ISOLATED TUMOR CELL MEMBRANES USED AS ANTICANCER IMMUNIZING AGENTS

By

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NOMENCLATURE

C	degree centigrade
X g	times gravity
Hepes	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic Acid
hrs	hours
I.P.	intraperitoneal
L1210	Leukemia L1210 cells
LM	fetal mouse cells, cultured
%	per cent
pH	negative log of hydrogen ion concentration
mg	milligram
min	minutes
ml	milliliter
NADH	Nicotinamide adenine dinucleotide (reduced)
SA 180	Sarcoma 180 ascites tumor cells

CHAPTER I

INTRODUCTION

Tumor immunology is presently one of the most promising areas of cancer research. The intrinsic qualities of the immune system suggest that it could be more effective in combating cancer than other methods which are now in use. First, it is the body's own natural defense mechanism, and second, it can reach all areas of the body. In instances of bacterial and viral invasion, the immune system of the host serves as an important line of defense, and in almost all cases of treatment of disease, the immune system is responsible for the final clearing of the infective agents.

The mechanisms utilized by the host in rejecting tumors is the same as those used in combating infection. The action of cell-mediated immunity can be demonstrated by (a) the adoptive transfer of lymphoid cells from an immune host to an infected host, or (b) by the in vitro mixing of sensitized lymphocytes and viable tumor cells. Antibody-mediated immunity is thought to be mainly G-type immunoglobulins which are cytotoxic by binding of complement (Bellanti, 1971).

Complexity is the rule in interactions between oncogenesis and immune reactions. It has often been observed that there is an increased incidence of tumors in patients who have undergone immunosuppression for an organ graft (Hellstrom and Hellstrom, 1974). A popular explanation for this phenomenon is called immune surveillance.

The different circulating components of the immune system are constantly monitoring the internal environment in which it functions. Marx (1974) suggests that neoplastic cells arise many times in the life of a complex organism but most are eliminated because of their foreign antigenic configuration.

Another aspect of the complex reaction between tumor and host is that in response to a weakly antigenic tumor, different immune reactions, which may or may or may not be effective, can be elicited. It has been observed that an early, weak immune response may be stimulatory while a later, stronger response is inhibitory (Prehn, 1972).

An alternate proposal to the immune surveillance theory is a theory supported by Marx (1974), which observes that close to half of all tumor types are associated with the lymphoreticular system, and that immunosuppression and immune deficiency diseases directly affect that system. Still, it is not clear how a developing tumor can escape detection by the immune system.

Tumor immunology became feasable with the demonstration that most, if not all tumors possess antigens not characteristic of their tissue of origin. These antigens are known as tumor specific antigens (TSA) or tumor associated transplantation antigens (TATA). These antigens may or may not cross-react with antigens of other tumors of the same type. Probably all tumors caused by the same type of virus do cross-react, whereas most chemically-induced and spontaneous tumors cannot be demonstrated to have cross-reacting antigens (Old and Boyse, 1964).

One antigenic difference is known as a fetal antigen. Coggin (1974) states that an antigenic difference might result from renewed

expression of genes normal to some previous stage of development. The presence of fetal antigens has been demonstrated by Ting(1972). Finely minced syngeneic fetal mouse tissue was injected intraperitoneally into inbred C3H/HeN male mice and their serum collected by retro-orbital bleeding. In a process similar to the indirect fluorescent antibody technique, tumor cells were first incubated with the antiserum, then with ^{125}I labeled antimouse gammaglobulin. The results were counted as positive when the radioactive count was more than double the count resulting from antisera produced from mice injected with syngeneic adult tissue. The test serum must also not react with normal host cells. The results indicated the presence of antigens which were common to the fetal cells and the tumor cells, and were recognized as foreign by the immune system of the host.

Viral antigens have also been shown to be expressed on some tumor cells. Roizman and Spear (1971) succeeded in using antiviral antibody to bind to cell surfaces and subsequently increase their density enough that they could be separated from unaltered membranes by centrifugation on a sucrose density gradient.

Three mechanisms have been suggested by Nowotny et al. (1974) by which a proliferating neoplasm can escape detection by the immune defenses of the host. First, the tumor cells might selectively lose their immunogenicity. Second, there could develop a growth enhancing immune response toward the tumor, or third, an " interference" could occur to disrupt the anti-tumor effect developed by the host. Although the third explanation is favored by their findings for their tumor-host system, other authors have found evidence to support the other two explanations too. The "interference" suggested by Nowotny

et al. (1974) was thought to be products of cell decay (PCD). Other authors such as Hellstrom and Hellstrom (1970) have suggested possible "serum blocking factors" that might be antigens which have been shed from the tumors and complex with antibodies in the medium.

Prehn (1972) suggests a similar mechanism which is analagous to the situation of a pregnancy during which an antigenic configuration foreign to the host is present inside the body but is protected by the shedding of antigens, in this case, fetal antigens.

Coggin and Anderson (1974) draw an analogy between the escape of a tumor and the escape of a fetus from immune rejection by the host. In both cases, the autochthonous lymphocytes have been shown to be cytotoxic to the respective cells, and sera from the pregnant and tumor-bearing hosts have been found to have blocking ability. They propose that the immune system of placental animals is poised to reject small numbers of cells exhibiting fetal antigens while allowing large masses of such cells to escape.

With the demonstration of antigenic differences on tumor cell surfaces, it became clear they could be used as immunizing agents or immunotherapeutic agents after being inactivated. The modified cells can be used alone as an immunizing agent or in conjunction with chemotherapy, radiation therapy, or surgery as immunotherapy.

A number of agents have been used to alter cells such as X-irradiation at doses sufficient to kill the cells (Kolsch et al. 1973), Vibrio cholera neuraminidase which cleaves sialic residues from the membrane surface (Kollmorgen et al., 1973), iodoacetamide which blocks sulfhydral groups, and mitomycin C, an inhibitor of DNA synthesis which may or may not lead to membrane alterations (Prager et al.,1974).

Prager et al. (1974) managed to achieve 80% survival of host organisms against a challenge of 1 X 10^3 tumor cells after vaccination with cells treated with iodoacetamide or mitomycin C. Kollmorgen et al. (1973) also achieved up to 80% success using neuraminidase treated cells after using chemotherapy to reduce the tumor burden to 1 X 10^5 or fewer cells.

Cell surface phenomena seem to account for many of the characteristics observed in a developing tumor. The process of transformation changes the extent of cell to cell interactions which account for the change in social behavior and metastases (Coggin and Anderson, 1974).

Even though internal cellular membranes may present antigens similar to the external ones, it is clear that it is the plasma membrane of the tumor cell which primarily comes in contact with the immune surveillance system of the host. The plasma membrane is also thought to play an important role in processes such as cohesiveness and contact inhibition.

It was because of these facts that experiments in this study were designed in which tumor cell plasma membranes were used to immunize animals against a subsequent administration of live tumor cells.

The hypothesis was that isolated membranes would present more of the biologically important antigens per unit of material than altered whole cells, and that better results could be achieved using immunization schemes similar to others used with whole cells.

CHAPTER II

MATERIALS AND METHODS

Tumor-Host System

The animal hosts used in this study were female white mice of an outbred strain designated HaM/ICR, obtained from the Charles Rivers Mouse Farms, Wilmington, Mass. They were always isolated for two weeks when received in this laboratory, and given food and chlorinated water ad libitum to check for disease and parasites. At the initiation of any experiment, the subject mice were at least six weeks of age and had obtained a weight of at least 25 grams.

In one experiment, the animal hosts were black female BDF_1 mice obtained from Sprague-Dawley Laboratory, Madison, Wisconsin, and used at an age of about six weeks and a weight of about 20 grams. The BDF_1 is an inbred strain.

The tumor cell line was Sarcoma 180. This tumor originated as a carcinoma in the axilla of an undesignated strain of white mouse. It was isolated in the Cancer Institute of Columbia University in 1914 (Stewart et al., 1959). It was originally grown as a solid tumor but has been adapted to grow as an ascites cell, and the conversion occurred sometime before 1919. It is carried in this laboratory by weekly intraperitoneal transfers of about 1×10^6 cells diluted in cold Hepes buffered saline.

The tumor apparently shows fewer histocompatability antigens than an unaltered cell because of its ability to grow in different strains of mice. This fact is of primary importance because the histocompatibility antigens are usually the most strongly expressed antigens. Their absence allows more detailed examination of reactions with tumor associated and fetal antigens.

Tumor Cell Membrane Isolation

The agent used as an immune stimulus in these experiments was tumor cell plasma membranes isolated by a procedure originated by Shin and Carraway (1973), using a modification of the Warren method (1969) of isolating membranes.

The method consists of removing the ascites Sarcoma 180 cells from freshly killed mice, washing four times in cold Hepes buffered saline at pH 7.2, and centrifuging at 210 X g for three min. The supernatant solution, which contained ascites fluid and erythrocytes, was discarded. The pellet was suspended in 10 volumes of 40 mM Tris buffer at pH 7.4, and allowed to stand for three min to swell the cells. At the end of the designated time, the pellet was centrifuged at 1200 X g for two min. The supernatant solution containing hemoglobin and some erythrocytes was also discarded. The pellet of about 3 ml volume of swollen cells was suspended in 15 ml of 40 mM Tris buffer containing 1.0 mM $MgCl_2$ or $ZnCl_2$ and allowed to stand at room temperature for 15 min and then at 4 C for 15 min in order to "harden" the cell membranes. The cell suspension was then treated in a Dounce hand homogenizer with a loose pestle until microscopic examination revealed that about half the cells had been broken. An equal volume

of 40 mM Tris buffer was added, and treated with a slow centrifugation of 210 X g for three min to spin down nuclei and whole cells. The membranes in the supernatant were centrifuged at 1200 X g for 10 min for $ZnCl_2$ treated fractions or 2400 X g for 10 min for the $MgCl_2$ treated fraction. The supernatant solution which contains soluble cytoplasmic constituents was discarded. This separation yielded a supernatant solution which contained some mitochondria and a pellet that was rich in plasma membranes. The pellet was resuspended in Tris and layered onto a discontinuous sucrose density gradient, with layers of 8 ml each of 40%, 45%, 50%, and 55% sucrose for the $ZnCl_2$ treated membranes, or 35%, 40%, 45%, and 50% for $MgCl_2$ treated membranes, and centrifuged at 4.1×10^4 X g for 1 hr, or the pellet of membranes can be purified by suspension in a two-phase mixture of Dextran T500 and Carbowax 6000 and centrifuged at 8000 X g for 15 min. Either procedure yielded a band of purified tumor cell plasma membranes. All membrane preparations were frozen until used.

Membrane Quantitation

The amount of membrane material available for use as an antigen was determined by the Lowry protein assay (Lowry et al., 1951). The purity of the membranes was checked by chemical and enzyme marker assays using lactoperoxidase, NADH diaphorase and adenosine triphosphatase, and also by SDS acrylamide electrophoresis (Shin and Carraway, 1973).

Erythrocyte Membrane Isolation

A readily accessible membrane to be used as a non-tumor immune stimulus may be obtained from mouse or human erythrocytes. Whole citrated human blood was obtained from the Community Blood and Plasma Service in Birmingham, Alabama, and mouse blood was collected in Alsever's solution (Barta, 1974).

Membranes were obtained by the method of Triplett, Wingate, and Carraway (1972). The whole blood was centrifuged at 14,500 X g for 10 min in a Sorvall SS-34 rotor to separate the cells from the serum, and washed three times in 10 mM Tris buffer pH 7.4 with 0.15 mM NaCl. The layer of white cells on the surface of the pellet was removed after each wash. To effect lysis, the cells were suspended in 10 volumes of Tris buffer containing no NaCl, and allowed to stand for 10 min at 4 C, and centrifuged at 14,500 X g for 10 min. The hemoglobin was removed by six washes using two to three volumes of Tris buffer each time. These membranes were also frozen until used.

Indirect Fluorescent Antibody Test

The humoral response of a host to a tumor or an immunization can be checked using antibodies conjugated with fluorescein, in a method similar to that used by Möller (1961). The indirect method uses either conjugated goat anti-rabbit immunoglobulin or conjugated rabbit anti-mouse immunoglobulin, obtained from Cappel Laboratories Inc., Downingtown, Pennsylvania, and Sylvana Laboratory, Millburn, New Jersey respectively.

Whole Sarcoma 180 cells were obtained fresh from a host, washed

and centrifuged twice in cold Hepes buffered saline. The dilutions of antiserum to be tested were then added to the pellet and allowed to incubate at room temperature for 15 min. The reaction was stopped by washing twice in cold Hepes buffer after which the pellets were incubated with a standard dilution of the proper fluorescein conjugated antiserum. The unbound serum was removed with three more washes in Hepes buffer and the pellet was suspended in 1 ml of buffer and observed under 500X magnification with a special fluorescein isothiocyanate-specific filter system, and darkfield conditions. Control slides were made using either normal mouse serum or non-tumor cells or both, instead of the test tumor cells and the primary antiserum.

Dose - Response Experiment

In order to detect any improvedment in the survival capabilities of the host CD_1 (HaM/ICR) mice as a result of immunization, it was first important to delineate the ordinary tumor-host interaction.

Seven different groups of 15 mice each were administered doses of 1×10^2, 1×10^3, 1×10^4, 1×10^5, 1×10^6, 1×10^7, or 1×10^8 viable Sarcoma 180 tumor cells by intraperitoneal injection, and the survival time of each mouse was noted. The mean survival time for each group was determined.

Immunization Experiments

Immunizations with Tumor Cell Membranes

In the first immunization experiment, four groups of five CD_1 females about six weeks old were established. Each group received

intraperitoneal injections three times per week for two weeks, with the protein dosage set at 0.4 mg per injection. The first group was given $ZnCl_2$ treated tumor cell membranes, the second group $MgCl_2$ treated tumor cell membranes, the third group received human red blood cell membranes type O+, and the fourth group received 0.2 ml of physiological saline as a control system. On day 18 of the test, each mouse was given 1 X 10^6 viable tumor cells as a challenge. The survival time of each of the animals was recorded.

A second immunization experiment was designed modifying the parameters used in the first experiment. Five groups of five mice each were set up. The protein dose was increased, and the tumor challenge was decreased by a factor of 10. However, 0.8 mg of protein injected three times per week is very close to the dose which can cause immune paralysis and allow normal rate of tumor growth, that is, one mg three times per week as reported by Mitchison (1968). Therefore the first injection remained at 0.4 mg of protein, and 0.8 mg on five subsequent injections, and total were given twice per week for three weeks to allow the immune system to develop a full response before the challenge dose was given.

In this experiment one group received $ZnCl_2$ treated tumor cell membranes, the second group received $MgCl_2$ treated membranes, the third group 1 X 10^6 viable fetal mouse cells grown in cell culture (LM cells), the fourth group mouse red blood cell membranes, and the fifth group was given 0.1 ml of Hepes buffered saline on each injection. The survival time of each mouse was recorded.

Test for Cross-Reacting Antigens

Another experiment was designed to test human erythrocyte membranes of different blood groups for cross-reacting antigens with immunizing capabilities against Sarcoma 180.

Four groups of five mice each were established. Three of the groups received human erythrocyte membranes, 0.8 mg of protein three times per week for two weeks. The first group was given type B+, the second type O-, and the third group type A-. The fourth group served as the control and received 0.2 ml of physiological saline each injection. A challenge dose of 1×10^5 viable Sarcoma 180 cells was given each mouse on day 14 of the test. The time of death of each mouse was noted and recorded.

Titration of Immune Response

A study was designed to determine the immunocompetence of host mice which had been immunized with tumor cell membranes. All test mice were immunized with six injections of Sarcoma 180 membranes, 0.4 mg of protein on the first dose and 0.8 mg on the succeeding five doses, administered twice per week for three weeks. Control mice were given 0.2 ml of Hepes buffered saline each time. Four groups of ten mice each were given $ZnCl_2$ treated tumor cell membranes, another four groups of ten each were given $MgCl_2$ treated membranes, and four groups of five each served as control animals, injected with 0.2 ml of Hepes buffered saline each time. On day 25 of the test, one group from the $ZnCl_2$ membrane category, one from the $MgCl_2$ membrane category, and one from the control category were challenged with 1×10^4 viable Sarcoma 180 cells. Other groups from each category were challenged

with 1 X 10^5, 1 X 10^6, or 1 X 10^7 cells. The individual survival times were recorded, and group mean survival times calculated.

Titration of Antigen

To accompany the previous titration of immune response, a titration of antigenic material was designed. Smaller doses of membrane material were given in an effort to determine the minimum protein concentration that could be given while still achieving maximal results.

Five groups of 10 CD_1 mice were set up with one group receiving 0.25 ml of Hepes buffered saline each time. In reference to the schedule of one injection of 0.4 mg of protein, and five injections of 0.8 mg, a total of twice per week for three weeks, the four test groups were given either 1/2, 1/4, 1/8, or 1/16 of the standard doses of $ZnCl_2$ treated tumor cell membranes. Respectively, the 1/2 group received one injection of 0.2 mg and five injections of 0.4 mg of membrane material, the 1/4 group received one 0.1 mg injection, and five 0.2 mg injections. The 1/8 group received one 0.05 mg injection, and five 0.1 mg injections, and the 1/16 group received one 0.025 mg injection, and five 0.05 mg injections. A viable challenge of 1 X 10^5 Sarcoma 180 was given on day 23 of the test. Again the survival times of each mouse was recorded, and the group mean survival times calculated.

BDF_1-SA 180 Trial System

In an attempt to see if the methods used with the Sarcoma 180 and CD_1 tumor-host system were applicable to another system, the

standard regimen was applied to the inbred BDF_1 strain of mouse.

Sarcoma 180 tumor cell membranes isolated by $ZnCl_2$ treatment from cells grown in CD_1 mice were used as the antigen for a group of 10 BDF_1 females at six weeks of age. They were given 0.4 mg protein on the first dose and 0.8 mg for the following five doses which were administered twice per week for three weeks. A group of four control mice received 0.2 ml of Hepes buffer each time. On day 24 of the test, each mouse received 1×10^5 viable Sarcoma 180 cells. The survival times were noted.

Intramuscular Challenge

To see if the immunity conferred by this immunization technique was successful with a challenge at a site other than the peritoneal cavity, a mouse which had previously been shown to be immune, and a mouse with no previous treatment were both injected intramuscularly in the right rear leg with 1×10^4 viable Sarcoma 180 cells. After seven days, the mice were sacrificed, autopsied, and histological sections taken from the muscle to check for tumor growth and host reaction.

CHAPTER III

RESULTS

Dose - Response Experiment

The basic survival response of the CD_1 host mouse to different SA 180 tumor cell inoculation densities was determined in the Dose - Response Experiment. Figure 1 shows the survival time of each mouse, plotted as a function of per cent cumulative mortality versus time in hours after tumor injection, with the dotted line indicating the mean survival time for each group.

For convenience in estimating the expected survival time or the effective inoculum, on which to base data interpretation, the log of the tumor cell inoculation is plotted as a function of the mean survival time of each inoculation group (Fig. 2). Even though no injection of smaller than 1×10^2 cells was administered, it appears that an injection of one viable SA 180 cell would cause death of the host.

Seven days following tumor implantation, one mouse was sacrificed from each group, and the serum collected and heat inactivated. A fluorescent antibody test was done with a dilution of 1:100, and evaluated on a scale of 1--4. A control slide using normal serum was also made. The titer for each of the groups was the same, about +1, indicating that antibodies were present in the system of each of the mice, directed against the tumor, but the level was very low. The control slide showed no non-specific staining.

Immunization Experiments

Immunizations with Tumor Cell Membranes

The results of the first of the immunizations experiments (Fig. 3) is plotted as the per cent surviving in each group versus the time after tumor cell injection. Line (a) represents the conbination of the control groups which were immunized with physiological saline, human red blood cell membranes, or $MgCl_2$ treated SA 180 membranes. Line (b) represents the test group that received $ZnCl_2$ treated SA 180 membranes. The mean survival times for each of the groups in hours were, control groups: saline--290.9, human erythrocyte membranes--336.2, and test groups: $MgCl_2$ sarcoma membranes--292.1, and in the $ZnCl_2$ sarcoma membrane group for those animals that died, the mean survival time was 332.2. Note also that two of the five animals, or 40% in the $ZnCl_2$ sarcoma membrane group survived the challenge.

The results of the second immunization are presented in Figure 4. Line (a) represents the combined survivals of the control group which received LM cells, the control group which received CD_1 mouse erythrocyte membranes, and the control group which received Hepes buffered saline. The mean survival times of those groups were 306.7, 304.7, and 298.2 hrs respectively. Line (b) represents the survival of the $ZnCl_2$ treated sarcoma membrane group. Line (c) represents the survival of the $MgCl_2$ treated membrane group. Note that 60% or three of five mice were able to completely reject their tumor load in the $ZnCl_2$ treated membrane group, and that 80%, or four of the five mice in the $MgCl_2$ treated membrane group rejected their tumor challenge of 1×10^5 viable sarcoma. The mean survival time of the $ZnCl_2$ treated membrane

immunized mice that died was 372.3 hrs, but only one mouse of the $MgCl_2$ membrane group died which made that number insignificant.

Test of Long-Term Immunity

In order to prove that the survivors were actually immunized, and that the immunity lasted at least 30 days, each mouse from the first two immunization experiments was re-challenged with 1 X 10^5 viable SA 180 cells on the thirtieth day following the initial tumor cell challenge. All mice tested were able to reject the challenge, whereas control mice given the same injection died within the expected time period.

To test their long-term immunocompetence, mice which had been immunized with $ZnCl_2$ or $MgCl_2$ treated SA 180 membranes, and had survived a challenge of live tumor cells were re-challenged. Mice were used from groups that had received their last immunizing injection six months, five months, or three months before this challenge of 1 X 10^5 viable SA 180 tumor cells. Four control mice were also included which died within the expected time period, but again all of the test mice survived the challenge, and showed no tumor development.

Test for Cross-Reacting Antigen

The possibility of a cross-reacting antigen was noted with the results of the first tumor cell membrane experiment. Therefore in the cross-reacting antigen experiment, types B+, O-, and A- human erythrocyte membranes were tested for immunizing capabilities. Despite previous indications, no protective ability was seen since the survival times of all groups were similar to the control (Table I).

Titration of Immune Response

In order to evaluate the effectiveness of immunization with tumor cell membranes on the schedule of two injections per week for three weeks with one injection of 0.4 mg protein and five injections of 0.8 mg protein, groups were organized which were injected with $MgCl_2$ treated tumor cell membranes, and others were injected with $ZnCl_2$ tumor cell membranes. The results in table II show the capabilities of these animals to reject tumor challenges between 1×10^4 and 1×10^7 viable SA 180. Figure 5 shows the same results with line (a) representing the per cent survival of each group immunized with $MgCl_2$ treated tumor cell membranes versus viable tumor challenge dose.

Titration of Antigen

The results of the antigen titration experiment are presented in Table III. The mean survival times of each of the groups are comparable within statistical limits. Note that there was one mouse in both the 1/2 and the 1/4 group which were able to survive the tumor challenge. These values should be compared with the mean survival time and number of surviving mice achieved in the immune response experiment with the group which was immunized with $ZnCl_2$ treated tumor cell membranes and challenged with 1×10^5 cells (Table II). That group received 0.4 mg of membrane protein on the first injection, and five injections of 0.8 mg of protein.

BDF_1 - SA 180 Trial System

The results of this experiment which was designed to test the capability of initiating immunity in an inbred strain of mouse with

isolated plasma membranes, and to test the specificity of such an immune response, are shown in Table IV. Neither of the test groups showed any improvement in mean survival time over the control groups.

Intramuscular Challenge

An intramuscular challenge was undertaken in this experiment to demonstrate that an immune host could reject a tumor challenge given at a site other than the peritoneal cavity. An autopsy on day seven showed no trace of tumor in the muscle of the immune animal, and there was no evidence of enlargement of regional lymph nodes. However, in the control animal, tumor cells had infiltrated between the muscle fibers, and the regional lymph nodes were greatly enlarged.

Figure 1. Cumulative Mortality of HaM/ICR (CD_1) Mice Following Injection of Different Cell Concentrations of SA 180

Seven groups of 15 mice each were challenged with viable SA 180 at concentrations of 1 X 10^2 through 1 X 10^8 cells. The number of animals dead from each group plotted as the per cent cumulative mortality is shown as a function of time following the tumor challenge.

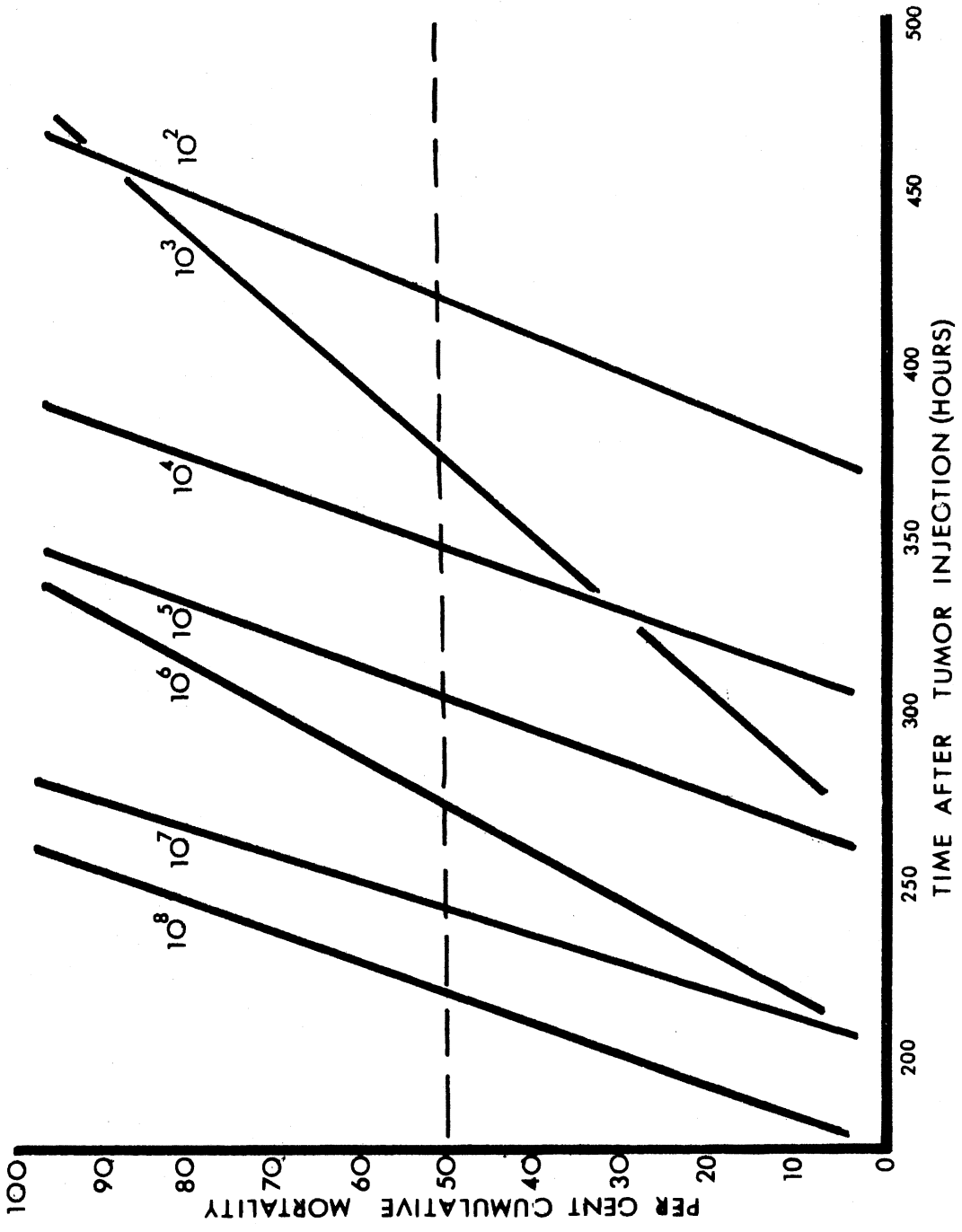
PER CENT CUMULATIVE MORTALITY
100
90
80
70
60
50
40
30
20
10
0
200
250
300
350
400
450
500
TIME AFTER TUMOR INJECTION (HOURS)
10^8
10^7
10^6
10^5
10^4
10^3
10^2

Figure 2. Tumor Cell Inoculation Concentration as a Function of the Host Mean Survival Time

Data derived from the Dose - Response experiment in which each point plotted represents the mean survival time of a group of 15 mice.

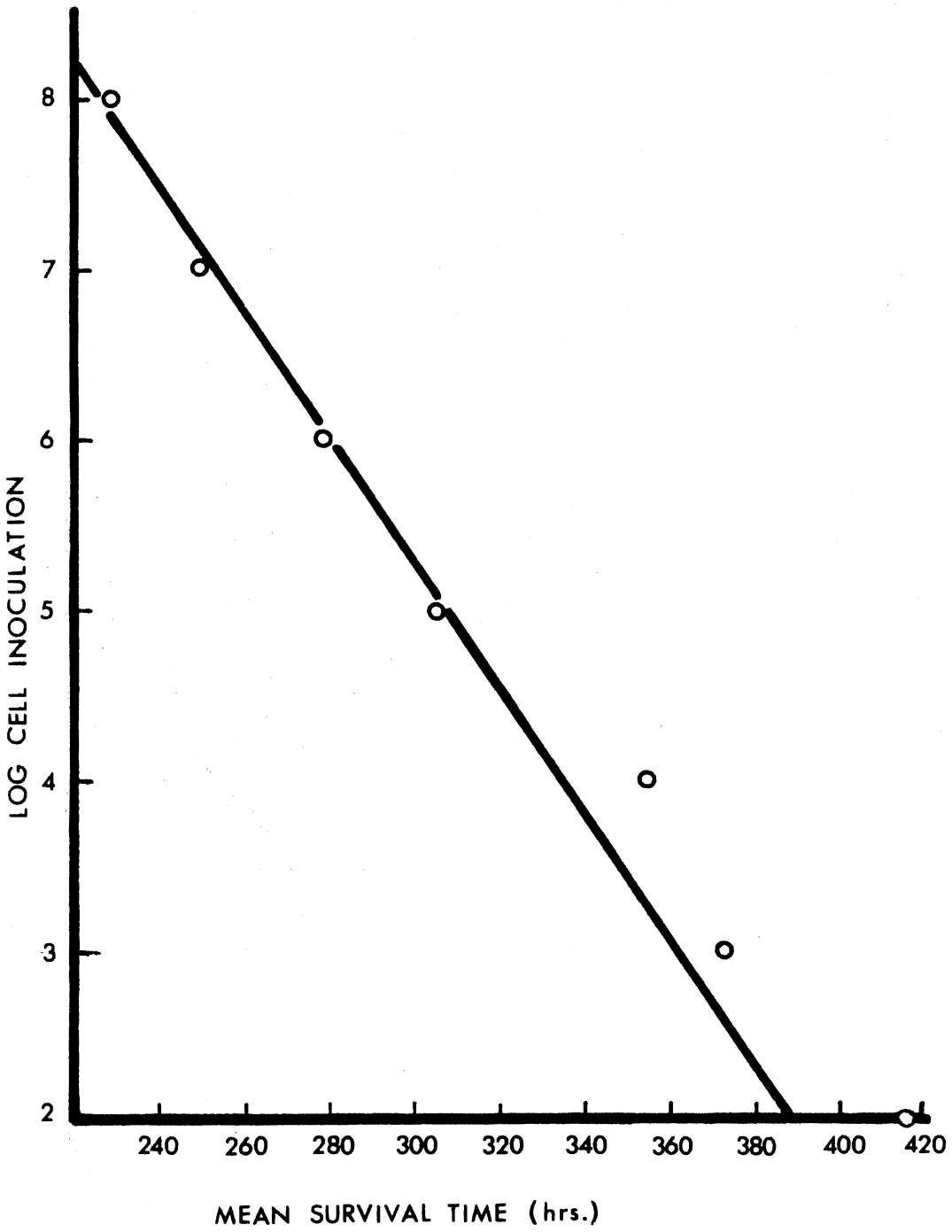
LOG CELL INOCULATION
8
7
6
5
4
3
2
240
260
280
300
320
340
360
380
400
420
MEAN SURVIVAL TIME (hrs.)

Figure 3. Per Cent of Animals Surviving At Times Following Injection of 1 X 10^6 SA 180 cells in the First Tumor Cell Membrane Immunization.

Group (a) received physiological saline, human erythrocyte membranes, or $MgCl_2$ treated SA 180 membranes. Group (b) received $ZnCl_2$ treated SA 180 membranes. Each immunization category was made up of five mice. All animals were challenged with 1 X 10^6 viable SA 180 cells.

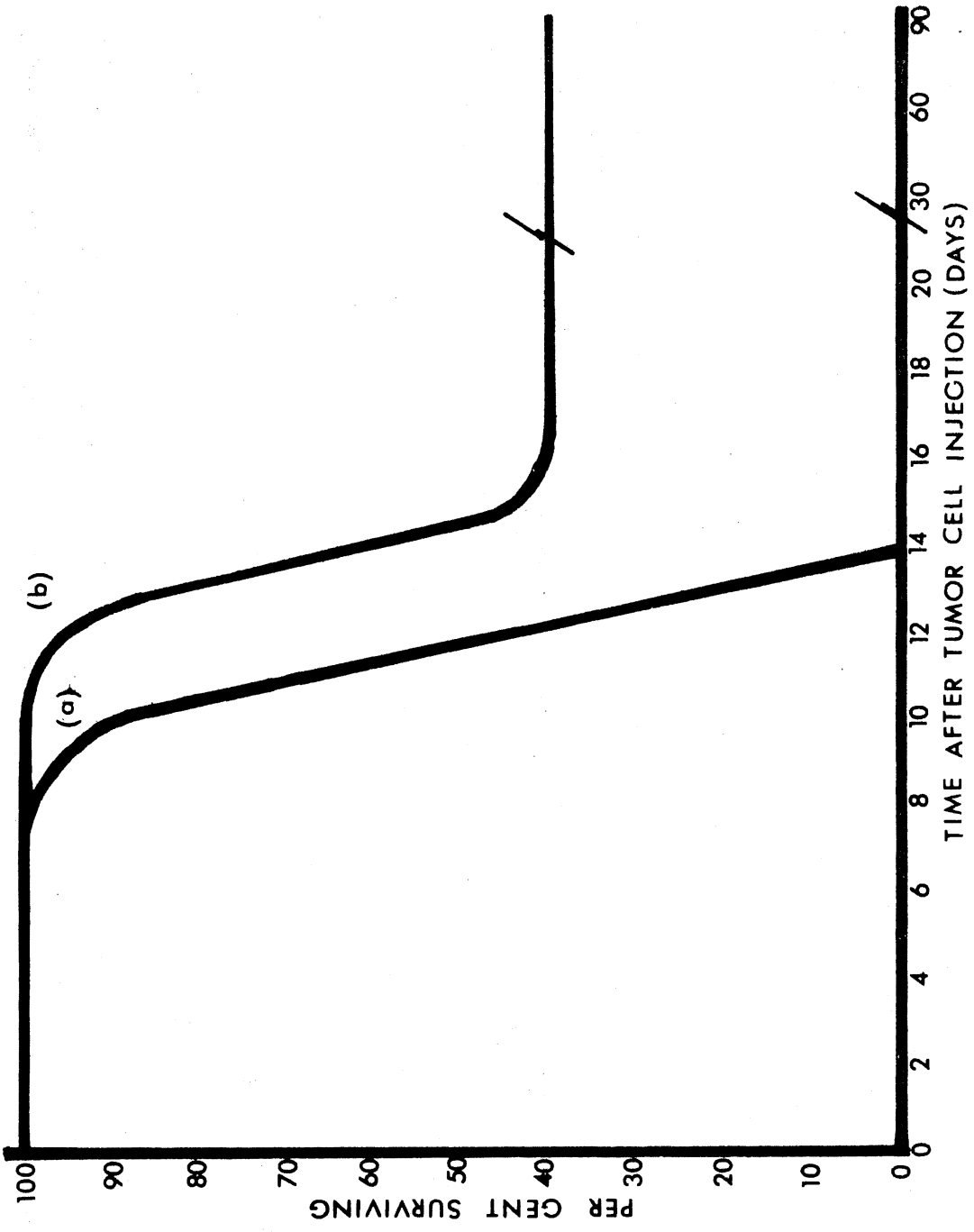
PER CENT SURVIVING
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
18
20
30
60
90
TIME AFTER TUMOR CELL INJECTION (DAYS)
(a)
(b)

Figure 4. Per Cent of Animals Surviving At Times Following Injection of 1 X 10^5 SA 180 Cells in the Second Tumor Cell Membrane Immunization.

Group (a) received mouse erythrocyte membranes, LM cells, or Hepes buffered saline. Group (b) received $MgCl_2$ treated SA 180 membranes. Group (c) received $ZnCl_2$ treated SA 180 membranes. Each immunization category was made up of five mice. All animals were challenged with 1 X 10^5 viable SA 180.

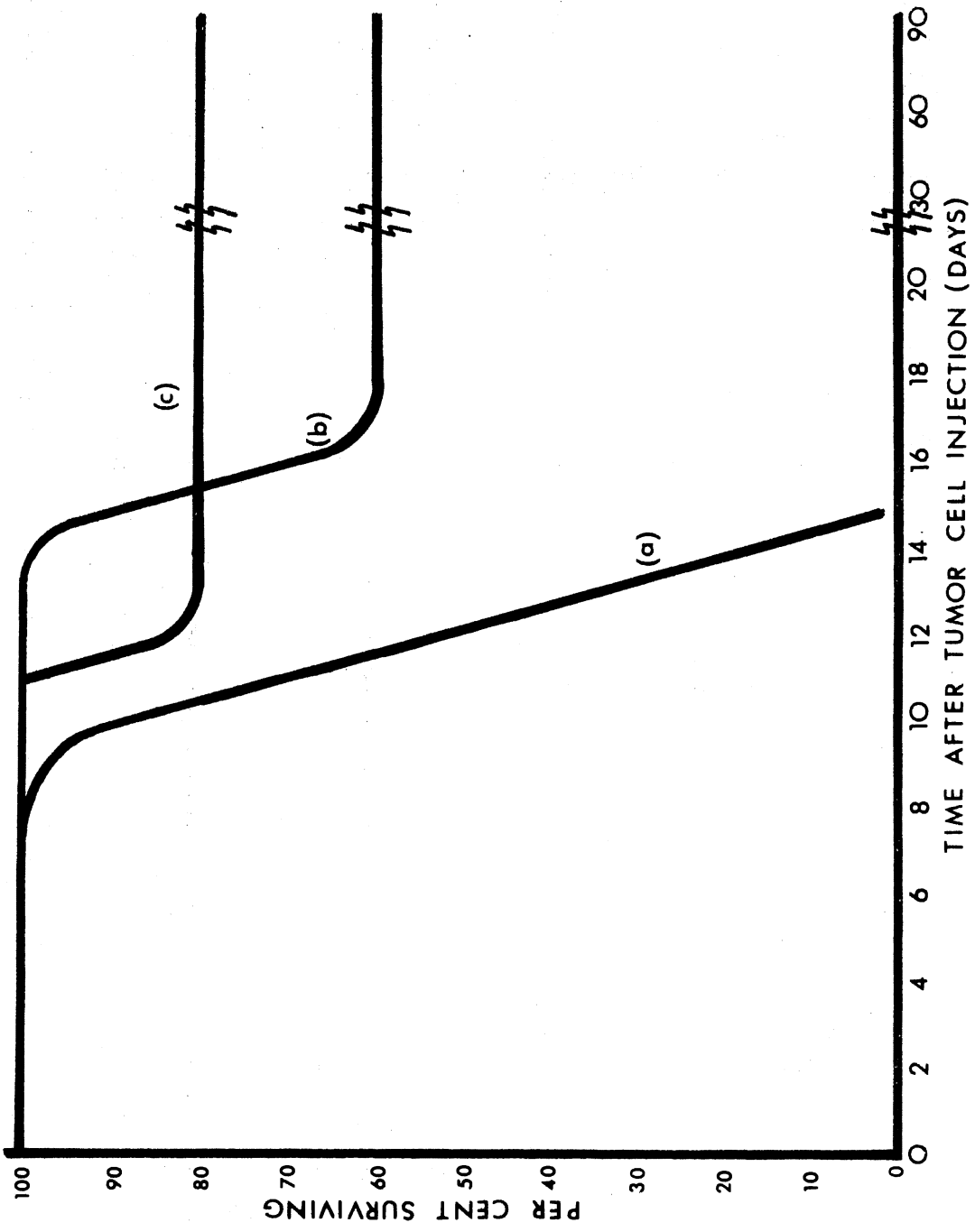
PER CENT SURVIVING
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
18
20
30
60
90
TIME AFTER TUMOR CELL INJECTION (DAYS)
(a)
(b)
(c)

Figure 5. Immune Titration.

All animals in line (a) were immunized with $MgCl_2$ treated SA 180 membranes. All animals in line (b) were immunized with $ZnCl_2$ treated SA 180 membranes. Tumor challenge is listed on the x-axis. There were 40 mice in each of the tumor membrane immunized categories and 20 control mice, which gives a total of 100 mice. No control mice survived any tumor challenge. Animals are labeled "survivors" if they live for 30 days after tumor injection.

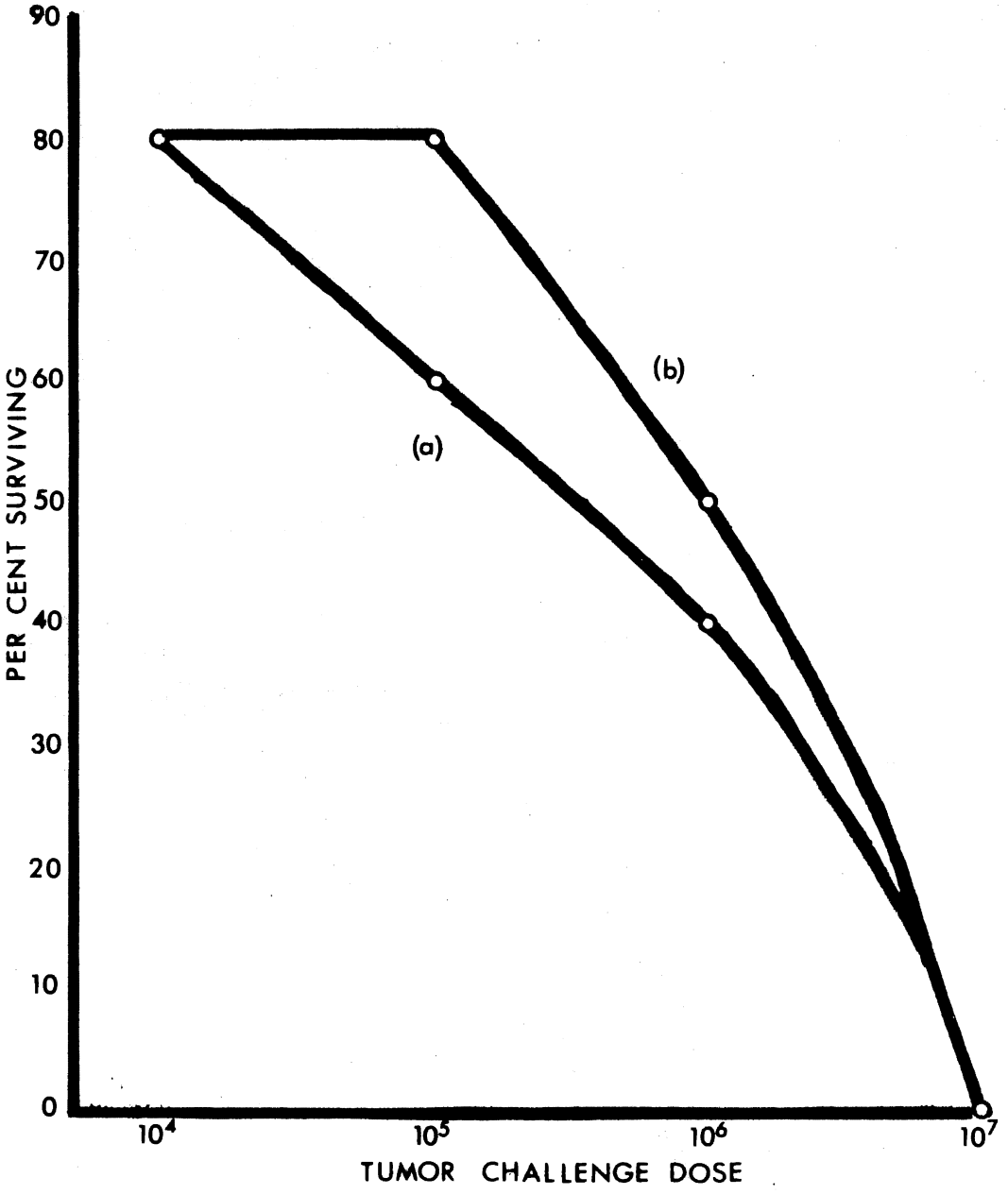
90
80
70
60
50
40
30
20
10
0
PER CENT SURVIVING
(a)
(b)
10^4
10^5
10^6
10^7
TUMOR CHALLENGE DOSE

TABLE I

MEAN SURVIVAL TIMES OF MICE IMMUNIZED WITH HUMAN ERYTHROCYTE MEMBRANES

	Immunizing Agent			
	Physiological Saline	Type B+	Type O-	Type A-
Mean Survival Time (hours)	356.7	329.2	317.3	358.8

Each animal was challenged with 1 X 10^5 viable SA 180 after the immunization procedure.

TABLE II

TITRATION OF MEMBRANE IMMUNIZATION BY CHALLENGING MICE WITH SA 180 AT CONCENTRATIONS OF 10^4 THROUGH 10^7

	Number of Viable SA 180 Injected Per Mouse							
	10^4		10^5		10^6		10^7	
IMMUNIZING AGENT	%S	MST	%S	MST	%S	MST	%S	MST
$ZnCl_2$ treated SA 180 Membranes	80	364	80	385	50	315	0	296
$MgCl_2$ treated SA 180 Membranes	80	391	60	368	40	305	0	259
Hepes Control	0	355	0	323	0	265	0	240

%S--Per cent of animals surviving 30 days following SA 180 challenge.

MST--Mean survival time (hours) for those animals in each group which died.

TABLE III

TITRATION OF ANTIGEN - MEMBRANE MATERIAL

Amount of Immunization

	control	1/2*	1/4*	1/8*	1/16*
MST	327.8	335.3	327.1	345.2	340.8
%S	0	10	10	0	0

*The fraction represents the portion of the schedule of one injection of 0.4 mg and five injections of 0.8 mg of $ZnCl_2$ treated SA 180 membranes that each mouse received.

TABLE IV

BDF$_1$ IMMUNIZATION WITH SA 180 $ZnCl_2$ TREATED MEMBRANES CHALLENGED WITH SA 180 OR WITH L1210

	Challenge	
	1 X 10^5 SA 180	1 X 10^5 L1210
Test Group[a]	235.8	202.8
Control Group[b]	313.6	193.5

[a]Both test groups immunized with one injection of 0.4 mg and five injections of 0.8 mg of $ZnCl_2$ treated SA 180 membranes

[b]Control groups received six injections of 0.2 ml of Hepes buffered saline.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

The CD_1 - SA 180 tumor - host system shows a similarity to the human tumor situation. The CD_1 strain of mouse shows more relevance to a human population since it is an outbred strain. One disadvantage of using an outbred strain of mouse is that each individual responds differently to antigenic stimulation. There is also some possibility that during growth, a transplantable tumor expresses a slightly different antigenic configuration in each animal.

The Sarcoma 180 being a tumor showing an apparently reduced number of histocompatibility antigens is one that allows more direct experimentation with tumor associated transplantation antigens because their effect is not masked by the more strongly expressed histocompability antigens.

In regard to the dose - response experiment, and by looking at the time needed for a ten-fold increase in cell number, the cell generation time can be determined as being about 9.4 hrs. Taking together the initial inoculum, the cell generation time, and the corresponding host survival time, it was determined that tumor burden at time of death is fairly constant regardless of inoculation size. The exact burden at time of death cannot be determined because of altered cell kinetics at higher cell densities. The dose - response experiment is a guideline for determining the success of later experiments

using immunization and challenges. The Fluorescent Antibody test performed on day seven in the Dose - Response experiment demonstrates the development of a weak immune response in all hosts with a tumor load between 1 X 10^4 and 1 X 10^7 cells. However, such a response cannot be termed effective since regression has not been observed in any normal host.

In comparison of the first and second tumor cell membrane experiments, the reasons for the increased number of survivors in groups immunized with $ZnCl_2$ and $MgCl_2$ treated membranes are evident. First, the protein dosage was increased from 2.4 mg to 4.4 mg. Secondly, the immune stimulation was administered over a period of three weeks, and thirdly, the tumor challenge was decreased by a factor of 10.

In the first tumor membrane experiment, a 40% survival rate was achieved utilizing $ZnCl_2$ treated tumor cell membranes as an immunizing agent. In the second experiment, using altered parameters, a 60% survival rate was seen using $ZnCl_2$ treated tumor cell membranes, and an 80% survival rate was seen using $MgCl_2$ treated tumor cell membranes. Obviously these results indicate that the isolated plasma membranes exhibit antigens accessible to the immune system of the host that are also present on a viable tumor cell surface.

Also in the second tumor membrane experiment, whole viable LM cells were tested for possible exhibition of fetal antigens that might be present on SA 180 cells. The mean survival time of the group suggested that no immunizing capability was present. However, Coggin and Anderson (1974) stated that no immunization could be demonstrated with whole viable fetal cells, because the cells proceed to mature inside a host. Immunization can only be demonstrated by using

X-irradiated fetal cells between 10 and 11 hrs of development after fertilization.

Human erythrocyte membranes were tested for the possibility of a cross-reacting antigen with Sarcoma 180 in the third experiment. Even though a lengthening of the survival time similar to animals immunized with $ZnCl_2$ treated tumor cell membranes was indicated by immunization with human red blood cell membranes type O+ in the first tumor membrane experiment, no success was seen in immunizing hosts against SA 180 with membrane types B+, O-, and A-. Although type O+ was not retested, very little possibility remains that any cross-reacting antigen is present on human erythrocyte membranes. However, the membranes used in the first experiment may have been biologically "different" enough to cause a non-specific stimulation of the immune system.

The titration of immune response experiment showed that with each challenge dose, animals immunized with $ZnCl_2$ treated tumor cell membranes were more capable of rejecting their tumor challenge, or were capable of a longer survival time (Table II). The results are consistent with the first experiment in which more survivors were achieved with $ZnCl_2$ treated membranes than with $MgCl_2$ treated membranes. The results of the second experiment appear inconsistent with this conclusion, but the difference between the groups was only one animal out of five, and the immune titration experiment, using 10 mice per group probably presented a more reproducable result.

Also evident from the results of the immune titration was the fact that mice which had received identical immunizations were more capable of rejecting or partially eliminating smaller tumor

challenge doses than large ones.

The antigen titration experiment was an attempt to achieve successful immunizations using smaller doses of immunizing material than had previously been tried. The results indicate that the schedule which had been adopted beforehand was the best compromise between reliability and economy, that is, injecting $ZnCl_2$ treated tumor cell membranes with one injection of 0.4 mg protein and five injections of 0.8 mg a total of twice per week for three weeks. A group which was immunized in such a manner in the immune titration demonstrated a survival rate of 80% against a challenge of 1×10^5 viable SA 180, whereas the next lower group in the antigen titration which received 1/2 as much protein dosage of membranes on each injection achieved only a 10% survival rate.

The next experiment was an attempt to immunize the inbred BDF_1 strain of mouse with $ZnCl_2$ treated tumor cell membranes and one group was challenged with 1×10^5 SA 180 and another group was challenged with 1×10^5 L1210 cells. The challenge with SA 180 cells was an attempt to check the immunization of an inbred strain of mouse against an allogeneic tumor, and the challenge with L1210 was to check the specificity of the immune response which was elicited by injection with sarcoma membranes.

However, there was no lengthening of survival time in the group challenged with Sarcoma 180, and also no lengthening of survival time in the group challenged with L1210. Therefore any possible cross-reactivity between the tumors was not evident since no immunity was demonstrated against SA 180. The reason for the lack of immunization was not immediately evident since the Sarcoma 180 tumor is not

strain specific and should have expressed as much antigenic "foreignness" in the BDF_1 host as in the CD_1 host. It does seem possible however, that a tumor might express different antigens in response to each type of host that it grows in, and the fact that the membranes used in this experiment were isolated from cells grown in CD_1 hosts might affect the outcome of the experiment.

A tumor challenge at a site other than the peritoneal cavity such as the intramuscular challenge experiment is important because of two reasons. First, the peritoneal cavity is the area in which the immunizing injections were administered, and successful rejection of a tumor at that sight might not mean the host is also capable of rejecting tumors from sites where immunization was not given, and secondly, the peritoneal cavity is an immunologically privileged site where macrophages and lymphocytes are in constant circulation.

Therefore, the results of the intramuscular challenge is a direct indication of the ability of the host to reject a tumor at any site. The autopsy of the control host showed enlarged regional lymph nodes and tumor infiltration of muscle tissue which indicated that the presence of the tumor had been detected by the immune system, but the reaction did not stop tumor growth. The absence of any lymph node enlargement or presence of any tumor cells in the immune host indicated that the animal mounted an effective immune response, and all tumor cells had been eliminated before the autopsy on day seven.

The immunity conferred by the procedures mentioned above are probably of the long-term type since animals which survived an initial tumor challenge at the end of each experiment were re-challenged with 1×10^5 viable SA 180 30 days after the first challenge. Also mice

were selected from previous experiments that had survived tumor challenges three months, five months, and six months before a final challenge of 1 X 10^5 SA 180. In every instance, each host was capable of rejecting the tumor challenge.

In conclusion, it has been shown that plasma membranes isolated from Sarcoma 180 tumor cells by the method of Shin and Carraway (1973) can be administered to host CD_1 (HaM/ICR) mice on schedules and quantities to induce immunity against a viable tumor challenge.

It has also been shown that neither non-tumor control membranes such as ones isolated from human or mouse erythrocytes nor whole viable LM cells induce any immunity when administered in a regimen identical to the one used for tumor cell membranes.

Results also indicated a distinct and repeatable difference in the success achieved when immunizing animals with sarcoma membranes isolated using $ZnCl_2$ compared with membranes isolated using $MgCl_2$. In most instances $ZnCl_2$ treated membranes are superior for immunizing capacity, but a survival rate of 80% against an intraperitoneal tumor challenge of 1 X 10^5 SA 180 has been achieved using both types.

Furthermore, the resulting immunity has been shown to be long-term type, lasting at least six months, and it has been shown to be effective against tumor challenges at sites other than the peritoneal cavity such as in a muscle.

It seems plausible that isolated tumor cell membranes could be used in remission therapy of cancer patients. If after surgery, membranes could be isolated from directly removed cells, or cultured cells they could be administered to the patient to induce immunity to eliminate any remaining cells at the primary, or possible metastatic sites.

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