation, the suspended cells were washed off, leaving the glass adherent macrophages intact. Such adherent cells of all the groups of mice were cultured and utilised as per requirement.

Sterilization

In the present study different types of sterilizing procedures were adopted: (1) the hot air sterilization by dry heat, (2) autoclaving or moist heat sterilization and (3) sterilization by filtration, depending on
nature of materials to be sterilized. Glass wares and other innate materials were sterilized using both dry and moist air. Whereas, for media, reagents and tissue components sterilization were done by filtration through millipore filters to prevent denaturation of biological materials and alteration in chemical properties of the reagents. All the reagents and media after sterilization were kept in sterile containers at 4°C.

Ultraviolet radiation (UV radiation) with a wavelength between 2400-2800 Å was used routinely for sterilization of laminar flow, uv hood and tissue culture room. UV irradiation was also used for sterilization of rubber teats, plastic tubing and animal dissecting board etc. Chemical sterilization was made by using ethyl alcohol and phenol and related compounds namely, phenyl, dettol, lysol, savlon etc.
3. PREPARATION OF EXPERIMENTAL ANIMALS

(A) Transplantation of Tumor

Propagation of a tumor line by tumor transplantation is a major tool in experimental cancer research. The technique has faithfully served investigations in several fields of cancer research—involving genetic, chemical, physical, hormonal, viral and also the biochemical and immunological aspects.

Arguments have been advanced both in favour and against the use of transplanted tumor in cancer research; but spontaneous or induced tumor will probably never be able to replace the transplantation technique because of the time involved in their production, their biological individuality and more pronounced internal heterogeneity (Klein, 1959). The preferential use of transplantation technique in cancer immunology, biochemistry, etc., is due to the fact that with suitable choice of material, it is relatively easy to obtain reasonably homogeneous samples of malignant tissue at a fairly predictable rate and in large quantities, provided, of course, appropriate host animals are available (Leibelt and Leibelt, 1967).

The history of transplantation of tumor has been reviewed by several authors (Eittner, 1931; Shimkin and Novinsky, 1955; Shimkin, 1960; Woodruff, 1960; Triolo, 1964). In 1876, M. Novinsky, was the first to carry out a successful transplantation of a lymphosarcoma and an anaplastic nasal tumor through two transplant generations in days. A rat sarcoma was transferred to other rats by Firket in 1892. In subsequent years considerable progress have been made in the field of transplantation of tumors in experimental models and at present, this has received the highest popularity for studying cancer biology. Velich in 1898 transferred sarcoma through eight generations in rats, but the transplant was lost when attempted in a foreign group of rats. The results were interpreted as being due to transfer of micro-organisms and not due to transfer of cells. Subsequently, other workers reported that tumorous
tissue could not be successfully transplanted into other members of the same species. The failures were identified with the involvement of immunity in such situations.

This prompted workers in the field of transplantation research to transplant a tumor in allogeneic strain of mice using various immuno-suppressive agents along with some immunological supplements.

What follows in the subsequent chapter is the methodology for growing and maintaining ascites fibrosarcoma tumor line in syngeneic (Swice) mice using serial transplantation technique.

Transplantation of Ascites Fibrosarcoma Tumor in Syngeneic Mice: A Dose Response Study

Maintenance of the tumor line requires inoculation of the tumor cells of the particular line in sensitive strain of mice in an optimum dose, i.e., a dose in which there will be a 90-95% tumor take with a mean survival time of 30-35 days. Further, for immunological studies, this optimisation is considered needful for (i) a reasonably long survival time and (ii) reasonably good tumor take. So, for a particular tumor line, optimisation of the dose becomes a critical factor and should be achieved by transplantation with variable tumor doses to different groups of mice. Attempts have been made in our laboratory for the present series of investigations to maintain ascites fibrosarcoma cell line in mice and to do this, a critical time-dose response study has been carried out for optimisation with the above objects in view.

Materials and Methods:

Ascites fibrosarcoma tumor line, maintained in our laboratory by serial transplantations, has been used as the tumor line of choice. The peritoneal fluid containing the ascites tumor cells were collected with the help of a sterile syringe using 20 gauge needle, washed thrice with 0.85% NaCl and finally the viable cells counted in a haemocytometer chamber using 0.4% trypan blue and the various doses adjusted with 0.85% NaCl. Inbred swiss mice susceptible to the tumor formed the material for the proposed study. The mice were divided into 2 groups consisting
of 10 mice each in which the following doses were injected intraperitoneally under asepsis: $2 \times 10^2$ and $2 \times 10^4$ cells.

After challenge, the mice were inspected regularly for changes in their general health conditions, behavioural changes (if any) and their body weights and abdominal circumference (giving a measure of the tumor growth rate) at every 5 days interval. In all the cases changes in body weight were expressed as percentile body weight (final body wt/original body wt. x 100).

**Results:**

The suitability of dose (S) has been evaluated from the extent of tumor take, growth of the tumor and the total survival after the tumor challenge together with a watch towards the general health condition of the animals involved. Of the several doses compared a steady tumor model was selected by comparing the parameter of two sets of animals, one inoculated with $2 \times 10^2$ cells, and the other inoculated with $2 \times 10^4$ cells respectively.

**2x10^2 Cells:**

The last animal in this group died on 49th day. There was a significant tumor take in 90% mice as indicated by an abrupt increase in body weight and presence of palpable tumors. Two animals suffered anal ulceration and finally died along with other eight mice between 20-26th day ($LD_{50}$26 days, Table I). In 90% of the animals tumor growth was observed as early as 15th day of challenge. Thus, on around 20±4 days the animal were ready for experimental purpose (Fig.1).

**2x10^4 Cells:**

90% of the animal died between 20th and 40th day of challenge ($LD_{50}$24 days, Table I). Onset of tumor growth was observed from 7th to 10th day and palpable tumor was obtained at about 10th day. Animals died mainly of anal ulceration. Though most the animals died within 35 days, a survivor showed metastatic solid tumor on its right hind leg and finally died on 103rd day (Fig.2a, 2b).
LEGENDS TO FIGURES
FIG. 1

![Graph showing body weight (percentile) vs. days. The graph includes lines for Swiss 2 x 10^2 cells.](image)
FIG. 2a

SWISS

$2 \times 10^4$ CELLS

PERCENTILE BODY WEIGHT

DAYS
Tumor growth pattern (AFS, $2 \times 10^4$) untreated control

Days after tumor challenge
A critical analysis of the data helped the present study to select the dose of choice for the induction of a steady tumor model in experimental animals. Although a dose of $2 \times 10^2$ cells showed a considerably longer survival period, the optimum period for a palpable tumor take is delayed. As per observation, it takes roughly $15 \pm 1$ days (Table I) for palpable tumor to occur with a longer survival period ($LD_{50/26 \text{ day}}$) whereas, a dose of $2 \times 10^4$ cells provides a steady tumor appearance at $10 \pm 1$ days, (Table I) the survival time being almost negligibly affected ($LD_{50/24 \text{ days}}$). The rate of tumor growth was also much faster with the latter group compared to that found in the former. Thus, considering easy and quicker tumor incidence with an almost parallel survival rate, a dose of $2 \times 10^4$ cells was considered as the ideal selection.
(B) Immunization with Particulate Antigens

The particulate antigens are sometimes referred to as corpuscular antigens although a wider range of antigens is reserved for the term "particulate." In fact, the particulate antigens "usually cover the corpuscular antigens, bacterial antigens or some particles derived thereof." For immunogenic purposes a number of particulate antigens including bacteria are used for prophylactic measure after attenuation or some other specific processing. Sheep erythrocyte (SRBC) and Bacillus Calmette Guérin (BCG) are the two materials that have been used for the purpose of immunisation throughout the course of studies. The purpose of administration of these particles in tumor bearing animals was to evaluate their role in the tumor growth kinetics in the host concerned.

Sheep Erythrocyte

These are used as a well recognized antigen for immunisation in several experimental animals. The material is highly immunogenic and found to stimulate the antibody synthesis in the B-cells, called "plaque forming cells" in the particular instance; the material is also reported to form rosettes particularly with T-lymphocyte. In number of cases, these have been reported to stimulate "Active T-cell formation." Importance of this antigen in immunity has been discussed by many.

Materials and Methods:

Preparation: Sheep blood collected in fresh, cold, sterile Alsever's solution was washed thrice in cold sterile fresh normal saline and suspended in the same to prepare 3%, 5% and 7% suspensions (PCV in ml/saline in ml).

Route of Administration:

The antigen was administered intra-peritoneally (i.p) which has been described as the best method for immunostimulation (Mathe, 1973). The inoculation was performed aseptically.
The amount and day of administration:

0.5 ml each of 3%, 5% and 7% SRBC suspensions was given i.p., and each dose was administered on various days with respect to the tumor challenge, taking the day of tumor challenge as the "0" (zero) day.

Comments:

The data obtained so far on the tumor growth rate and survival rate (LD_{50}/day) after SRBC administration has been carefully evaluated. Emphasis has been given to the effect of SRBC administration on the "0" day of tumor challenge (Table I & II). Comparing the effective SRBC concentration starting from 3%, 5% and 7% (PCV/Saline volume), it has been found that a 7% concentration exhibited its greater therapeutic effect both in terms of "occurrence of tumor" or "tumor take" and the "total survival time" or LD_{50} in days. Details have been discussed in individual chapter.

Bacillus Calmette Guérin (BCG):

This attenuated bovine tubercle bacillus is the biological adjuvant which has received most attention so far. Early studies indicated that BCG is a powerful stimulator of humoral immunity (Lewis and Loomis, 1924) and that of cell mediated immunity (Diener, 1936). A number of subsequent studies revealed that the material has got a wide-open immunogenic property which was later considered as a very competent immunostimulant. The recent studies on BCG administration in tumor bearing subjects revealed that it can also act as antitumor agent (Biziozi et al., 1954; Baldwin and Pimm, 1973; Jelum et al., 1985).

Materials and Methods

Preparation and route of administration:

Lyophilized samples were obtained from the BCG vaccination laboratory, Guindy, Madras, each ampoule containing 2 mg (dry weight) of the organisms. The sample was resuspended in 2 ml of fresh, sterile and chilled 0.9% saline per ampoule. 0.1 ml of the suspension was admi-
nistered i.p. to each animal, receiving either $2 \times 10^2$ or $2 \times 10^4$ cells of AFS, on various days with respect to the day of tumor challenge ("0" day).

Comments

It is observed that BCG exhibits both preventive and therapeutic effects on neoplasia. The effect, of course, is dose and tumor load dependent. With low ($2 \times 10^2$ cells) tumor load, the tumor appearance is delayed (compared to control neoplastic animals, viz. 15 ± 1 days) to about 27 days, $LD_{50}$/days, is raised from 26 to 52 days. With higher tumor load, the effect is again dependent on the day of administration, $LD_{50}$, however, is lowered in all these cases. Details have been discussed in individual chapter concerned.

(C) Groups maintained

The inbred swiss mice thus formed different groups of animals inoculated with either tumor cells, BCG or SRBC, or any of the particulate antigens with tumor. The purpose of the inoculation has been to induce some immunoregulatory effects while combating the tumor development. Thus there are some animals in which tumor and antigen administration has been done simultaneously. Accordingly, the role of these particulate antigens have been studied. The following groups of animals were maintained:

1. Tumor bearing control (TC): Mice receiving $2 \times 10^4$ AFS cells.
2. BCG control (C-BCG): Mice receiving i.p. 0.1 mg of lyophilised BCG.
3. Animals receiving tumor and BCG (T-BCG): Mice receiving $2 \times 10^4$ AFS cells and 0.1 mg of BCG simultaneously.
4. SRBC control (C-SRBC): Mice receiving 0.5 ml of 7% SRBC intra-peritoneally.
5. Animals receiving Tumor and SRBC (T-SRBC): Mice receiving $2 \times 10^4$ AFS cell and 0.5 ml SRBC simultaneously.

A normal control group of animals has been utilised whenever felt necessary.
All the parameters in support of investigation conducted so far were subjected to the above groups of animals either in terms of *invivo* or *in vitro* condition after isolating the required effector *cell line* from the groups concerned.