ISOLATED TUMOR CELL PLASMA MEMBRANES AND MODIFIED WHOLE TUMOR CELLS AS ANTICANCER IMMUNIZING AGENTS

By

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Bachelor of Science

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1975

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
MASTER OF SCIENCE
December, 1976

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ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to my major adviser, Dr. Norman N. Durham, for his guidance, counsel, understanding and patience throughout the course of the investigations and in the preparation of this thesis.

I also want to thank Drs. George V. Odell, Jr., Norman N. Durham, and Mark Sanborn for their valuable time spent as members of the advisory committee and for their contributions towards the preparation of this thesis.

The author wishes to especially thank Drs. Bob Chestnut and John Huggins for their valuable help and advice, as well as Sandy Cantwell, Tim Trenbeth, Glendon Jett and Pam Moore for their assistance in some of the work included in this study. I want to also thank Kevin Castleberry for his excellent drafting work included in this thesis.

Special thanks is given to Dr. Lawrence Evans in the Department of Medicine and Surgery at the College of Veterinary Medicine for the irradiation of the tumor cells used in one immunization.

Financial assistance furnished by the Oklahoma State University Microbiology Department is deeply appreciated.

Finally, special appreciation is extended to my husband, Ron, and our parents, Mr. and Mrs. E. D. Edmisten and Mr. and Mrs. W. H. Neafus, Jr. for their continual encouragement, understanding and financial help and sacrifices that made this study possible.

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NOMENCLATURE

C	degree centigrade
g·min	gravity times minutes
HBS	Hepes Buffered Saline
hrs	hours
i.p.	intraperitoneal
L1210	Leukemia L1210 cells
mg	milligram
ml	milliliter
min	minutes
mM	millimolar
%	per cent
PBS	Phosphate Buffered Saline
SA-180	Sarcoma 180 ascites tumor cells

CHAPTER I

INTRODUCTION

General Immunology

A contemporary definition of the term immunity would be "all those physiological mechanisms which endow the animal with the capacity to recognize materials as foreign to itself and to neutralize, eliminate or metabolize them with or without injury to its own tissues" (7).

The responses of immunity may be classified into two categories: nonspecific immunologic responses and specific immunologic responses. Specific immune responses depend on prior exposure to an antigen and the subsequent recognition of and reaction to it. On the other hand, nonspecific responses occur following initial and subsequent exposure to an antigen, and while selective in differentiating "self" from "non-self", are not dependent upon specific recognition (7). These immunologic responses serve three major functions: defense, homeostasis and surveillance. The first is involved in resistance to infection by microorganisms, the second, in removal of effete (worn out) self components, and the third, with the detection and destruction of mutant cells. Failure of surveillance, which recognizes abnormal cell types which constantly arise within the body either spontaneously or induced by certain viruses and chemicals, has been assigned a causal role in the development of malignancy (7).

Lymphocytes are fairly small (5-15 um diameter), round, nondescript cells that are ubiquetous in blood, lymph and connective tissues. The two fundamentally different kinds, B and T cells, differ in origin, in surface macromolecules, in circulation patterns, and above all, in the mode and consequences of their interaction with antigens (17).

Besides circulating through blood, lymph, and tissue spaces, lymphocytes are aggregated into primary and secondary lymphatic structures, where different stages in their differentiation are carried out. In the primary organs (thymus, bursa of Fabricius or its analog in non-avian species) lymphocytes become committed to react specifically with particular antigens, and in the secondary lymphatic organs the committed cells react with antigens, which stimulate their terminal differentiation with different functions. The B cells differentiate into antibody-secreting plasma cells, and T cells become effectors of cell-mediated immunity; also, both cell types differentiate into their respective memory cells (17).

Specific Immunity

The specific immune responses are concerned with the recognition of foreign traits in a highly discriminatory way. Results of the subsequent reaction between host and foreign configuration depend on properties of the substance (size, structure, amount) and also on the properties of the host (age, genetic make up) (7). The specific immune response is the host's subsequent reaction to a foreign substance and encompasses a series of cellular interactions expressed by the elaboration of specific cell products. There are three general characteristics

of the specific immune response which distinguish it from the nonspecific response: specificity, heterogeneity and memory.

Specificity is the highly discriminatory selectivity with which the products of the immune response will react solely with the configuration identical or similar to that which caused the initial response (7). Nonspecific responses represent the initial encounter with foreign traits, which upon subsequent encounter merely repeat the same general response to that substance. Therefore, they lack specificity. Specificity is the character of the immune response which distinguishes one antigen from another (7).

The second characteristic of the immune response is heterogeneity, in which a wide array of cell types and cell products are induced to interact with a diversity of responses with the variety of cell types. Unlike the nonspecific response of phagocytosis in which there is a limited number of pre-existent cell types, the specific responses are characterized by the induction and interaction of a variety of new cell types specific for the inducing antigen (7). Heterogeneity of the cell types, T and B cells, gives rise to elaboration of an equally heterogeneous population of cell products. This heterogeneity of antibody contributes a fine degree of homeostatic control with which the host may respond in a highly variable and specific manner with foreign structures (7).

The third property of the specific immune response is that of anamnestic response or memory. Memory results in augmentation of the response through proliferation and differentiation of cells upon subsequent exposure to an immunogen. This leads to an increased elaboration of cell products. The nonspecific immune responses do not include

the property of memory (7).

The lymphoid cells of the immune system have the ability to react specifically with antigens and to elaborate specific cell products. The lymphoid cells include plasma cells and lymphocytes. These cells, once sensitized, become "committed" and are referred to as immunocytes. The two main functions of lymphocytes are antibody production and cell-mediated interactions, as directed by thymic-independent and thymic-dependent influences, respectively. It is believed that the thymus influence leads to the production of cells equipped to handle cell-mediated events. The lymphocytes considered to be thymic-independent, and whose known function is concerned with antibody synthesis are also part of the recirculating pool of lymphocytes.

Mature B plasma cells have been shown to store and release antibody and are believed to be of primary importance in antibody synthesis. While the B cell lymphocyte is involved in antibody synthesis, the T cell produces a variety of factors which trigger inflammatory or cell-damaging reactions leading to cell-mediated events. These factors include the migration inhibitory factor (MIF), a cytotoxic factor capable of injuring a variety of cell types, interferon and several other factors whose biological roles are not yet well defined. Some are released upon interaction of sensitized B lymphocytes with appropriate antigens; others may remain cell-bound. In either case, they lead to the destruction of foreign target cells or to the damage and destruction of host tissues. Thus, it is apparent that the lymphocytes possess the most diversified function of all cells of the immune system (7).

The induction of antibody formation by many immunogens requires specific interaction with both B and T cells, with the T cells somehow regulating the proliferation and differentiation of B cells into antibody-secreting plasma cells (17).

Studies have shown that the active cells in bone marrow and thymus are precursors of B and T cells, respectively, and that the antibody-secreting cells are derived from the marrow and not from the thymus cells (17). B and T lymphocytes look alike and both are mobile, nonphagocytic cells of varying size.

Specific binding of antigen by membrane-bound receptors on the cell surface can stimulate transformation of small B cell lymphocytes into larger ones whose more abundant cytoplasm contains endoplasmic reticulum and a prominent Golgi apparatus, and is richer in mitochondria and polysomes. In accordance with the appearance of a secretory system (endoplasmic reticulum, Golgi apparatus) some of the large lymphocytes of B type secrete antibodies. The large cells also divide more rapidly and some of those of B lineage differentiate into mature plasma cells, the most active of all lymphoid cells in synthesis and secretion of immunoglobulins. Many large lymphocytes also revert back eventually into small ones, which probably function as "memory cells". Small lymphocytes (except for a few exceptions) rarely divide unless stimulated by antigen (17).

Transplantation experiments in chickens have shown that B cells arise from migrant bone marrow stem cells (primitive precursors of hematopoietic and lymphoid cells) that lodge in the bursa of Fabricius where they begin to synthesize immunoglobulins. In mammals, which lack

a bursa, it is not certain where the B cell precursors become committed to synthesize a particular immunoglobulin. This could happen in the bone marrow itself or in lymph nodes or lymphoid structures, such as the tonsils or appendix (17).

T lymphocytes also originate from bone marrow stem cells, which migrate to the thymus where they divide rapidly. Most of the rapidly dividing cells die without leaving the thymus. The survivors, mature T cells, differ from entering stem cells in several important properties that are acquired in the thymus, or possibly in other tissues under direction of a thymus hormone. They develop characteristic surface antigens. They can react specifically with one or a few antigens and they become antigen-sensitive (immunologically committed) lymphocytes. Besides acquiring the capacity to regulate B cell responses to antigens, specifically reactive T lymphocytes can become specific effector cells for cell-mediated immune responses. They can destroy tumor cells, cause rejection of allografts, and promote the differentiation of resting macrophages into highly bacteriocidal cells capable of destroying bacterial pathogens. T cells are heterogeneous and it is likely that T cell effectors of cell-mediated immune reactions differ from those that regulate the differentiation of B cells into antibody-secreting cells (17).

T cells exercise their effects both through contact with B cells and the release of diffusible factors that act at short range on nearby B cells. Thus T cells can aid B cells when they react at the same time and in the same locale. The highest antibody yields are obtained when cooperating B and T cells are specific for determinants on the same immunogenic particle. There seem to be two diffusible factors: a low-molecular-weight (dialyzable) nonspecific substance that enhances the

response of B cells of any specificity and a high-molecular-weight (non-dialyzable) specific factor that augments only those B cells that can react specifically with the same immunogen that activated the T cells release of the factor (17).

Though T cells exercise an important regulatory role on responses of B cells to T-dependent immunogens, it is important to note that antigens can induce B cells, in the absence of T cells, to make antibodies of the IgM class, and to differentiate into memory cells whose response to a subsequent introduction of the immunogen elicits the augmented secondary response. However, the further maturation of the primary response, usually characterized by the production of IgG antibodies, seems not to occur unless T cells are engaged (17). With antigens whose induction of antibody synthesis in primary spleen cell culture requires accessory T cells, there is a further requirement for adherent cells, probably macrophages, and a complex of B and T cells bind to the antigen on the sticky surface of the macrophage. While each of the cooperating B and T cells react specifically with an antigenic determinant of the immunogen, the macrophages act nonspecifically (17).

Antibodies combat the antigens in one of several ways. They may combine with the antigen and complement and neutralize them; they may cause the invading microorganism to break up and dissolve (a phenomenon known as lysis); or they may make the invaders more susceptible to phagocytosis (63).

The two effector mechanisms which mediate specific immune responses are those mediated by a cell product of the lymphoid tissues referred to as antibody (humoral immunity) and those mediated by specifically

sensitized lymphocytes themselves (cell-mediated immunity or delayed hypersensitivity). Antibody is the product of the lymphoid series which is either cell-bound or secreted as an extracellular product. It has the capability of reacting with the configuration responsible for its production (7). The cell-mediated response is the second major effector mechanism underlying specific immunity. It is recognized to be a response important in recovery from many infectious diseases and important in surveillance against neoplasms (7). The effector mechanism seems to be monitored by the thymus and mediated by the thymic-dependent processes (7). Cell-mediated reactions are initiated by the recognition of antigen by the cell surface receptor on the lymphocyte. The morphologic changes of lymphocytes consist of blast cell formation and subsequent mitosis. Several agents have been known to induce these changes (mitogens, Bacillus Calmette Guerin (BCG), Corynebacterium parvum, tumors invading an organism, antibodies against foreign structures) (7, 8, 21, 35, 50, 67).

Nonspecific Immunity

Nonspecific immunity involves an inflammatory response and phagocytosis (7). The first encounter of the host with a foreign configuration leads to a stereotyped response. This consists of the mobilization of phagocytic elements into areas that a foreign configuration has been introduced. This can occur as an isolated event or as a part of the inflammatory response (7). Once mobilized, the phagocytic cells mount an attack on their target by a process called phagocytosis. It requires recognition of the material to be ingested, movement toward the object (chemotaxis), and ingestion and subsequent intracellular digestion by

lysosomal products. It includes utilization of humoral antibodies (opsonins) and enhancement of intracellular metabolic events.

Following injury or invasion, systemic events are also triggered, which involve fever and a series of hematologic phenomenon (7).

Tumor Immunity

The host possesses both specific and nonspecific mechanisms of response to tumor formation. In tumor rejection, as in protection from infecting agents, the host immune response is directed toward the maintanence of homeostasis, the tendency to maintain uniformity or stability in the internal environment of the organism. This homeostasis may be altered toward the establishment of the tumor or in favor of the host (7). Most investigators believe that the host's immune system plays a major role in the defense against neoplasms (10, 12, 14, 24, 25, 29, 52, 53). The immunogenicity of the cell surface is extremely complex because of the large number of expressed and potentially expressed antigenic determinants (14).

Surgery, radiotherapy and chemotherapy are all vital techniques required to reduce tumor burden, but none of these has the sufficient specificity to recognize and discriminately destroy widely distributed metastatic cells.

Infection of a newborn animal with DNA or RNA viruses may lead to development of tumors because of immunologic immaturity of the newborn (7). Such nonspecific factors as phagocytosis, chemotaxis, and the inflammatory response are incompletely developed in the young host (7).

The fundamental role of cell-mediated immunity is clearly shown by the ability to transfer a tumor immune response from an immune to a

nonimmune animal by adoptive transfer of lymphoid cells. Also, mixing tumor cells in vitro with sensitized lymphocytes leads to the destruction of tumor cells by the lymphocytes. Thymectomized animals, patients with thymic-dependent immunologic deficiencies and patients who have been immunosuppressed, all show an increased incidence of malignancy (6). In patients with Burkitt's lymphoma and malignant melanoma, a loss of tumor-specific cell-mediated immunity has been demonstrated in those who were in the active phase of disease. After removal of these malignancies, there was a corresponding return of tumor-specific delayed hypersensitivity (7). Cell-mediated immunity is a primary response to tumor antigens. Cell-mediated immunity is the prime mode of destruction of tumor cells (7, 9, 10).

The distribution and concentration of antigens found on the tumor cell surfaces vary with the type of tumor (7). There appears to be a relationship between the relative concentration of antigen and the responsiveness of tumor tissue to antibody (7).

Tumor-specific antibodies, in the presence of complement, lyse tumor cells by perforation of the cell membrane. Most are of the IgG class (7, 10). However, both circulating antibodies and cell-mediated immunity are implicated in tumor growth and regression (9, 10). Experimental evidence indicates that circulating antibodies against tumor antigens may play a role in prolonging tumor survival. Humoral antibodies may protect cells from destruction by immune lymphoid cells by blocking receptor sites on target cells, which masks the antigenic sites to which immune B lymphocytes are directed (9). So, in some cases, immunization of experimental animals with tumor cells or tumor antigens

causes that animal to produce circulating antibodies that block cytolytic effects of B lymphocytes on the tumor and allows the growth of tumor cells (9, 56). Cellular immunity, though, plays the major role in the rejection of cancer (9, 56). Chemotherapy is not able to eradicate most tumors even when cytostatic drugs are given in large, toxic doses. Also, many patients with localized tumors relapse after apparently complete surgical resection or radiotherapeutic destruction, indicating that these two weapons often leave residual disease. Therefore, a search for treatments using the host's own immune system to kill the last tumor cell has been promoted (37).

Tumor immunology is 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 in use. First, it is the body's own natural defense, and second, it can reach all areas of the body. In instances of viral and bacterial infection, the immune system is responsible for the final clearing of the infective agents from the host.

Tumor immunology became feasible with the demonstration that most 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) and they may or may not cross react with antigens of other tumors of the same type (10). Probably 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 (43).

The primary goal of immunotherapy is to use the host's immune system to prevent manifestation of the tumor by specifically destroying all

neoplastic cells even though these cells are disseminated throughout the host's body. Evidence indicates that tumor destruction could be accomplished by immunization with modified tumor cells, whose surfaces have been altered to enhance tumor-specific immunogenicity without provoking autoimmune rejection of normal host tissue (54).

In addition to the search for superior immunizing agents, one must also consider the immunological capabilities of the host prior to tumor development (normal status), during active tumor growth, and at different times following anticancer therapy. Many aspects of the immunological scheme are only partially resolved. The humoral and cell-mediated components, once believed to be separate and rather independent faculties of the immune system, now appear to vary in their degree of interaction from nearly independent activity against some types of stimulation to very close interdependence in reaction to other stimuli (12, 14, 24, 25, 45). Once the tumor cell population reaches a mass of between 10^3 and 10^4 cells in mice, it appears to be sufficiently large to nullify those immunological responses of the host capable of inhibiting tumor growth (29). Investigators now believe that autosolubilized antigens, mostly of fetal origin, are released from the tumor cell. These antigens, free in circulation, are then able to interact with the host's lymphoid cells causing the loss of killer activity. These soluble antigens may also stimulate the production of specific immunoglobulins which apparently cover the tumor surface and protect it from immune destruction (14, 24, 25). Therefore, tumor burden must be reduced below this nullifying level to allow immunotherapy to proceed. Surgery, radiotherapy or chemotherapy are therefore required to reduce tumor burden, but they must be

timed correctly to allow the host's immune system to fully benefit from these treatments (38).

When rabbit antisera prepared against extracts of gastrointestinal tract carcinomas are absorbed with extracts of normal intestinal mucosa, the residual antibodies react with an antigen (a glycoprotein with 50% carbohydrate; MW 1 x 10^5 to 2 x 10^5) that is present not only in gastrointestinal mucosa, liver and pancreas (which are endodermal derivatives of the gut). This carcinoembryonic antigen (CEA) or fetal antigen appears to be specified by a normal gene that is expressed transiently in endodermal cells during fetal development, and in adult life if these cells undergo malignant change (17).

Serum from nearly all patients with colon or rectal adenocarcinoma contains CEA, and many also contain antibodies to CEA. Both disappear with successful surgical removal of the cancer, and reappearance of CEA can be the first diagnostic clue to the tumor's recurrence. With widespread metastases, the CEA concentration rises and anti-CEA disappears (masked by antigen excess). With highly sensitive radioimmunoassays that can detect 1 ng, CEA or a substance that cross-reacts with it has also been detected in sera of some persons with carcinoma of lung or breast. Other fetal antigens are associated with certain other human cancers: alpha-fetoprotein is found in serum of patients with hepatomas or embryonal carcinomas, gamma-fetoprotein, a serum protein of fetal blood, is found in subjects with various types of cancer. The detection in adult serum of fetal antigens, by sensitive and rapid serological tests, promises to provide screening assays for the early detection of human cancers (17).

With the demonstration of antigenic differences on tumor cell surfaces, it was proposed that 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 whole tumor cells prior to immunization regimes. Cells can be injected into the host mixed with a nonspecific immune stimulant such as BCG (6, 19, 29). Treatments such as X-irradiation at doses sufficient to kill tumor cells (30), treatments with Vibrio cholera neuraminidase which cleaves sialic residues from the membrane surface (10, 29, 62), repeated freeze-thawing, exposure to iodoacetamide which blocks sulfhydral groups (13, 27, 42), and mitomycin C, an inhibitor of DNA synthesis which may or may not lead to membrane alterations (45, 46), can enhance tumor cell immunogenicity in certain tumor-host systems. These findings encourage the notion that efficient and effective immunizing agents do exist and can be obtained through suitable manipulation (37).

Cell surface phenomena 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 (14).

Even though internal cellular membranes may present antigens similar to external ones, it is the plasma membrane of the tumor cell which comes in contact with the immune surveillance system of the host. Also, the plasma membrane is thought to play an important role in processes such as cohesiveness and contact inhibition (42).

It is most likely that tumor cell membranes can be made more antigenic and effective immunizing agents by the enzymatic and/or chemical modifications similar to those described for whole cells. The use of membranes as immunizing agents is very desirable and represents a logical extension of current research efforts for several important reasons. First, the cell membrane surface is that portion of the tumor cell which interacts with the host's immune system. Second only tumor material contains the specificity required for effective tumor rejection. Third, since the host's immune system has a finite response capability which has already been diminished by tumor burden, it should be presented with the maximum quantity of surface immunogen (cell membrane) while minimizing the addition of non-surface antigen (DNA, RNA, enzymes, mitochondria, etc.). The immune system of a mouse is paralyzed by administration of greater than 1 mg irradiated BSA protein given 3 times a week for 3 weeks (40). Fourth, the administration of membranes minimizes the risk of infecting the host with some viruses (intracellular nonmembrane-associated) or other foreign agents which reside in cells both in vivo and in vitro and often remain active after the cells become non-viable.

The hypothesis was that certain modifications of whole tumor cells (cells treated with $ZnCl_2$, mitomycin C, or X-irradiated cells) and isolated $ZnCl_2$-treated tumor cell membranes enhance the immunogenicity of these immunizing agents, and lead to the development of a more effective tumor-specific immunotherapy agent.

CHAPTER II

MATERIALS AND METHODS

Tumor-Host System

The animal hosts used for all immunizations and used for growing some of the tumor cells were white mice of an outbred strain designated HaM/ICR (CD_1) originally obtained from the Charles Rivers Mouse Farms, Wilmington, Mass. They were isolated for two weeks when received, and given food and tap water ad libitum. Some were retained as breeders and their offspring used in later experiments. The mice used in experiments were at least six weeks of age. In some experiments, the animals used for growing the SA-180 cells were black BDF_1 mice of both sexes. They were obtained from Sprague-Dawley Laboratory, Madison, Wisconsin, and used at an age of about six weeks. The BDF_1 is an inbred mouse strain.

Sarcoma 180 ascites (SA-180) tumor cells originated as a carcinoma, a malignant tumor made up of connective tissue enclosing epithelial cells. It was originally grown as a solid tumor but was adapted to grow as an ascites cell sometime before 1919 (31). The tumor has been maintained at Oklahoma State University since 1965 by weekly intraperitoneal (i.p.) injections of approximately 1×10^5 cells in 0.1 ml of Hepes (N-2 hydroxyethyl piperazine-N'-2, ethane sulfonic acid) buffered saline (HBS) in HaM/ICR (CD_1) mice of both sexes. After seven days,

approximately 8 x 10^7 cells were recovered from the peritoneal cavity of the mouse by aspiration, washed with HBS and used for reinjection.

This tumor may possibly show fewer histocompatibility antigens than most cells because of its ability to grow in different strains of mice. This is important because the histocompatibility antigens are usually the most strongly expressed antigens. Their absence allows a more detailed examination of reactions with tumor-associated and fetal antigens.

Procedures for Stock Solutions

All solutions used were made up in doubled distilled (dd) water.

Hepes Buffered Saline (HBS)

8.00 gms NaCl
0.40 gms KCl
0.10 gms Na_2HPO_3
1.00 gms Dextrose
2.30 gms Hepes
Add H_2O q.s. 1 liter (1.)
Adjust to pH 7.4 with NaOH and autoclave or filter.

Phosphate Buffered Saline (PBS) (pH 7.2)

24 ml of 0.15 M KH_2PO_4
76 ml of 0.15 M Na_2HPO_4
100 ml of 0.15 M NaCl
Autoclave or filter and store at 4° C.

The following solutions were maintained: 0.15 M KH_2PO_4, 0.15 M NaCl, 0.15 M Na_2HPO_4.

40 mM Tris (hydroxymethyl) aminomethane (Tham) MW 121.14

Stock solutions of 0.1 N HCl and 0.2 M Tris were prepared. These were used to prepare the various tris buffer solutions.

0.01 N HCl

0.2 M Tris

0.05 M Tris

0.04 M (40 mM) Tris

Preparation of Two-Phase Reagent Stock Solutions

Stock solutions of two-phase reagents (500 ml) were made of 30% w/w Carbowax 6000 (Applied Sciences Laboratories, Inc., State College, Pa.) and 20% Dextran T-500 (Pharmacia, Uppsala, Sweden) and mixed with 0.2 M $NaPO_4$ buffer (pH 6.5) and 10 mM $ZnCl_2$. The mixture was allowed to stand overnight in a separatory funnel in the cold room (4° C). The next morning, the upper phase and lower phase were separated into individual bottles, autoclaved and stored in the cold until used.

30% Carbowax 6000

20% Dextran T-500

10 mM $ZnCl_2$

0.2 M $NaPO_4$ Buffer (pH 6.5)

For this buffer 500 ml each of 0.2 M $NaPO_4$ and 0.2 M Na_2PO_4, pH 6.5 are made and mixed together until pH 6.5 is reached.

The following volumes of the various solutions were combined into a large beaker, mixed and allowed to settle in the cold overnight. It separated into two phases, a lower phase and an upper phase.

77.4 gms 30% Carbowax
150.0 gms 20% Dextran
249.9 ml 0.2 M $NaPO_4$ buffer (pH 6.5)
60.0 ml 10 mM $ZnCl_2$

Preparation of Glycine-EDTA (Ethylenediaminetetra-acetic Acid) and Glycine-EDTA-2, Mercaptoethanol

Various stock solutions (5 mM glycine, 1 mM EDTA and 5 mM 2, mercaptoethanol) were used to prepare the solution of glycine-EDTA-2, mercaptoethanol. The glycine-EDTA solution was made by omitting the 2, mercaptoethanol.

5 mM Glycine and 1 mM EDTA

Glycine (0.375 gms) and EDTA (0.336 gms) were added to 1 liter water. The pH was adjusted to 8.6 with either HCl or NaOH before the addition of mercaptoethanol.

5 mM 2, Mercaptoethanol

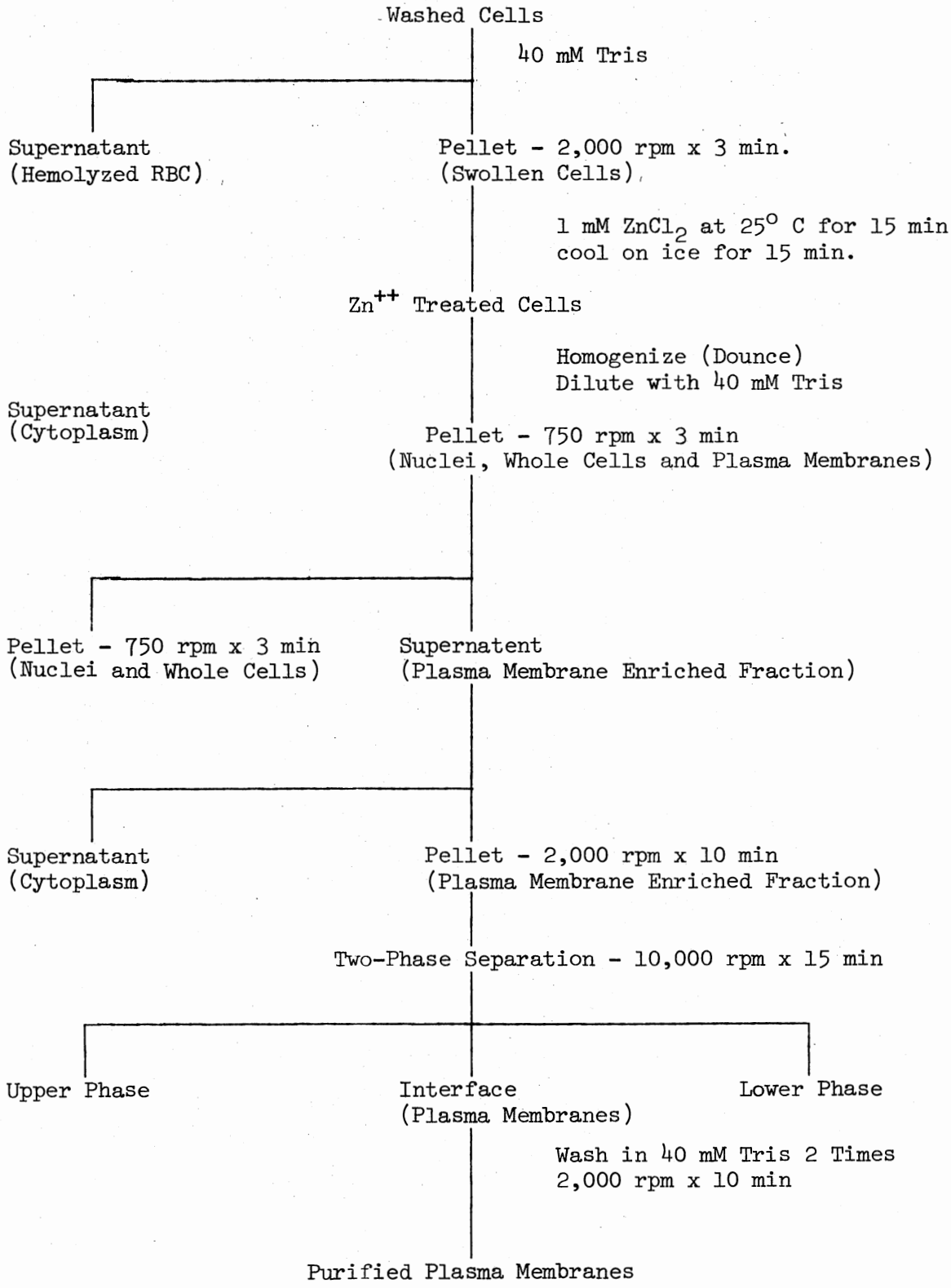
For the solution used in the third extraction of membranes with EDTA, 2, mercaptoethanol (0.078 gms) was added to 1 liter of the prepared glycine-EDTA solution. The solution was sterilized when used at room temperature.

Isolation of SA-180 Plasma Membranes by a Zn^{++} Stabilization Method

The agent used as the immune stimulus in most of the experiments was SA-180 tumor cell plasma membranes isolated by a procedure described by Shin and Carraway (1973), using a modification of the Warren method (1969). (See Figure 1.) Tumor cells were collected in a laminar flow hood.

Figure 1. Membrane Isolation Scheme for the SA-180 Tumor Using the Stabilization Technique

Details of the isolation are given in the methods section.


Washed Cells
40 mM Tris
Supernatant
(Hemolyzed RBC)
Pellet - 2,000 rpm x 3 min.
(Swollen Cells)
1 mM $ZnCl_2$ at 25° C for 15 min
cool on ice for 15 min.
Zn^{++} Treated Cells
Homogenize (Dounce)
Dilute with 40 mM Tris
Supernatant
(Cytoplasm)
Pellet - 750 rpm x 3 min
(Nuclei, Whole Cells and Plasma Membranes)
Pellet - 750 rpm x 3 min
(Nuclei and Whole Cells)
Supernatent
(Plasma Membrane Enriched Fraction)
Supernatant
(Cytoplasm)
Pellet - 2,000 rpm x 10 min
(Plasma Membrane Enriched Fraction)
Two-Phase Separation - 10,000 rpm x 15 min
Upper Phase
Interface
(Plasma Membranes)
Lower Phase
Wash in 40 mM Tris 2 Times
2,000 rpm x 10 min
Purified Plasma Membranes

Washed ascites SA-180 cells from 20-25 freshly killed mice were suspended in 10 volumes of cold 40 mM Tris and the cells allowed to swell for 3 minutes at 4° C. The swollen cells were centrifuged at 1446 g·min (2000 rpm x 3 min) in a Sorvall SS-34 head. This procedure hemolyzes erythrocytes and leaves their ghosts in the supernatant solution which is discarded. The pellet of swollen cells was suspended in 10 volumes of 1 mM $ZnCl_2$ at 25° C for 15 min and cooled in an ice bucket for an additional 15 min in order to "harden" the cell membranes. The cell suspension, which appeared swollen but intact under phase contrast microscopy, was then homogenized in a Dounce hand homogenizer (about 13-18 strokes) until microscopic examination revealed that most of the cells had been broken, the membranes were visible as sheets and intact envelopes and the nuclei were not ruptured. An equal volume of 40 mM Tris buffer was added to the homogenate solution. The homogenate was subjected to slow centrifugation (210 g·min) (750 rpm x 3 min) in an SS-34 head to spin down nuclei and whole cells. The whole cells were homogenized and centrifuged a second time to produce a higher yield of membranes. The membranes, which were left in the supernatant, were pelleted at 1200 g·min (2000 rpm x 10 min) in a Sorvall HB-4 head. The supernatant solution, which contained soluble cytoplasmic constituents, was discarded. The pellet, rich in plasma membranes and containing a few contaminating nuclei, was washed once more in 10 volumes of 40 mM Tris and centrifuged at 4820 g·min (2000 rpm x 10 min) in a Sorvall HB-4 head.

Purification by the Two-Phase Method

The washed pellet of crude plasma membranes was purified by partition between two immiscible phases composed of dextran and polyethylene glycol (Carbowax 6000) polymers in a phosphate buffer with 1 mM $ZnCl_2$ as described by Brunette and Till. The pellet was suspended in 4 volumes of the upper phase, mixed with 4 volumes of the lower phase and centrifuged at 244 Kg·min (10,000 rpm x 15 min) in a Sorvall HB-4 swinging bucket head to separate the two phases. The interface band of membranes was further purified by repeating the two-phase separation. The interface band, composed of large plasma membrane fragments, was decanted into a new tube and washed twice in 40 mM Tris at 4820 g·min (2000 rpm x 10 min) in a Sorvall SS-34 head. The purified membrane sheets were suspended in 10 ml 40 mM Tris and a Lowry protein assay performed to determine protein concentration (32). The membranes were frozen at -30° C until used.

Protein Quantitation Determined by the Lowry Method

The protein concentration of membrane or whole SA-180 cell protein was determined by the method of Lowry et al. (32).

Reagents

Folin Reagent = 50 ml Folin A + 0.5 ml Folin B-1 + 0.5 ml Folin B-2

Folin A = 2% Na_2CO_3 in 0.1 N NaOH

Folin B-1 = 2% Na Tartrate
Folin B-2 = 1% $CuSO_4$:5 H_2O

2 N Phenol Reagent

BSA Protein Standard = 500 ug/ml

Sample Preparation for the Lowry Protein Analysis

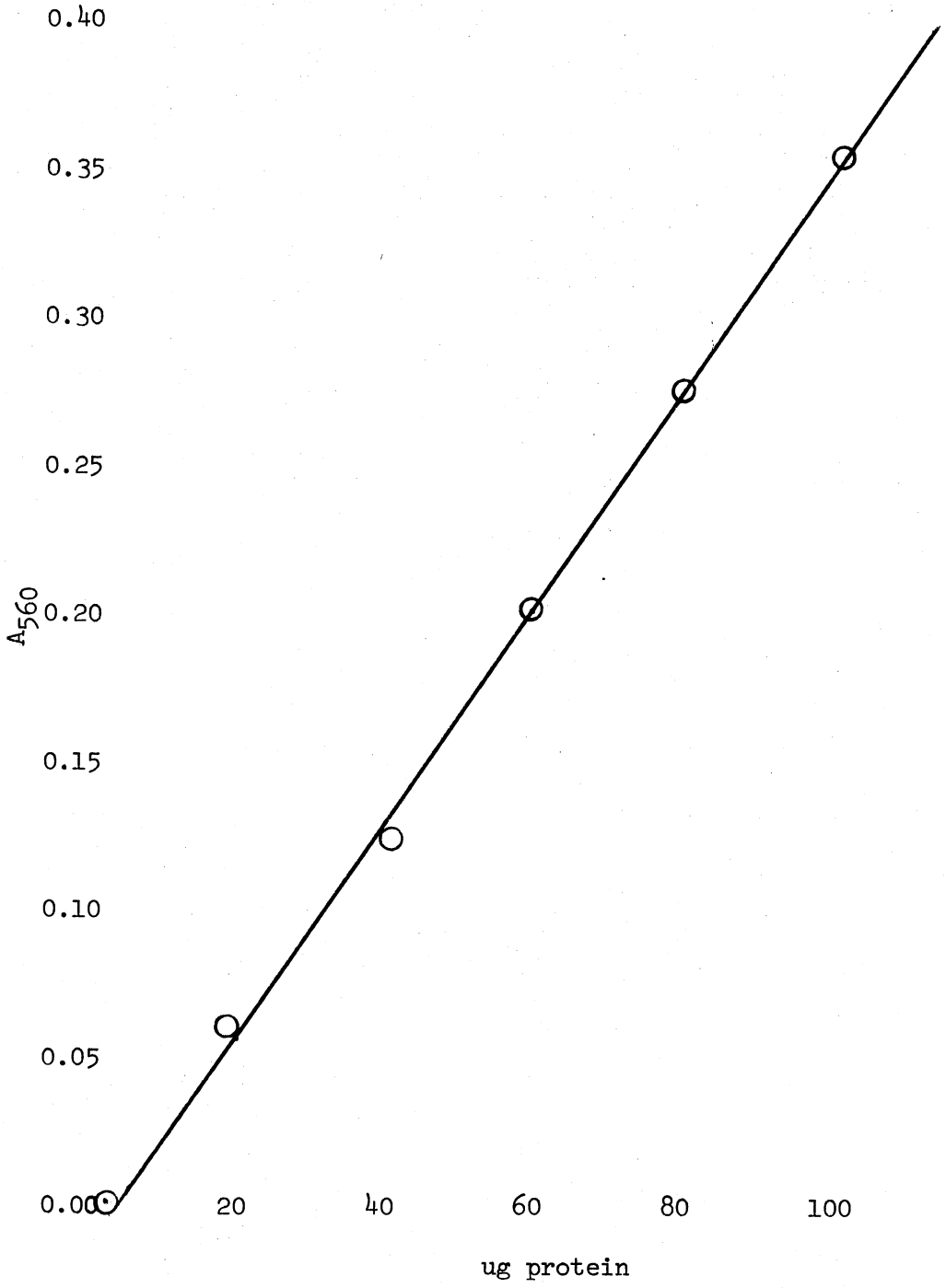
Sample could be solubilized by making to 4% in SDS (16% SDS, 5 mM EDTA, 1 mM PO_4 pH 7.4) and boiling sample for 10 min with a marble on top to stop evaporation. The solution was cooled and samples removed. Originally samples were solubilized, but results indicated that this was not necessary since the procedure itself solubilizes, and in later experiments it was omitted. One 5 ul, 10 ul and 20 ul samples were taken for the assay. All samples were made to 0.50 ml with H_2O. A standard curve was constructed with varying amounts of standard BSA protein and H_2O as seen in Figure 2.

Assay Procedure

The procedure is timed with a stopwatch to keep operations at the same time interval. To each tube add 2.0 ml Folin reagent. Wait 10 min (time is critical) and add 0.100 ml 2 N Phenol reagent. Vortex immediately. Wait 30 min and read at Absorbance 560 nm against a 0 ug BSA standard as blank.

The protein concentrations of the immunizing agents were determined by the Lowry method. Samples of membranes or treated whole tumor cells were suspended in HBS to the desired protein concentration for the injections in the immunization scheme.

Figure 2. Standard Curve for Lowry Protein Assay


0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
A_{560}
20
40
60
80
100
ug protein

Immunization of CD_1 Mice with Zn^{++} Treated SA-180 Cell Membranes Isolated from Tumor Cells Grown in BDF_1 Mice

The subjects used in all immunizations were HaM/ICR (CD_1) mice. The immunization schemes used in all experiments were devised to take full advantage of the capacity of the host's immune system. In general, the scheme consisted of 2 injections (i.p.) per week for 3 weeks. The protein dose for the first injection was 0.4 mg protein per mouse in the immunized group. The 5 subsequent injections contained 0.8 mg protein per mouse. The total protein given per mouse in the immunized group was 4.4 mg. The control mice received an equivalent volume of HBS. This scheme was chosen to allow the immune system to develop a full response before the challenge dose of viable tumor was given 1 week after the last injection. The general immunization scheme is shown in Table I and a summary sheet of the immunizing agents is given in Table II.

Membranes were isolated from cells grown in BDF_1 mice by the Zn^{++} two-phase stabilization method. Membranes isolated in this fashion will hereafter be designated as $ZnCl_2$ or Zn^{++} treated SA-180 membranes. A Lowry protein assay was performed and the membranes alliquoted into one tube of HBS-membrane solution containing 4 mg protein and 5 tubes containing 8 mg protein. The immunization scheme previously described was followed. The 7 mice in the immunized group and the 10 mice in the control group were challenged i.p. with 1.1×10^4 viable SA-180 tumor cells. One month following the initial challenge, the survivors were rechallenged with 1.25×10^5 live SA-180 cells per animal and the

TABLE I

GENERAL IMMUNIZATION SCHEME FOR MICE WITH MODIFIED SA-180 TUMOR MATERIAL

IMMUNIZED GROUP	CONTROL GROUP
Immunizing Agent	Hepes Buffered Saline
0.4 mg/mouse	equivalent volume
0.8 mg/mouse	equivalent volume
0.8 mg/mouse	equivalent volume
0.8 mg/mouse	equivalent volume
0.8 mg/mouse	equivalent volume
0.8 mg/mouse	equivalent volume

TABLE II

SUMMARY SHEET OF IMMUNIZING AGENTS

Immunizing Agent*	Cell Source Mice CD_1	Cell Source Mice BDF_1	Zn^{++} Trt.	Special Treatment	Admin. w/1st Injection Frozen	Admin. w/1st Injection Unfrozen
SA-180 Membranes (BDF_1 grown cells)		X	X		X	
SA-180 Membranes (Group 1 extracted)	X		X	Extracted w/ EDTA-glycine	X	
SA-180 Membranes (Group 2 extracted)		X	X	Extracted w/ EDTA-glycine	X	
SA-180 Membranes (Group 3 extracted)		X	X	Extracted w/ EDTA-glycine-2, mercaptoethanol	X	
Immunotherapy w/SA-180 Membranes (Group 1)		X	X	Preceeded w/a live 2.5×10^2 SA-180 inj.	X	
Immunotherapy w/SA-180 Membranes (Group 2)		X	X	Preceeded w/a live 2.0×10^2 SA-180 inj.	X	
Whole SA-180 Zn^{++} Cells (Group 1)	X		X			X
Whole SA-180 Zn^{++} Cells (Group 2)	X		X	4 mg protein given for 1st injection	X	
Whole SA-180 Zn^{++} Cells (Group 3)	X		X		X	
Whole SA-180 Zn^{++} Cells (Group 4)	X		X			X
Whole SA-180 Zn^{++} Cells (Group 5)	X		X		X	
Mitomycin C Trt Cells	X			Trt w/Mit. C	X	
Irradiated SA-180 Cells	X			Irradiated	X	

*All agents were given in the same schedule. The immunizing regimine used consisted of 0.4 mg protein per mouse for the first injection, and 0.8 mg protein per mouse for the 5 subsequent injections. The challenge dose of live tumor cells was given 1 week after the last immunizing injection in all immunizations. For the immunotherapy groups, the challenge was given 24 hours before therapy began.

survival times checked. Four months following the second challenge, those survivors were injected with 1 x 10^5 viable SA-180 cells and the survival times noted.

Immunization with Zn^{++} Stabilized SA-180 Plasma Membranes Extracted with Glycine-EDTA and Preliminary Evidence of Extraction with Glycine-EDTA-2, Mercaptoethanol

Another experiment involved extracting Zn^{++} stabilized SA-180 membranes with glycine-EDTA (in immunizations 1 and 2 with glycine-EDTA extracted SA-180 membranes) or with glycine-EDTA-2, mercaptoethanol (in immunization 3 with extracted membranes). In this type of isolation, small membrane vesicles are formed without the high-molecular-weight proteins that are thought to stabilize the membranes (27). If the proteins on the membrane are held together by disulfide bonds, the 2, mercaptoethanol will disrupt the bond and allow the protein to open up. For every mg of protein present in the isolated Zn^{++} two-phase membrane pellet, 2 ml glycine-EDTA (-2, mercaptoethanol in immunization group 3 with extracted membranes) solution was added. The membrane-glycine-EDTA (-2, mercaptoethanol) solution was mixed overnight at 4° C. The solution was next centrifuged in an SS-34 head at 945 Kg·min (15 K rpm x 35 min). The pellet of extracted membranes was suspended in a small volume of 40 mM Tris and the protein concentration determined as described earlier. The extracted membranes were alliquoted into concentrations for injections and the immunization scheme previously mentioned was followed. One week after the last injection, all mice

were challenged i.p. with a viable SA-180 tumor dose. The survivors were rechallenged one month following the initial challenge with a live tumor dose. In immunization 1 with EDTA-extracted membranes, the challenge was 1×10^4 SA-180 cells per animal and the rechallenge for all but one of the survivors was 1×10^6 live tumor cells. The one animal not injected with 1×10^6 cells was challenged with 1×10^5 tumor cells. For the second group of mice immunized with EDTA-extracted membranes, the challenge for all animals and the rechallenge for those survivors was 1×10^5 viable SA-180 cells. In group 3, the animals were challenged with 1.25×10^5 SA-180 cells per mouse.

Immunization with Zn^{++} Treated SA-180 Cells

Whole cells of SA-180 from 2 freshly killed animals were washed free of serum and red blood cells in HBS at 210 g·min (750 rpm x 3 min in an SS-34 head). Ten volumes of cold 40 mM Tris was added to the pellet of washed cells, and the cells were allowed to swell in the cold for 3 min. The cells were centrifuged at 1446 g·min (2000 rpm x 3 min), 10 volumes of 1 mM $ZnCl_2$ was added to the pellet, mixed and allowed to stand at room temperature for 15 min. The mixture was then placed in ice for an additional 15 min. The Zn^{++} treated cells were centrifuged at 210 g·min (750 rpm x 3 min in an SS-34 head). Ten ml of 40 mM Tris was added to the pellet and the protein concentration determined. The cells were divided for injection and the immunization scheme described was followed. One week after the last injection, all mice were challenged with live SA-180 cells. One month following the initial challenge, the survivors were rechallenged with viable SA-180 cells.

In immunizations 1 and 4 with Zn^{++} treated whole SA-180 cells, the modified cells were not frozen before the first injection was given, whereas in immunizations 2, 3 and 5 with Zn^{++} whole cells, the first injection was frozen before administered. In all immunizations with Zn^{++} treated SA-180 tumor cells, the 5 subsequent injections were frozen before injected. For group 1, the challenge given to all animals was 1×10^4 live SA-180 cells per animal. The rechallenge for the survivors was 1×10^6 cells per animal. In group 2, the challenge was 1.3×10^5 cells per animal. In group 3, the challenge was 1×10^5 cells per animal, and in groups 4 and 5 the challenge was 1.1×10^5 viable SA-180 cells per animal.

Immunization with Whole Irradiated SA-180 Cells

Whole SA-180 cells, harvested from HaM/ICR mice were washed and resuspended in HBS to a final concentration of 1×10^7 cells per ml. The cell suspension (10 ml) was placed into Falcon culture dishes and the open dishes were irradiated with U. V. light for 30 min at 50 rad/min (1500 rads total). The cells were stored 1 ml per tube (1×10^7 cells) in the freezer and were used to immunize mice. Each mouse in the immunized group received 10^6 irradiated cells in each of the 6 i.p. injections, with a total of 6×10^6 cells administered per animal. The control group received equal volumes of HBS. All mice were challenged with 1×10^6 viable SA-180 cells per mouse 1 week after the last injection.

Immunization with Mitomycin C Treated SA-180 Cells

For one experiment, SA-180 tumor cells were treated with mitomycin C and then used to immunize mice. The SA-180 cells (4×10^7) were put into 3 ml of medium 199 (Difco) and 10% calf serum, 500 ug of mitomycin C was added and the cell suspension allowed to incubate for 45 min in a 37° C water bath. At the end of the designated time, the cells were washed by centrifugation at 210 g·min (750 rpm x 3 min in an SS-34 head) three times in 5 mM EDTA and 0.9% NaCl, and the cells suspended in 10 ml of PBS pH 7.4. They were stored in the refrigerator for less than 10 days. Treated cells (1×10^5) were injected into a group of ten mice in the immunized group twice per week, while the 10 control mice were injected with an equal volume of HBS. All mice were challenged with 1×10^5 viable SA-180 cells one week after the last injection of the mitomycin C treated cells.

Immunotherapy Trials

Two schemes with 20 mice each were used in an attempt to find the level of SA-180 Zn^{++} two-phase stabilized membrane antigen that would most effectively enhance an immune response to a preadministered dose of live SA-180 cells.

Immunotherapy Scheme 1

Twenty mice were given a live SA-180 tumor cell injection of 2.5×10^2 cells. Immunizations with SA-180 Zn^{++} two-phase stabilized membranes were started 24 hours later for the group to be immunized, where-

as the control group received equal volumes of HBS. The immunization scheme followed the general immunization scheme already described and the survival times of the mice were noted.

Immunotherapy Scheme 2

Twenty mice were given an injection of 1 x 10^2 viable SA-180 cells. Immunizations with SA-180 Zn^{++} two-phase stabilized membranes were started 24 hours later for the group to be immunized, and the control group received equal volumes of HBS. The injection scheme used followed the general immunization scheme discussed previously. The survival times were noted.

CHAPTER III

RESULTS

Plasma Membrane Purification

The isolation of plasma membranes from the Sarcoma-180 ascites tumor was based on a stabilization of the cell membrane by the divalent metal ion Zn^{++} followed by gentle homogenization to yield large fragments of plasma membranes of reasonable purity.

Immunization of CD_1 Mice with Zn^{++} Treated SA-180 Cell Membranes Isolated from Tumor Cells Grown in BDF_1 Mice

This experiment was designed to see if membranes isolated from SA-180 tumor cells grown in the inbred BDF_1 mouse strain would immunize mice of the outbred CD_1 strain. Since the SA-180 Zn^{++} treated membranes isolated from cells grown in BDF_1 mice had been unable to immunize the BDF_1 mice against the tumor, either different antigens were expressed by the tumor when growing in the BDF_1 mice, or there was a difference in the capability of the BDF_1's immune system.

The results of this experiment are shown in Figure 3 and Table III. Of the immunized animals, 100% survived the challenge dose of 1.1×10^4 viable SA-180 tumor cells.

Figure 3. Per Cent of Animals Surviving Following Injection of 1.1 x 10^4 Viable SA-180 Cells in the Immunization Scheme Using BDF_1 Grown Zn^{++} Treated SA-180 Membranes to Immunize CD_1 Mice

Group (a) was the control group and received injections of HBS. The immunized Group (b) received $ZnCl_2$ treated SA-180 membranes. All animals were challenged with 1 x 10^4 viable SA-180 cells on day 0. Survivors of the challenge were rechallenged on day 31 with 1.25 x 10^5 live SA-180 cells per animal. Survivors of this were rechallenged with 1 x 10^5 viable SA-180 cells per animal on day 138.

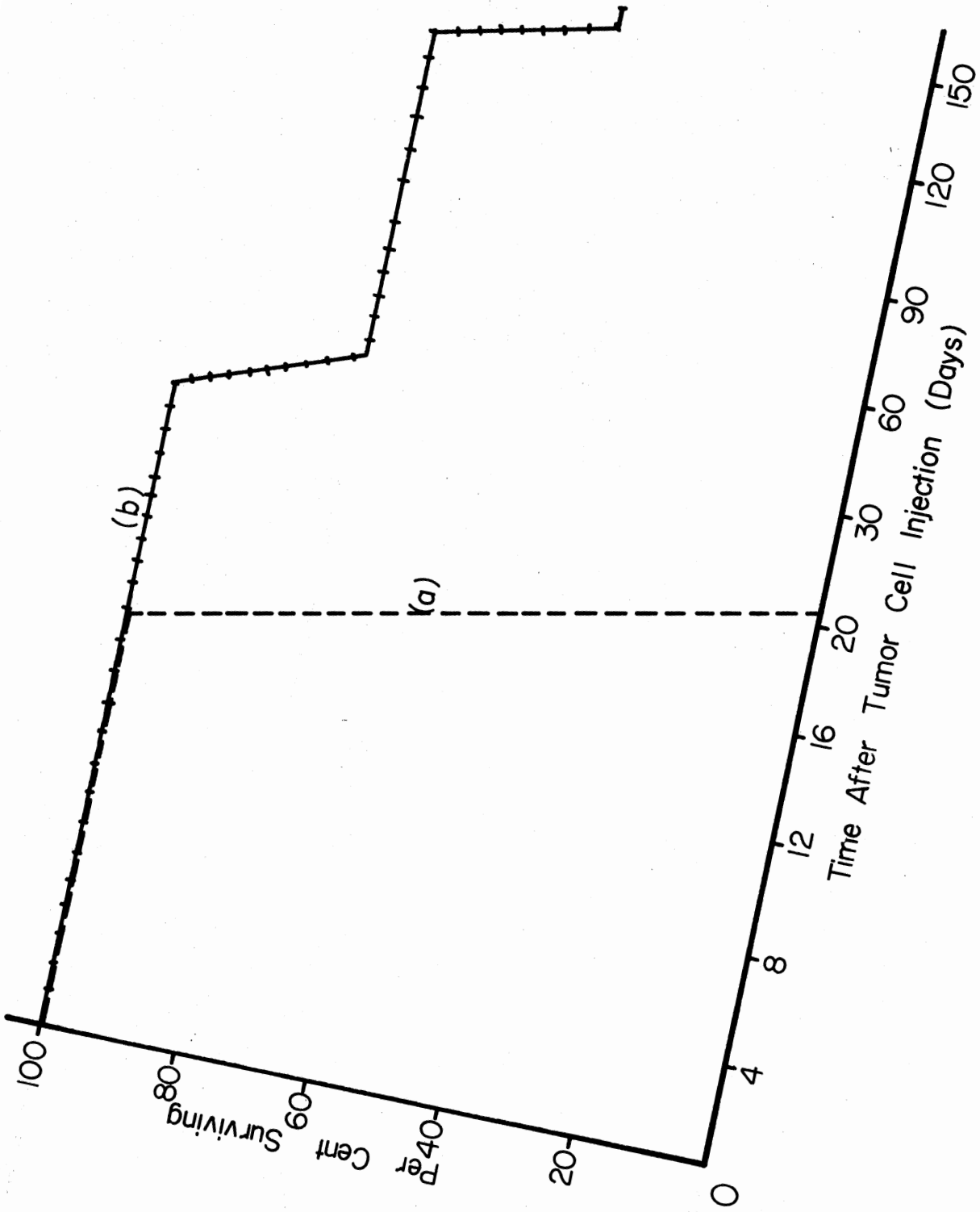

Per Cent Surviving
100
80
60
40
20
0
4
8
12
16
20
30
60
90
120
150
Time After Tumor Cell Injection (Days)
(a)
(b)

TABLE III

IMMUNIZATION SCHEME USING BDF_1 GROWN Zn^{++} TREATED SA-180 MEMBRANES TO IMMUNIZE CD_1 MICE

Date	IMMUNIZED MICE (Zn^{++} Membranes)	CONTROL MICE (HBS)
10-27-75	0.4 mg/mouse	0.1 ml/mouse
10-30	0.8	0.2
11-3	0.8	0.2
11-6	0.8	0.2
11-10	0.8	0.2
11-13	0.8	0.2

On November 20, 1975 all mice were challenged with 1.1×10^4 viable SA-180 cells per animal. The MST (Mean Survival Time) for the control mice was 20.8 days after the challenge. One hundred per cent of the immunized mice survived the challenge and on 12-21-75 they were rechallenged with 1.25×10^5 viable SA-180 cells. Twenty-eight and one-half per cent (2 mice) from the immunized mice died but 71% survived the rechallenge. The 5 survivors were rechallenged with 1×10^5 viable SA-180 cells on 4-5-76. Twenty-eight and one-half per cent (2 mice) died with a MST of 19.5 days. However 43% of the original 7 mice were able to neutralize all of the tumor dose administered 5 months since their immunization.

In Figure 3 the per cent surviving in each group versus the time in day after tumor cell injection is portrayed. Table III is a summary of the injection scheme and challenges, and shows that 100% of the 7 mice in the immunized group were able to neutralize all of the 1.1×10^4 live SA-180 tumor cells in the challenging dose. All of the mice in the control group died, with a mean survival time (MST) of 20.8 days. Thirty-one days after the challenge, the survivors of the immunized group were rechallenged with 1.25×10^5 viable SA-180 cells. Twenty-eight and one-half per cent (2 mice) of the original 7 mice died with a MST of 17 days, but 71% survived the rechallenge. The 5 survivors were rechallenged with 1×10^5 live SA-180 cells 4 months after the second rechallenge, and although it had been over 5 months since their immunization, 43% (3 mice) of the original 7 mice were able to neutralize all of the tumor dose administered. Those that died had a MST of 19.5 days after the challenge.

Immunization with Zn^{++} Stabilized SA-180 Plasma Membranes Extracted with Glycine-EDTA and Preliminary Evidence of Extraction with Glycine-EDTA-2, Mercaptoethanol

Another experiment involved modifying Zn^{++} two-phase membrane sheets with EDTA. In this type of procedure, small membrane vesicles are formed without the high-molecular-weight proteins that stabilize the membranes. Stabilize membranes are the products of Zn^{++} or other divalent metal treated SA-180 membrane isolations. Unstabilized membranes are the products of a microsomal type isolation or are stabilized

membranes that have been EDTA-extracted. In stabilized membranes, antigenic conformation and distribution are thought to be locked into place, or are not free to move. In unstabilized membranes, antigenic conformation and distribution are presumed to be free to move. An attempt was made to elucidate the effect on immunogenicity of the membrane sheets that removal of these high-molecular-weight proteins had.

Immunization with EDTA-Extracted Membranes

Group 1

The results in Figure 4 and Table IV show that 80% of the animals immunized with EDTA-extracted membranes were able to effectively resist a challenge of 1 x 10^4 viable SA-180 tumor cells. The control mice were all dead 19.5 days after tumor cell injection. The 2 immunized mice that died had a MST of 18.5 days. One month after the initial challenge, 7 of the 8 survivors were rechallenged with 1 x 10^6 live SA-180 tumor cells, and all died with a MST of 20 days. The one mouse which had not been rechallenged with the other immunized mice was injected with 1 x 10^5 viable SA-180 cells (rather than 1 x 10^6 cells) 3 months after the immunization and survived. This could indicate that it was the large rechallenging dose of 1 x 10^6 tumor cells for the rest of the immunized animals that had overwhelmed the animals immune system rather than an effect on long term immunity that removal of the high-molecular-weight proteins had. Since 10^6 tumor cells is a rather large challenge, even for some completely immunized animals to neutralize (8), the experiment was repeated and the challenge and rechallenge were kept at about 10^5 viable SA-180 tumor cells.

Figure 4. Per Cent of Animals Surviving Following Injection of 1 x 10^4 Viable SA-180 Cells in the Immunization Scheme Using EDTA-Extracted Zn^{++} Treated SA-180 Membranes to Immunize Mice (Group 1)

Group (a) was the control group, and received injections of HBS. Group (b) received $ZnCl_2$ treated SA-180 membranes that had been extracted with EDTA-glycine. All animals were challenged with 1 x 10^4 viable SA-180 cells on day 0. All but one survivor was rechallenged with 1 x 10^6 live SA-180 cells on day 30. On day 90, the one mouse that had not been rechallenged was injected with 1 x 10^5 viable SA-180 cells and it was the only mouse which survived.

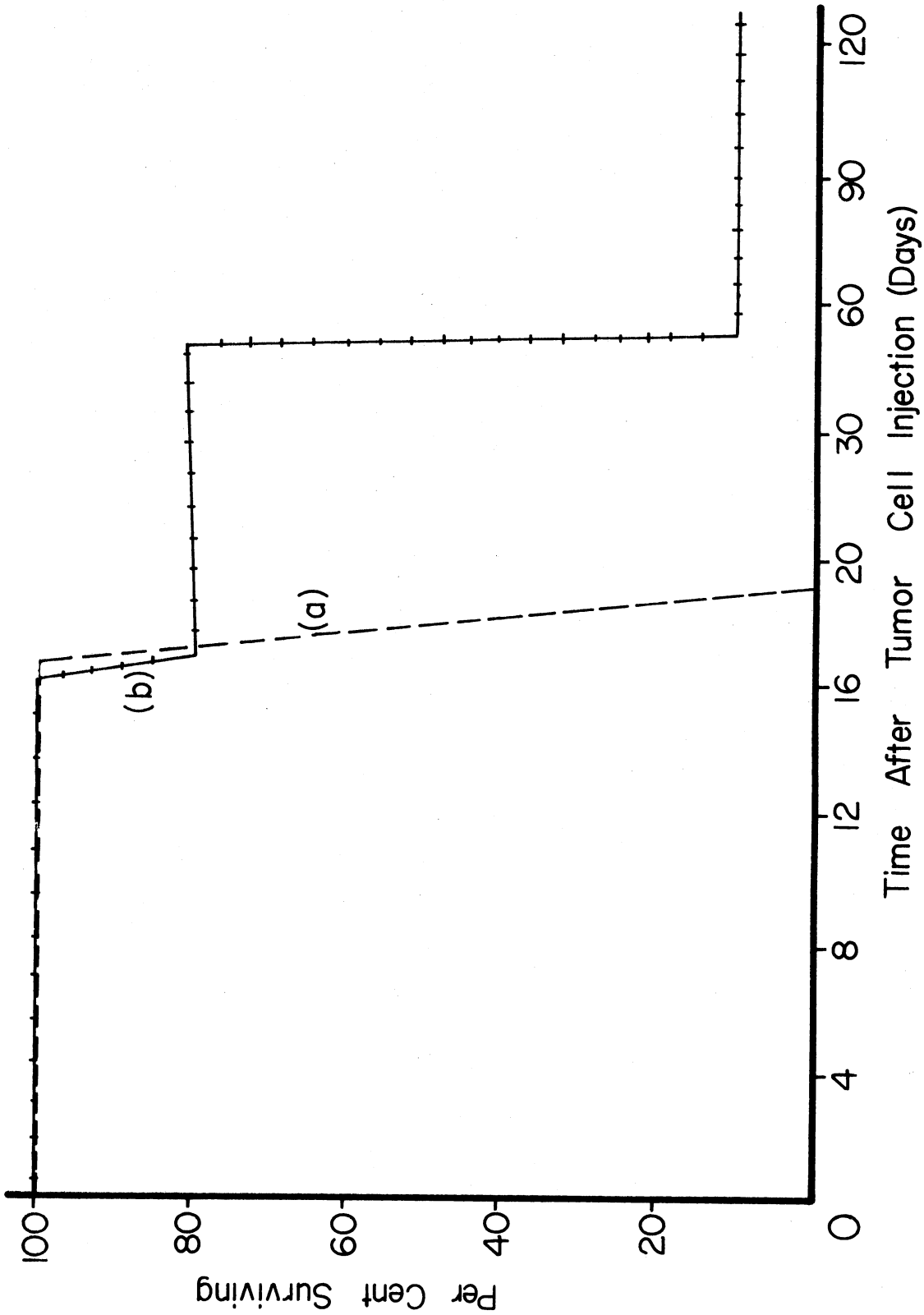

Per Cent Surviving
100
80
60
40
20
0
4
8
12
16
20
30
60
90
120
Time After Tumor Cell Injection (Days)
(a)
(b)

TABLE IV

IMMUNIZATION SCHEME USING EDTA-EXTRACTED Zn^{++} TREATED SA-180 MEMBRANES (GROUP 1)

Date	IMMUNIZED MICE (EDTA-extracted Membranes)	CONTROL MICE (HBS)
12-8-75	0.4 mg/mouse	0.4 ml/mouse
12-11	0.8	0.8
12-15	0.8	0.8
12-18	0.8	0.8
12-22	0.8	0.8
12-26	0.8	0.8

On January 5, 1976, all mice were challenged with 1×10^4 viable SA-180 cells. All of the control group died with a MST of 19.5 days after tumor cell injection. Eighty per cent of the immunized mice survived the challenge (2 died with a MST of 18.5 days) and 7 of the 8 survivors were rechallenged with 1×10^6 cells on February 11. All mice rechallenged died with a MST of 20 days. The one mouse which had not been rechallenged with the rest was rechallenged with 1×10^5 viable SA-180 cells on 4-5-76 and survived the challenge.

Immunization with EDTA-Extracted Membranes

Group 2

The results in Figure 5 and Table V confirm that the glycine-EDTA extracted membranes will effectively immunize mice, with 88% of the immunized mice surviving a 1 x 10^5 viable SA-180 tumor cell challenge per mouse. The one mouse in the immunized group that died had a MST of 22 days. All control mice died with a MST of 17.7 days. The survivors in the immunized group were rechallenged with 1 x 10^5 live SA-180 cells per animal one month after the initial challenge. All mice were able to survive the rechallenge.

Immunization with EDTA-Extracted Membranes

Group 3

In this immunization, membranes were used which had been extracted with glycine-EDTA solution in which 2, mercaptoethanol had been added. The results show in Figure 6 and Table VI that only 10% (1 mouse) in the immunized group was able to neutralize all of the live tumor challenge of 1.25 x 10^5 viable SA-180 cells per animal. The 9 immunized mice that died had a MST of 20.9 days. All control mice died with a MST of 18.5 days. The one immunized survivor was rechallenged with 1.05 x 10^5 viable SA-180 cells one month after the initial challenge and survived. The results show that under these conditions and with the immunization scheme used, the glycine-EDTA-2, mercaptoethanol-extracted SA-180 Zn^{++} stabilized membranes failed to illicit a protective immune response following a live tumor challenge.

Figure 5. Per Cent of Animals Surviving Following Injection of 1 x 10^5 Viable SA-180 Cells in the Immunization Scheme Using EDTA-Extracted Zn^{++} Treated SA-180 Membranes to Immunize Mice (Group 2)

Group (a) was the control group and received injections of HBS. Group (b) received $ZnCl_2$ treated SA-180 membranes that had been extracted with EDTA-glycine. All animals were challenged with 1 x 10^5 viable SA-180 cells per animal on day 0. The survivors were rechallenged with 1 x 10^5 viable SA-180 cells per animal on day 33.

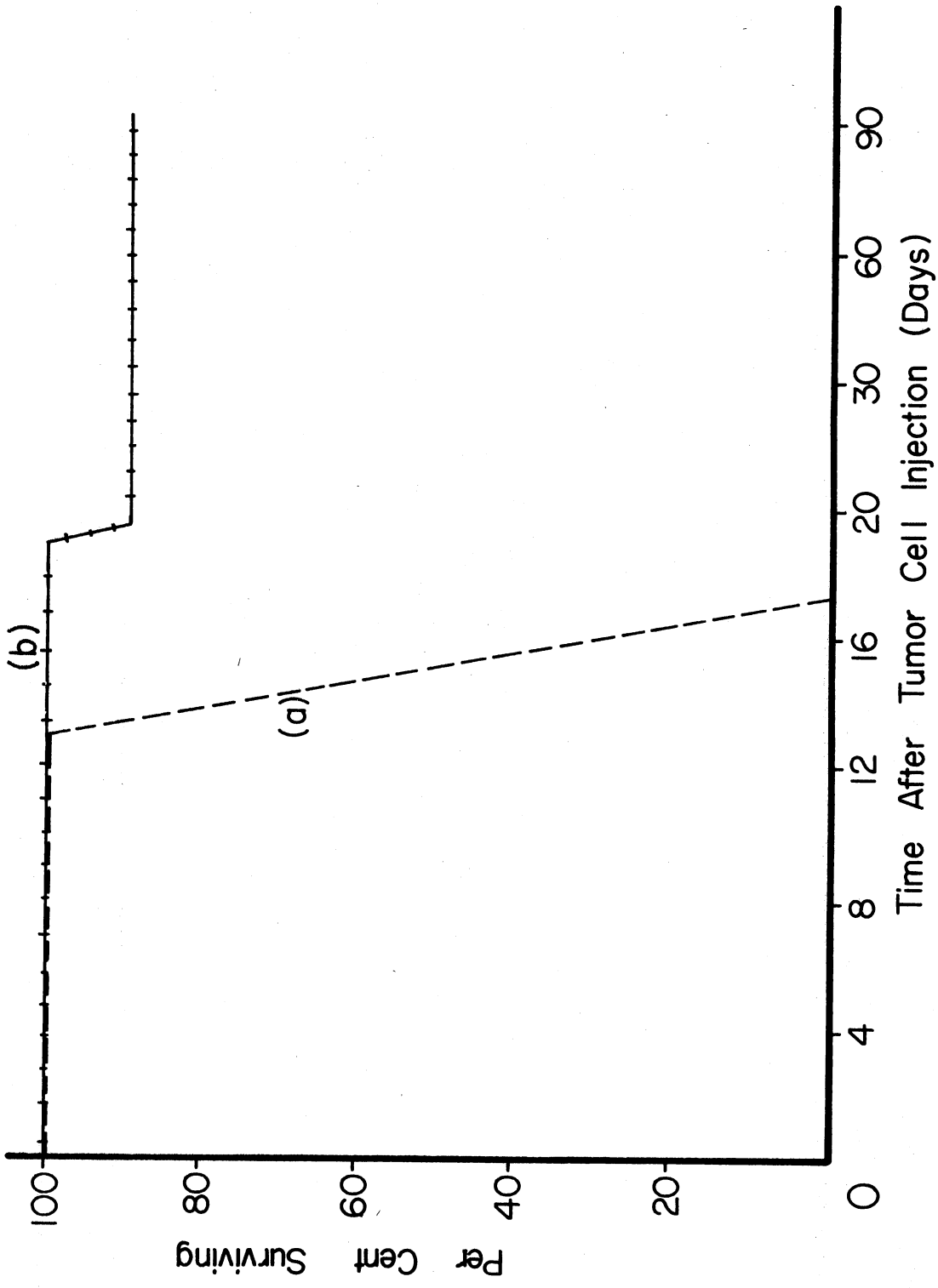

Per Cent Surviving
100
80
60
40
20
0
4
8
12
16
20
30
60
90
Time After Tumor Cell Injection (Days)
(a)
(b)

TABLE V

IMMUNIZATION SCHEME USING EDTA-EXTRACTED Zn^{++} TREATED SA-180 MEMBRANES (GROUP 2)

Date	IMMUNIZED MICE (EDTA-extracted Membranes)	CONTROL MICE (HBS)
4-28-76	0.4 mg/mouse	0.1 ml/mouse
5-2	0.8	0.2
5-5	0.8	0.2
5-11	0.8	0.2
5-13	0.8	0.2
5-18	0.8	0.2

On May 25, 1976, all mice were challenged with 1 x 10^5 viable SA-180 cells. All mice in the control group died with a MST of 17.7 days. In the immunized group, 88% (8 mice out of the 9) survived. The 1 mouse that died had a MST of 22.0 days. The mice were rechallenged on day 33 with 1 x 10^5 viable SA-180 cells per animal and all were able to combat this dose.

Figure 6. Per Cent of Animals Surviving Following Injection of 1 x 10^5 Viable SA-180 Cells in the Immunization Scheme Using EDTA-Extracted Zn^{++} Treated SA-180 Membranes to Immunize Mice (Group 3)

Group (a) was the control group and received injections of HBS. Group (b) received $ZnCl_2$ treated SA-180 cell membranes that had been extracted with EDTA-glycine-2, mercaptoethanol. All animals were challenged with 1.25 x 10^5 viable SA-180 cells on day 0.

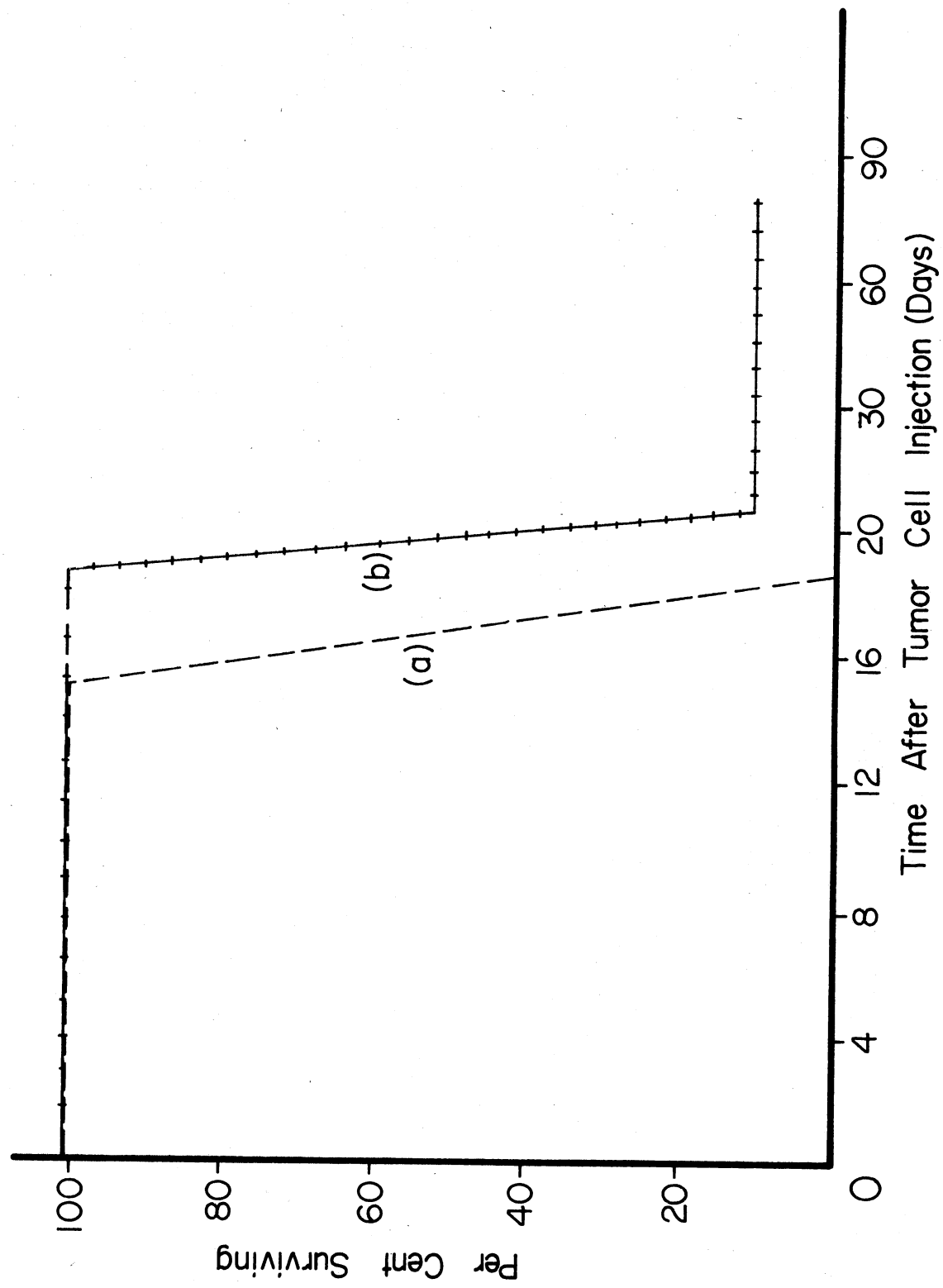

Per Cent Surviving
100
80
60
40
20
0
4
8
12
16
20
30
60
90
Time After Tumor Cell Injection (Days)
(a)
(b)

TABLE VI

IMMUNIZATION SCHEME USING EDTA-EXTRACTED Zn^{++} TREATED SA-180 MEMBRANES (GROUP 3)

Date	IMMUNIZED MICE (EDTA-extracted Membranes)	CONTROL MICE (HBS)
5-20-76	0.4 mg/mouse	0.1 ml/mouse
5-24	0.8	0.2
5-27	0.8	0.2
6-2	0.8	0.2
6-4	0.8	0.2
6-7	0.8	0.2

All mice were challenged on June 14, 1976, with 1.25×10^5 viable SA-180 cells per animal. The control mice all died with a MST of 18.5 days. In the immunized group, 9 of the 10 mice died with a MST of 20.9 days. One mouse (10% of the immunized group) was able to neutralize all of the challenge dose. This mouse was rechallenged on July 16, 1976, and survived.

Immunization with Whole Zn^{++} Treated SA-180 Cells

Immunization with Whole Zn^{++} Treated SA-180 Cells Group 1

The results of the first immunization with whole Zn^{++} treated SA-180 tumor cells are presented in Figure 7 and Table VII. Forty-five per cent of the immunized group neutralized all of the challenge dose of 1×10^4 viable SA-180 cells. The 5 immunized mice that died had a MST of 15 days, whereas the control mice had a MST of 14.8 days after tumor cell injection. The survivors of the first challenge were re-challenged one month after the initial challenge with 1×10^6 viable tumor cells. The 2 mice that died has a MST of 16 days, but 20% (2 mice) were able to combat this dose. The large challenge of 1×10^6 cells could have accounted for a higher rate of death than if 1×10^5 cells had been used. As stated earlier, 1×10^6 tumor cells is a rather large tumor burden to clear, even for some fully immunized animals. In repeats of this experiment the rechallenge was held at 1×10^5 cells per animal. Almost 3 months after the initial challenge the 2 survivors were challenged with 1×10^5 live SA-180 cells and both were able to combat all of this tumor dose.

The results show that Zn^{++} treatment of the whole SA-180 tumor cells enhanced the whole cell's immunogenicity when the first injection was not frozen before use and following an injection scheme as described. Even upon two challenges up to 4 months after immunization, 20% of the animals survived. This indicates that the whole Zn^{++} treated cells appear to be capable of illiciting a long term immune

Figure 7. Per Cent of Animals Surviving Following Injection of 1 x 10^4 Viable SA-180 Cells in the Immunization Scheme Using Whole Zn^{++} Treated SA-180 Cells to Immunize Mice (Group 1)

Group (a) was the control group and received HBS. Group (b) received $ZnCl_2$ treated whole cells. All animals were challenged with 1 x 10^4 viable SA-180 cells on day 0. On day 32, the survivors were rechallenged with 1 x 10^6 viable SA-180 tumor cells per animal. The survivors of this trial were again rechallenged and were given 1 x 10^5 viable SA-180 cells per animal on day 83.

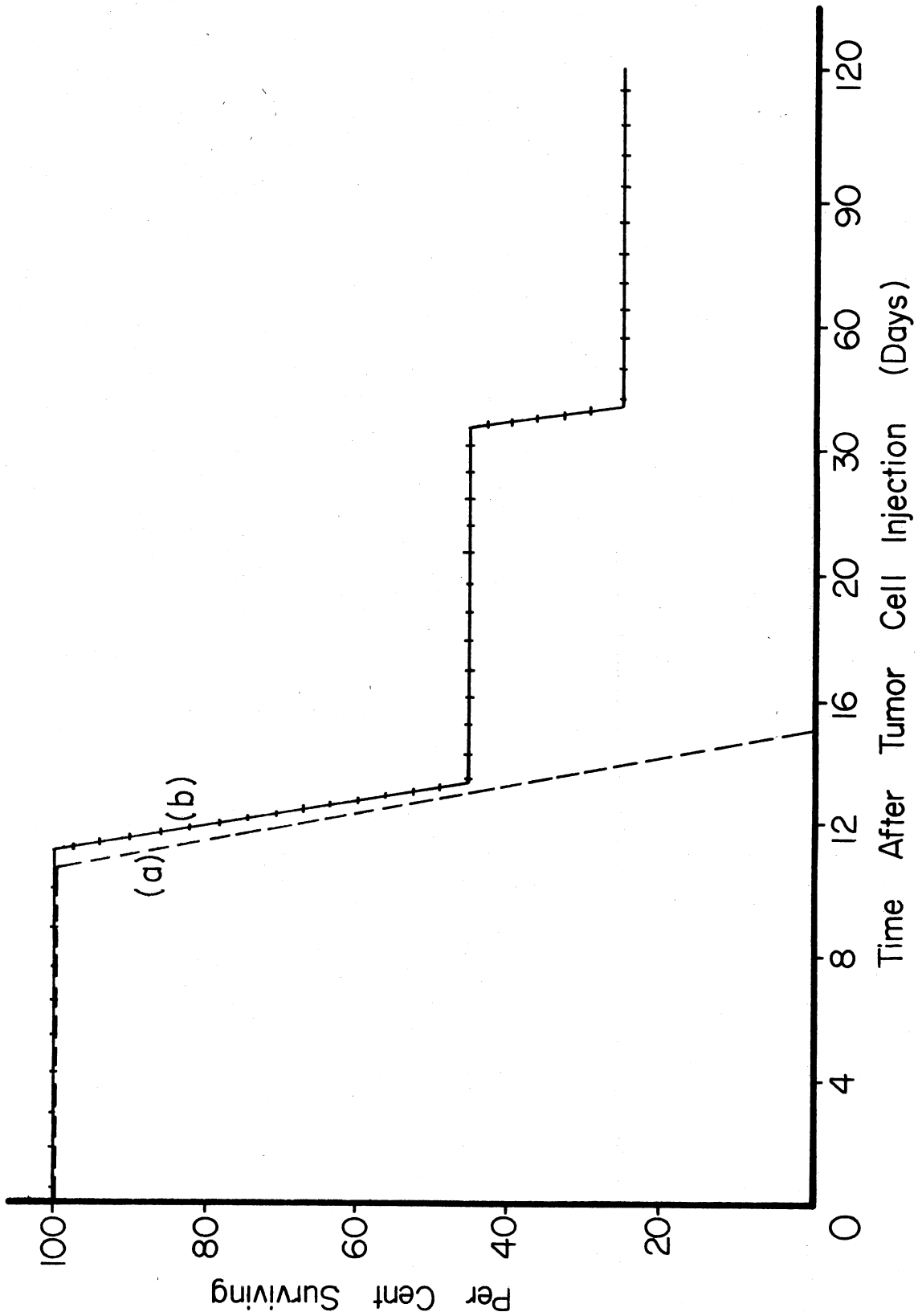

Per Cent Surviving
100
80
60
40
20
0
4
8
12
16
20
30
60
90
120
Time After Tumor Cell Injection (Days)
(a)
(b)

TABLE VII

IMMUNIZATION SCHEME USING WHOLE Zn^{++} TREATED SA-180 CELLS (GROUP 1)

Date	IMMUNIZED MICE (Zn^{++} Cells)	CONTROL MICE (HBS)
12-22-75	0.4 mg/mouse	0.1 ml/mouse
12-26	0.8	0.2
12-29	0.8	0.2
1-1-76	0.8	0.2
1-5	0.8	0.2
1-8	0.8	0.2

On January 11, 1976, all mice were challenged with 1×10^4 viable SA-180 tumor cells per animal. The control group had a MST of 14.8 days after tumor cell injection. Of the 9 immunized mice, 5 died with a MST of 15 days. There were 4 mice that survived, and they were rechallenged one month after the initial challenge (2-11-76) with 1×10^6 live SA-180 tumor cells per animal. The MST for the 2 mice that died was 17.5 days. The 2 mice that survived were rechallenged with 1×10^5 viable cells per animal almost 3 months after the initial challenge (4-5-76) and both mice survived.

response. It is possible that Zn^{++} treatment of the whole cells produces a different cell surface.

Immunization with Whole Zn^{++} Treated SA-180 Cells Group 2

The results of this immunization with whole Zn^{++} treated SA-180 cells, with all injections frozen before administered, are shown in Figure 8 and Table VIII. In this immunization, all mice in the immunized group received a first injection of 4 mg whole Zn^{++} treated cell protein rather than 0.4 mg per mouse. The control group had a MST of 18.2 days, whereas the immunized group had a MST of 18.1 days after a live SA-180 cell challenge of 1.3×10^5 cells per animal. The 10 times more antigen presented in the first injection made a total of 8.0 mg whole cell protein administered in the immunization scheme as compared to the 4.4 mg total protein of whole Zn^{++} treated cells presented to the animals in immunizations 1, 3, 4 and 5 with Zn^{++} treated whole cells.

The higher amount of antigen presented to the animals in this immunization could have blocked the immune response by paralyzing the animal's immune system and allowing normal tumor growth. The results show that under these conditions (with 8.0 mg protein given) and with all injections frozen before administered, the Zn^{++} treated whole SA-180 cells failed to illicit a protective immune response after live tumor challenge.

Figure 8. Per Cent of Animals Surviving Following Injection of 1.3 x 10^5 Viable SA-180 Cells in the Immunization Scheme Using Whole Zn^{++} Treated SA-180 Cells to Immunize Mice (Group 2)

Group (a) was the control group and received injections of HBS. Group (b) received $ZnCl_2$ treated whole SA-180 cells. All animals were challenged with 1.3 x 10^5 viable SA-180 cells on day 0.

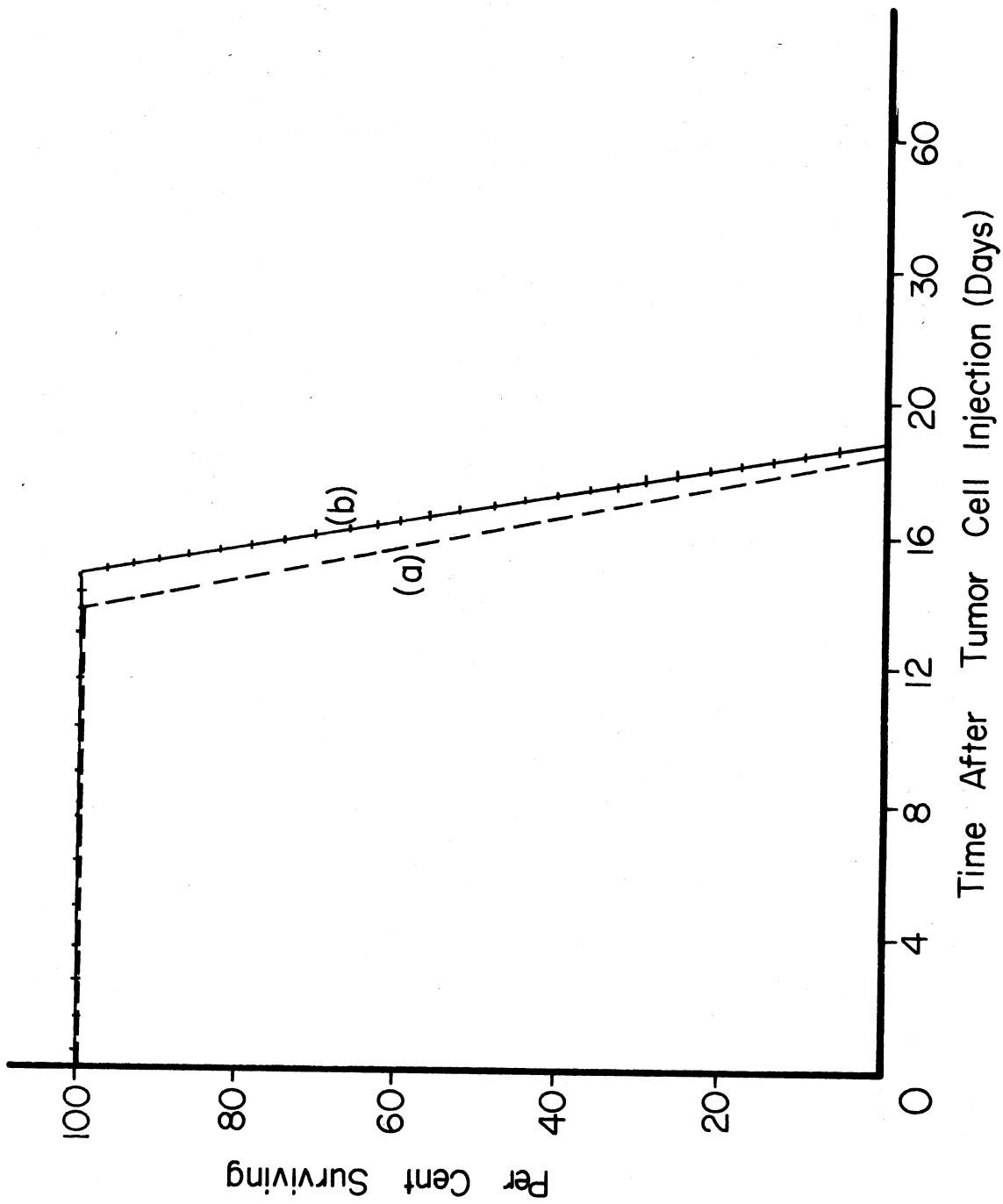

Per Cent Surviving
100
80
60
40
20
0
4
8
12
16
20
30
60
Time After Tumor Cell Injection (Days)
(a)
(b)

TABLE VIII

IMMUNIZATION SCHEME USING WHOLE Zn^{++} TREATED SA-180 CELLS (GROUP 2)

Date	IMMUNIZED MICE (Zn^{++} Treated Whole SA-180 Cells)	CONTROL MICE (HBS)
4-7-76	4.0 mg/mouse	0.1 ml/mouse
4-10	0.8	0.2
4-16	0.8	0.2
4-19	0.8	0.2
4-21	0.8	0.2
4-26	0.8	0.2

On May 2, 1976, all mice were challenged with 1.3×10^5 cells per animal. The MST for the control mice was 18.2 days. The MST for the immunized mice was 18.1 days.

Immunization with Whole Zn^{++} Treated SA-180 Cells Group 3

The results of this immunization, with all injections of Zn^{++} treated SA-180 cells frozen before administered, are shown in Figure 9 and Table IX. All mice were challenged with 1×10^5 viable SA-180 cells per animal. The immunized mice all died with a MST of 21.8 days, whereas all control mice died with a MST of 20.6 days. The results indicate that under these conditions and using the general immunization scheme already described, the Zn^{++} treated whole SA-180 cells did not illicit a protective immune response against a live tumor challenge.

Immunization with Whole Zn^{++} Treated SA-180 Cells Group 4

The results of this immunization, with the first injection of Zn^{++} treated SA-180 cells not frozen before administered and all subsequent injections frozen, are shown in Figure 10 and Table X. All mice were challenged with 1.1×10^5 viable SA-180 cells per animal. The immunized mice that died (6 out of the 8 mice) did so before the challenge was given (using the challenge date as day 0, the mice died at a calculated average MST of -4.3 days). There were 2 survivors that were able to combat the challenge dose. The control mice all died with a MST of 19.5 days. The results show that the Zn^{++} treated whole SA-180 cells, when given under these conditions, were effectively able to immunize mice against a live tumor challenge.

Figure 9. Per Cent of Animals Surviving Following Injection of 1 x 10^5 Viable SA-180 Cells in the Immunization Scheme Using Whole Zn^{++} Treated SA-180 Cells to Immunize Mice (Group 3)

Group (a) was the control group and received injections of HBS. Group (b) received $ZnCl_2$ treated whole SA-180 cells. All animals were challenged with 1 x 10^5 viable SA-180 cells on day 0.

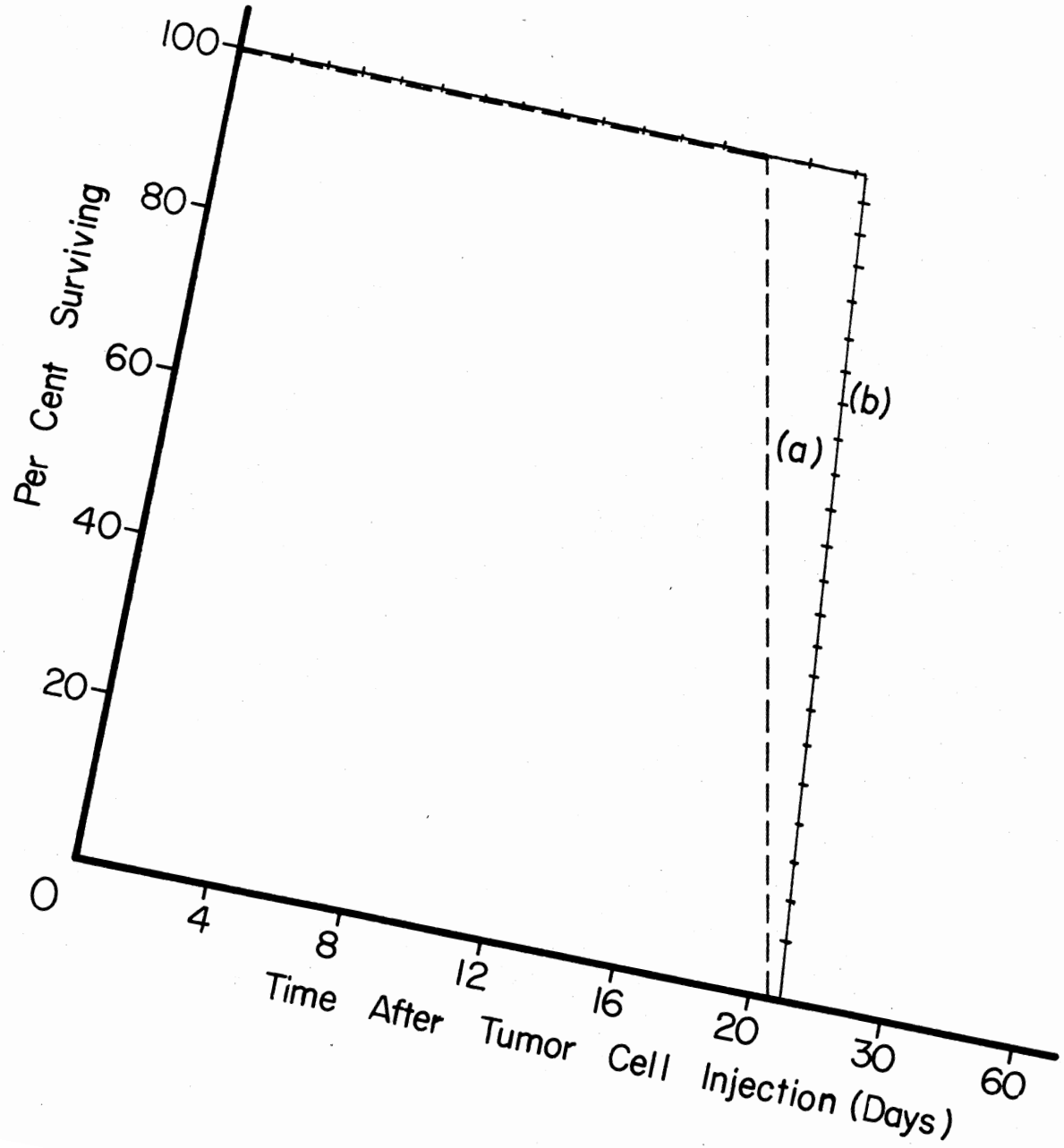

100
80
60
40
20
0
Per Cent Surviving
4
8
12
16
20
30
60
Time After Tumor Cell Injection (Days)
(a)
(b)

TABLE IX

IMMUNIZATION SCHEME USING WHOLE Zn^{++} TREATED SA-180 CELLS (GROUP 3)

Date	IMMUNIZED MICE (Zn^{++} Treated Whole SA-180 Cells)	CONTROL MICE (HBS)
5-25-76	0.4 mg/mouse	0.4 ml/mouse
5-27	0.8	0.8
6-2	0.8	0.8
6-4	0.8	0.8
6-7	0.8	0.8
6-9	0.8	0.8

On June 16, 1976, all mice were challenged with 1 x 10^5 viable SA-180 cells per animal. All mice in the immunized group died with a MST of 21.8 days. All mice in the control group died with a MST of 20.6 days.

Figure 10. Per Cent of Animals Surviving Following Injection of 1.1 x 10^5 Viable SA-180 Cells in the Immunization Scheme Using Whole Zn^{++} Treated SA-180 Cells to Immunize Mice (Group 4)

Group (a) was the control group and received injections of HBS. Group (b) received $ZnCl_2$ treated whole SA-180 cells. All animals were challenged with 1.1 x 10^5 viable SA-180 cells per animal on day 0. In Group (b), the mice that died (6 out of the 8) did so before the challenge dose was given.

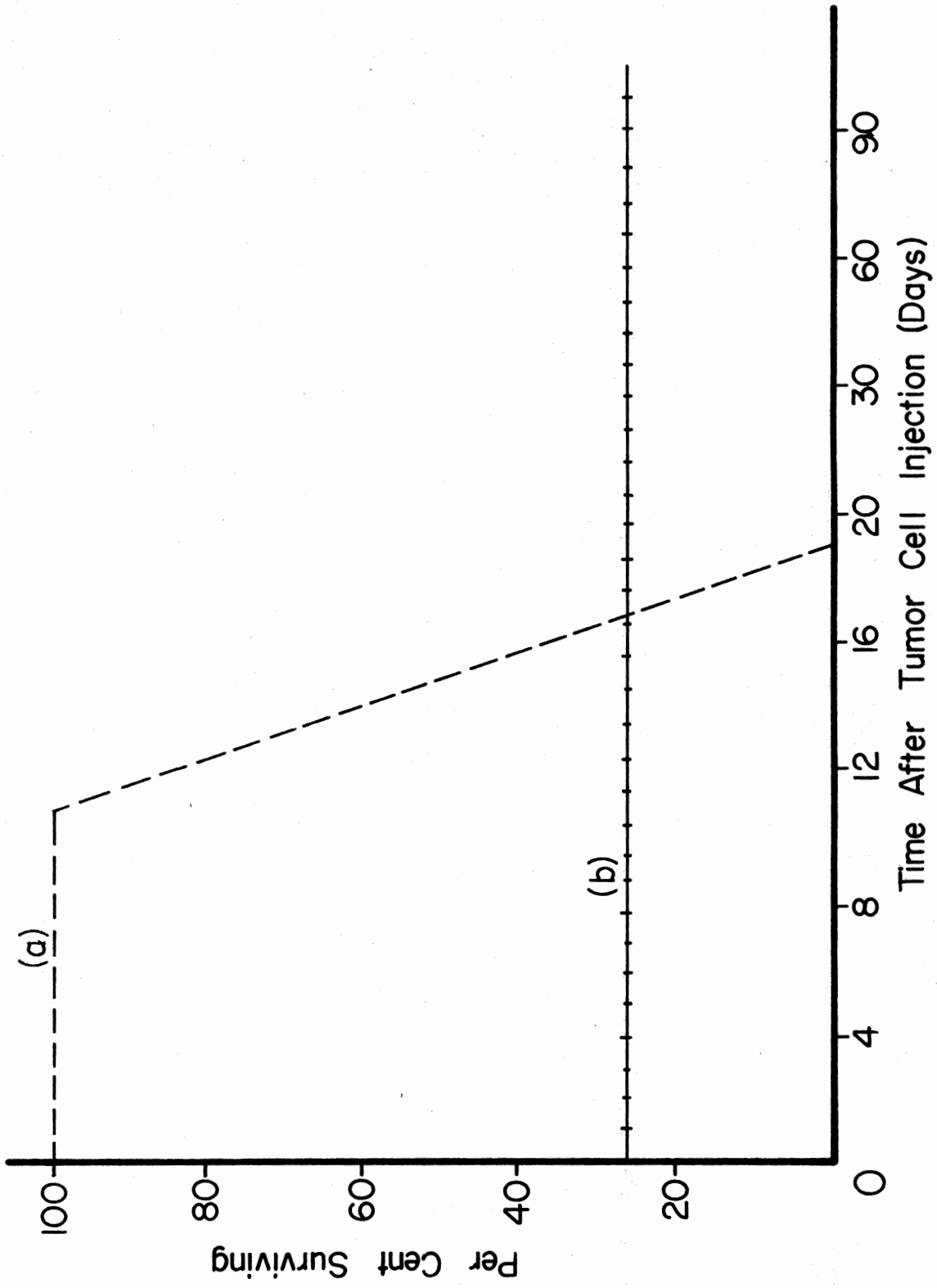

Per Cent Surviving
100
80
60
40
20
0
4
8
12
16
20
30
60
90
Time After Tumor Cell Injection (Days)
(a)
(b)

TABLE X

IMMUNIZATION SCHEME USING WHOLE Zn^{++} TREATED SA-180 CELLS (GROUP 4)

Date	IMMUNIZED MICE (Zn^{++} Treated Whole SA-180 Cells)	CONTROL MICE (HBS)
7-8-76	0.4 mg/mouse	0.1 ml/mouse
7-13	0.8	0.2
7-15	0.8	0.2
7-20	0.8	0.2
7-22	0.8	0.2
7-28	0.8	0.2

On August 4, 1976, all mice were challenged with 1.1×10^5 viable SA-180 cells per animal. All control mice died with a MST of 19.5 days. In the immunized group there were 2 survivors (25%), while 75% (6 mice) died with a MST of -4.3 days. (These mice developed the tumor after the first injection was given in which some of the Zn^{++} treated tumor cells were still viable since they were not frozen).

Immunization with Whole Zn^{++} Treated SA-180

Cells Group 5

The results of this immunization, with all injections of Zn^{++} treated SA-180 cells frozen before administered, are shown in Figure 11 and Table XI. All mice were challenged with 1.1 x 10^5 viable SA-180 cells per animal. The immunized mice that died (8 out of the 10) had a MST of 19 days, but 2 of the mice (20%) were able to survive the live tumor challenge. All control mice died with a MST of 19.5 days. The results show that under these conditions, the Zn^{++} treated whole SA-180 cells were able to effectively immunize mice.

Immunization with Irradiated

Whole SA-180 Cells

The results of the immunization with whole irradiated SA-180 tumor cells are shown in Figure 12 and Table XII. The control mice had a MST of 12.3 days, while the immunized mice had a MST of 14.6 days after injection of 1 x 10^6 viable SA-180 cells per animal. In this tumor system, the 2.3 day lengthened survival time of the test mice is not meaningful.

Immunization with Mitomycin C

Treated Whole SA-180 Cells

The results of the immunization with mitomycin C treated whole SA-180 cells are summarized in Figure 13 and Table XIII. The control mice and the immunized mice both had a MST of 18.5 days after a 1 x 10^5 viable SA-180 tumor cell challenge per animal. The results

Figure 11. Per Cent of Animals Surviving Following Injection of 1.1 x 10^5 Viable SA-180 Cells in the Immunization Scheme Using Whole Zn^{++} Treated SA-180 Cells to Immunize Mice (Group 5)

Group (a) was the control group and received injections of HBS. Group (b) received $ZnCl_2$ treated whole SA-180 cells. All animals were challenged with 1.1 x 10^5 viable SA-180 cells per animal on day 0.

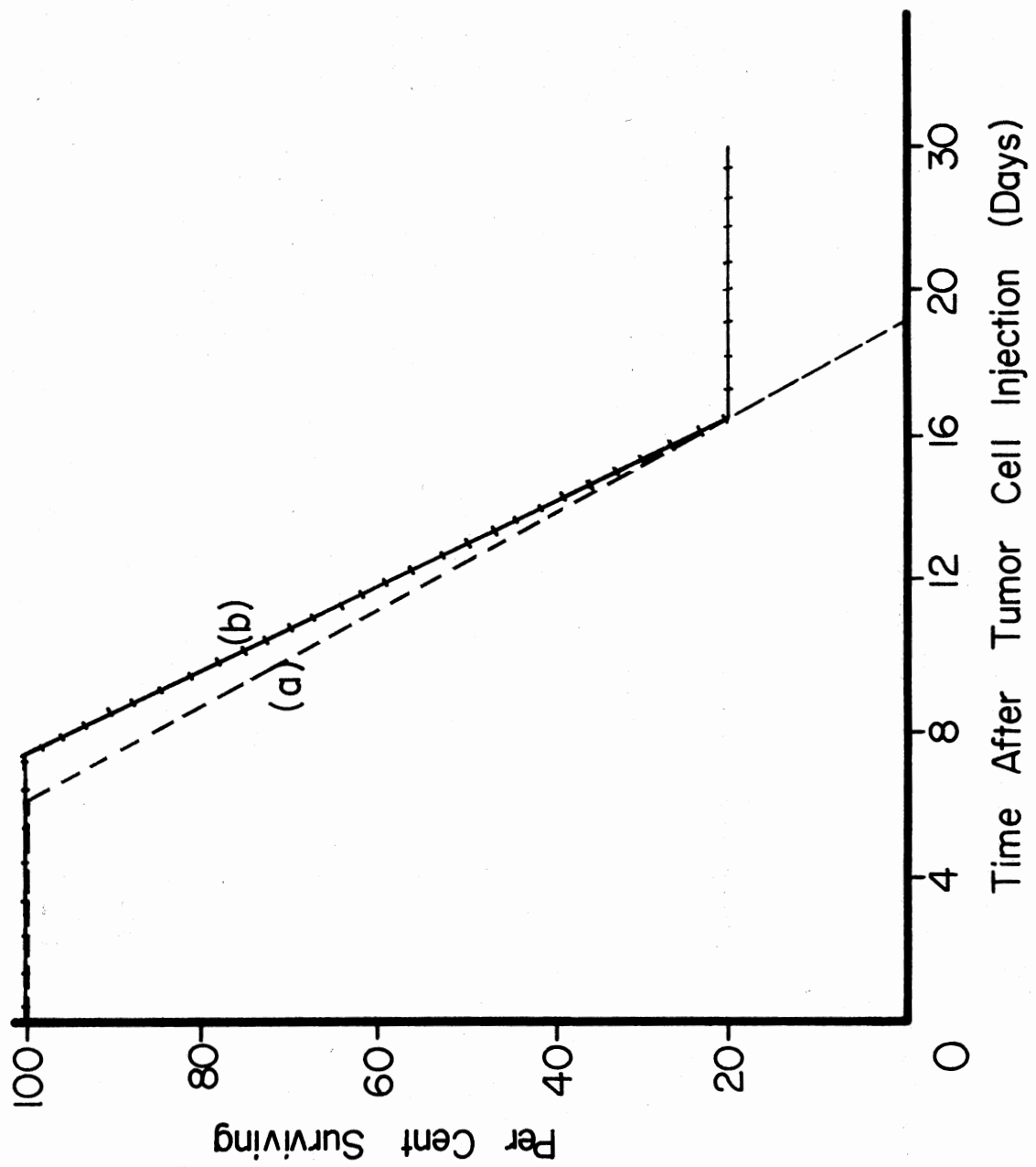

Per Cent Surviving
100
80
60
40
20
0
4
8
12
16
20
30
Time After Tumor Cell Injection (Days)
(a)
(b)

TABLE XI

IMMUNIZATION SCHEME USING WHOLE Zn^{++} TREATED SA-180 CELLS (GROUP 5)

Date	IMMUNIZED MICE (Zn^{++} Treated Whole SA-180 Cells)	CONTROL MICE (HBS)
7-8-76	0.4 mg/mouse	0.1 ml/mouse
7-13	0.8	0.2
7-15	0.8	0.2
7-20	0.8	0.2
7-22	0.8	0.2
7-28	0.8	0.2

On August 4, 1976, all mice were challenged with 1.1×10^5 viable SA-180 cells per animal. All control mice died with a MST of 19.5 days. In the immunized group, the 8 out of 10 mice that died had a MST of 19 days. Two of the mice were able to combat the challenge dose and survived.

Figure 12. Per Cent of Animals Surviving Following Injection of 1 x 10^6 Viable SA-180 Cells in the Immunization Scheme Using Whole Irradiated SA-180 Cells to Immunize Mice

Group (a) was the control group and received injections of HBS. Group (b) received whole SA-180 cells that had been irradiated. All animals were challenged with 1 x 10^6 viable SA-180 cells on day 0.

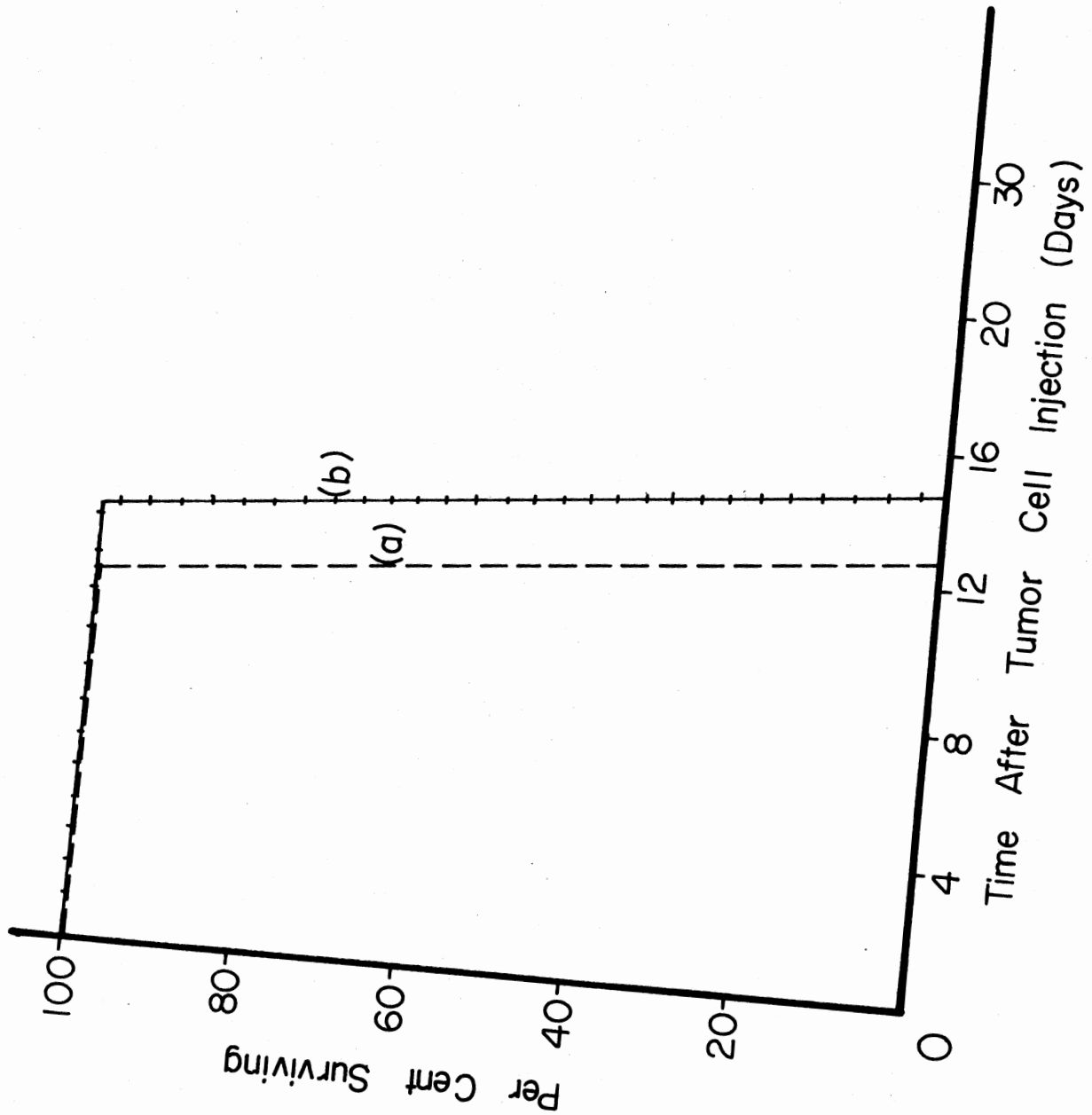

Per Cent Surviving
100
80
60
40
20
0
4
8
12
16
20
30
Time After Tumor Cell Injection (Days)
(a)
(b)

TABLE XII

IMMUNIZATION SCHEME USING WHOLE IRRADIATED SA-180 CELLS

Date	IMMUNIZED MICE (10^6 Irradiated Cells/Animal)	CONTROL MICE (HBS)
9-11-75	10^6	0.1 ml/mouse
9-15	10^6	0.1
9-18	10^6	0.1
9-22	10^6	0.1
9-25	10^6	0.1
9-29	10^6	0.1

On October 6, 1975, all mice were challenged with 10^6 viable SA-180 cells per animal. The control mice had a MST of 12.3 days. The immunized mice had a MST of 14.6 days.

Figure 13. Per Cent of Animals Surviving Following Injection of 1 x 10^5 Viable SA-180 Cells in the Immunization Scheme Using Whole Mitomycin C Treated SA-180 Cells to Immunize Mice

Group (a) was the control group and received HBS. Group (b) received whole mitomycin C treated SA-180 cells. All animals were challenged with 1 x 10^5 viable SA-180 cells on day 0.

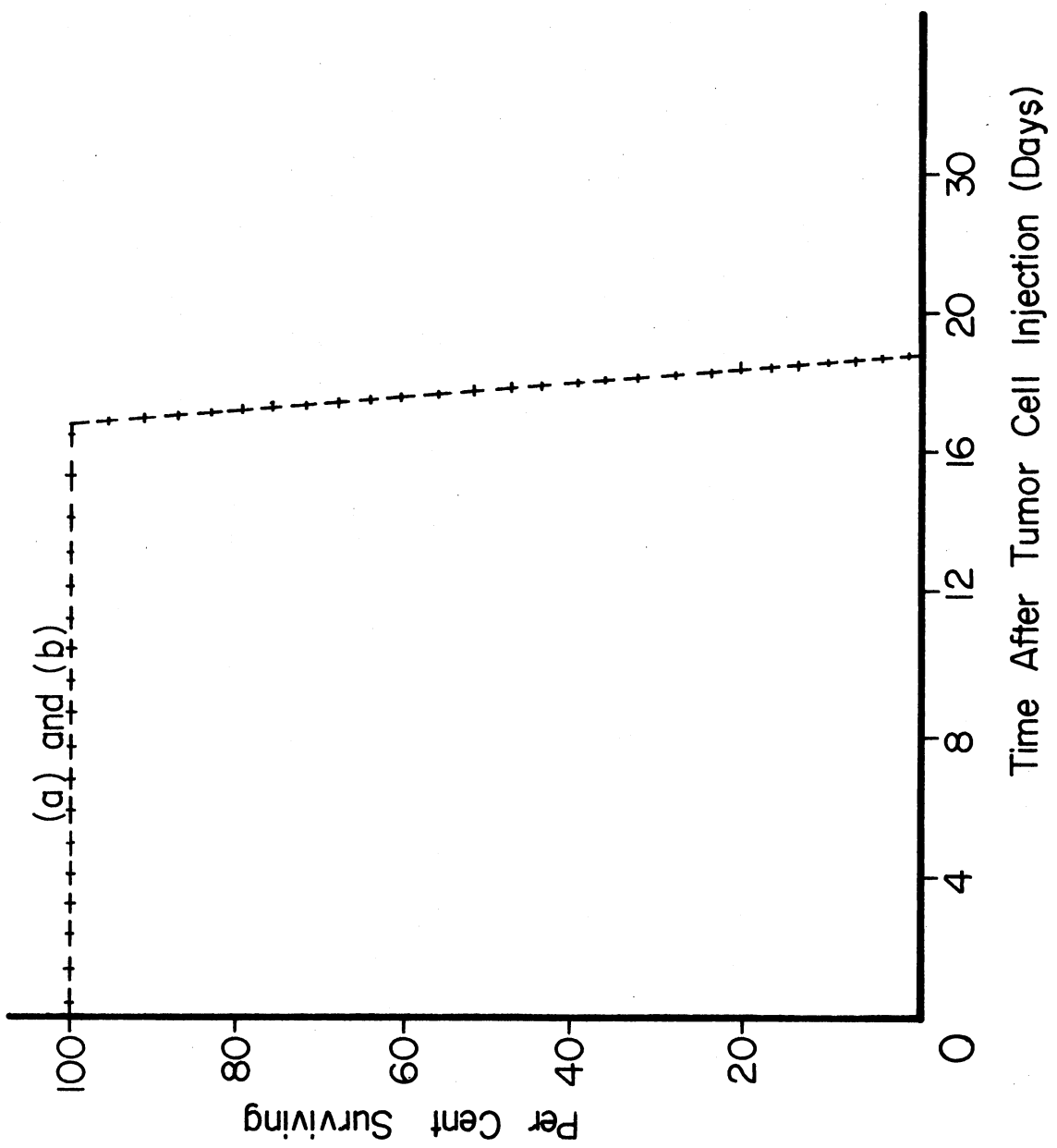

(a) and (b)
100
80
60
40
20
0
Per Cent Surviving
4
8
12
16
20
30
Time After Tumor Cell Injection (Days)

TABLE XIII

IMMUNIZATION SCHEME USING WHOLE MITOMYCIN C TREATED SA-180 CELLS

Date	IMMUNIZED MICE (Mitomycin C Treated Cells)	CONTROL MICE (HBS)
7-8-75	10^5	0.1 ml/mouse
7-10	10^5	0.1
7-15	10^5	0.1
7-17	10^5	0.1
7-22	10^5	0.1
7-25	10^5	0.1

All mice were challenged on July 31, 1975 with 1 x 10^5 viable SA-180 cells. Both control and immunized mice had a MST of 18.5 days.

indicate that the whole mitomycin C treated SA-180 cells did not stimulate a protective immune response with the injection scheme used.

Immunotherapy Trials

Immunotherapy Scheme 1

The results of the first immunotherapy trial are shown in Figure 14 and Table XIV. The control mice had a MST of 22.0 days after the time of injection of 2.5×10^2 viable SA-180 tumor cells. The immunized mice had a MST of 23.25 days after tumor cell injection. The immunization scheme used, with the injections of Zn^{++} treated SA-180 membranes beginning 24 hours after the injection of the tumor cells, did not aid the animals in combating the tumor challenge.

Immunotherapy Scheme 2

The second immunotherapy results are summarized in Figure 15 and Table XV. The control group outlived the immunized group, and it is possible that the higher amount of antigenic stimulus presented to the immunized group during the immunizations could have blocked the immune response by paralyzing the animal's immune system. Other experiments are needed to draw any conclusions except that under these conditions, with the immunizations with Zn^{++} treated SA-180 plasma membranes beginning 24 hours after a viable dose of 1×10^2 SA-180 tumor cells, the immunized mice died sooner than the control mice. The test mice had a MST of 33.1 days after the live tumor cell injection, and the control group had a MST of 37.9 days. The lengthened survival time of this group of immunized mice over group 1's immunized mice is apparently

Figure 14. Per Cent of Animals Surviving Following Injection of 2.5 x 10^2 Viable SA-180 Cells, with Immunotherapy with Zn^{++} Treated SA-180 Membranes Beginning 24 Hours After Tumor Cell Injection (Immunotherapy Group 1)

A pre-administered dose of 2.5 x 10^2 viable SA-180 cells was given to all animals on day 0. The immunized group (b) started immunotherapy with $ZnCl_2$ treated SA-180 membranes 24 hours later. Group (a) was the control group and received injections of HBS.

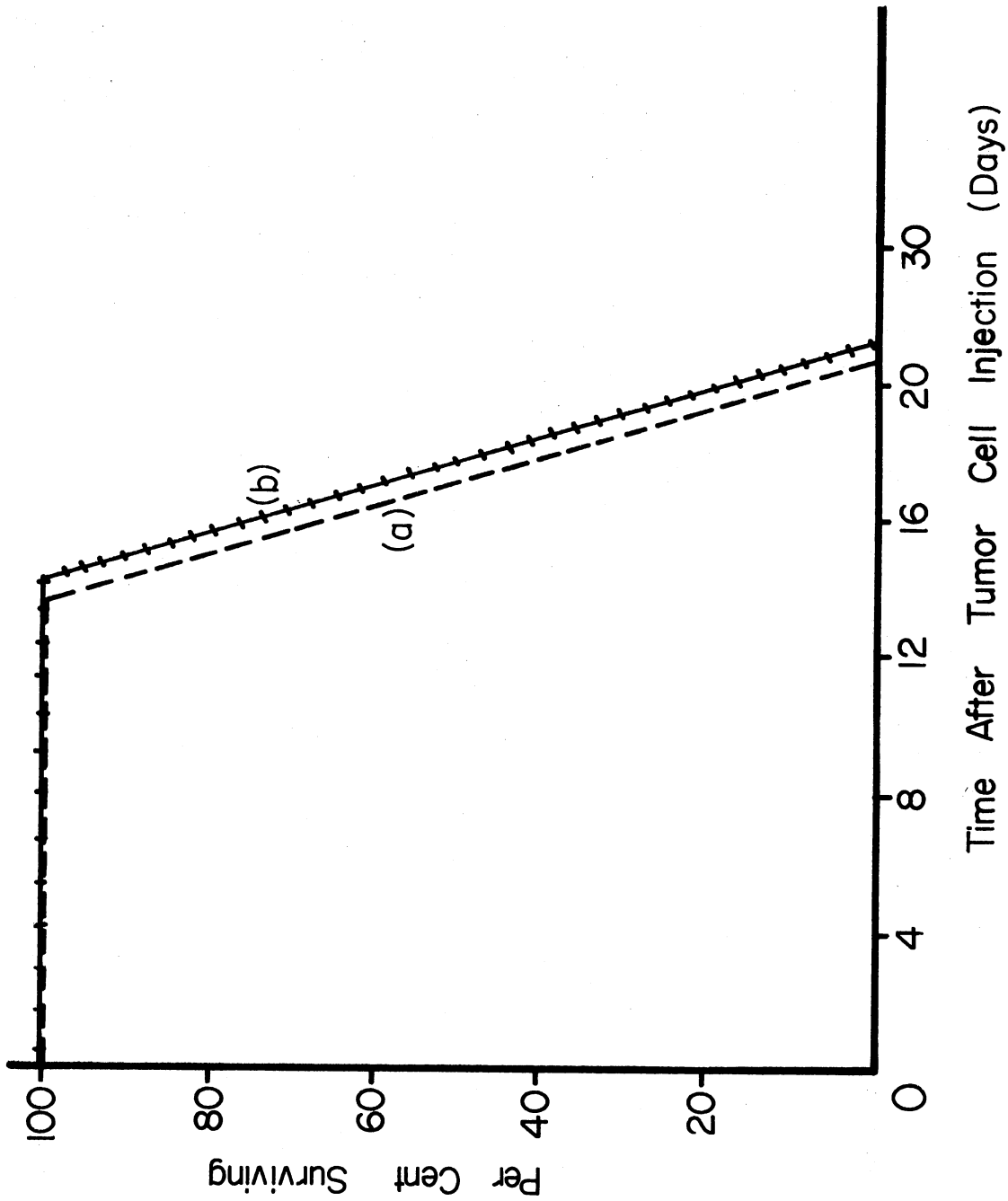

Per Cent Surviving
100
80
60
40
20
0
4
8
12
16
20
30
Time After Tumor Cell Injection (Days)
(a)
(b)

TABLE XIV

IMMUNOTHERAPY SCHEME USING Zn^{++} TREATED SA-180 MEMBRANES (GROUP 1)

Date	IMMUNIZED MICE (Zn^{++} Membranes)	CONTROL MICE (HBS)
2-12-76	0.4 mg/mouse	0.1 ml/mouse
2-16	0.8	0.2
2-19	0.8	0.2
2-23	0.8	0.2
2-26	0.8	0.2
3-2	0.8	0.2

On February 11, 1976, all mice were injected with 2.5×10^2. Immunotherapy with Zn^{++} treated SA-180 membranes began 24 hours later for the immunized group. Injection of HBS were given to the control mice. The control mice had a MST of 22.0 days after tumor injection, whereas the immunized mice had a MST of 23.25 days.

Figure 15. Per Cent of Animals Surviving Following Injection of 2.0 x 10^2 Viable SA-180 Cells, with Immunotherapy with Zn^{++} Treated SA-180 Membranes Beginning 24 Hours after Tumor Cell Injection (Immunotherapy Group 2)

A pre-administered dose of 2.0 x 10^2 viable SA-180 cells was given to all animals on day 0. The immunized group (b) started immunotherapy with $ZnCl_2$ treated SA-180 membranes 24 hours later. Group (a) was the control group and received injections with HBS.

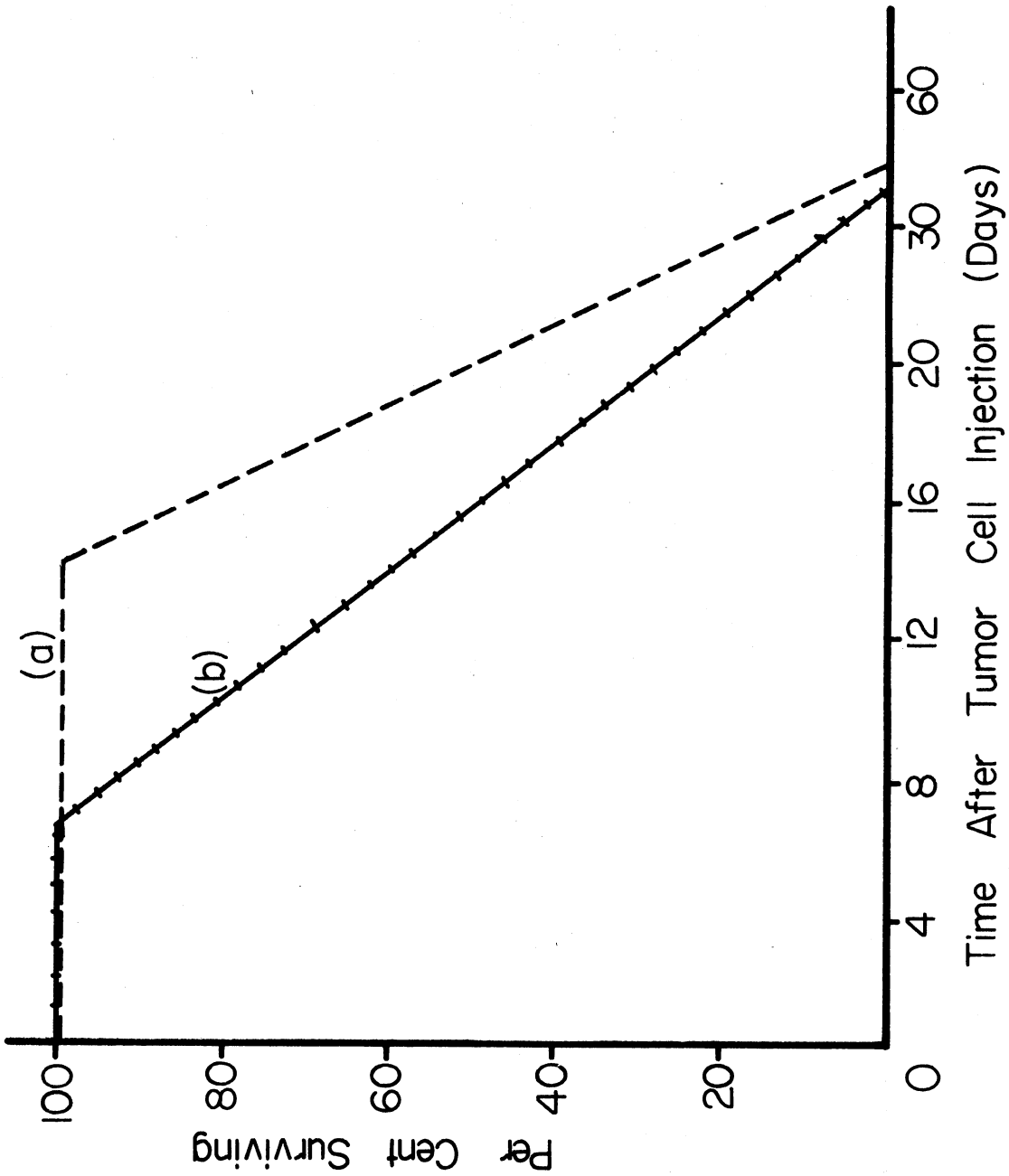

Per Cent Surviving
100
80
60
40
20
0
4
8
12
16
20
30
60
Time After Tumor Cell Injection (Days)
(a)
(b)

TABLE XV

IMMUNOTHERAPY SCHEME USING Zn^{++} TREATED SA-180 MEMBRANES (GROUP 2)

Date	IMMUNIZED MICE (Zn^{++} Membranes)	CONTROL MICE (HBS)
2-19-76	0.4 mg/mouse	0.1 ml/mouse
2-22	0.8	0.2
2-26	0.8	0.2
2-29	0.8	0.2
3-4	0.8	0.2
3-7	0.8	0.2

On February 18, 1976, all animals were injected with 1×10^2 viable SA-180 cells per animal. For the immunized group, immunizations with Zn^{++} treated SA-180 membranes began 24 hours later. The control group received injections of HBS. The control group had a MST of 37.9 days and the immunized group had a MST of 33.1 days.

from the 2½ times lower pre-administered live tumor dose presented to this group.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

The results summarized in Figures 16 and 17 show that SA-180 tumor cell membranes and various modified whole tumor cells can effectively immunize CD_1 mice against homologous tumor with the injection scheme used.

The results of the work done in this study with the BDF_1 mouse-SA-180 tumor cell system verified the immunizing potential of Zn^{++} treated SA-180 membrane sheets. Membrane sheets were isolated from tumor cells grown in BDF_1 mice and used to immunize CD_1 mice. All (100%) of the immunized group were able to neutralize all of the initial challenge dose of viable tumor cells, so it is evident that membranes isolated from the BDF_1 mice could satisfactorily immunize mice of another strain (CD_1). This indicates that the tumor must express much the same antigenic configuration while growing in the BDF_1 as it does growing in the CD_1 mice. Therefore, I conclude that there appears to be a difference in the immune capability of the BDF_1 mice that will not allow immunity to develop against a live tumor challenge after immunization with the SA-180 tumor cell membranes.

These findings agree with the work done by Mullins (42) and co-workers in which attempts were made to immunize inbred BDF_1 mice with $ZnCl_2$ treated SA-180 membranes. One group was challenged with viable SA-180 cells, and one group was challenged with viable L1210 tumor

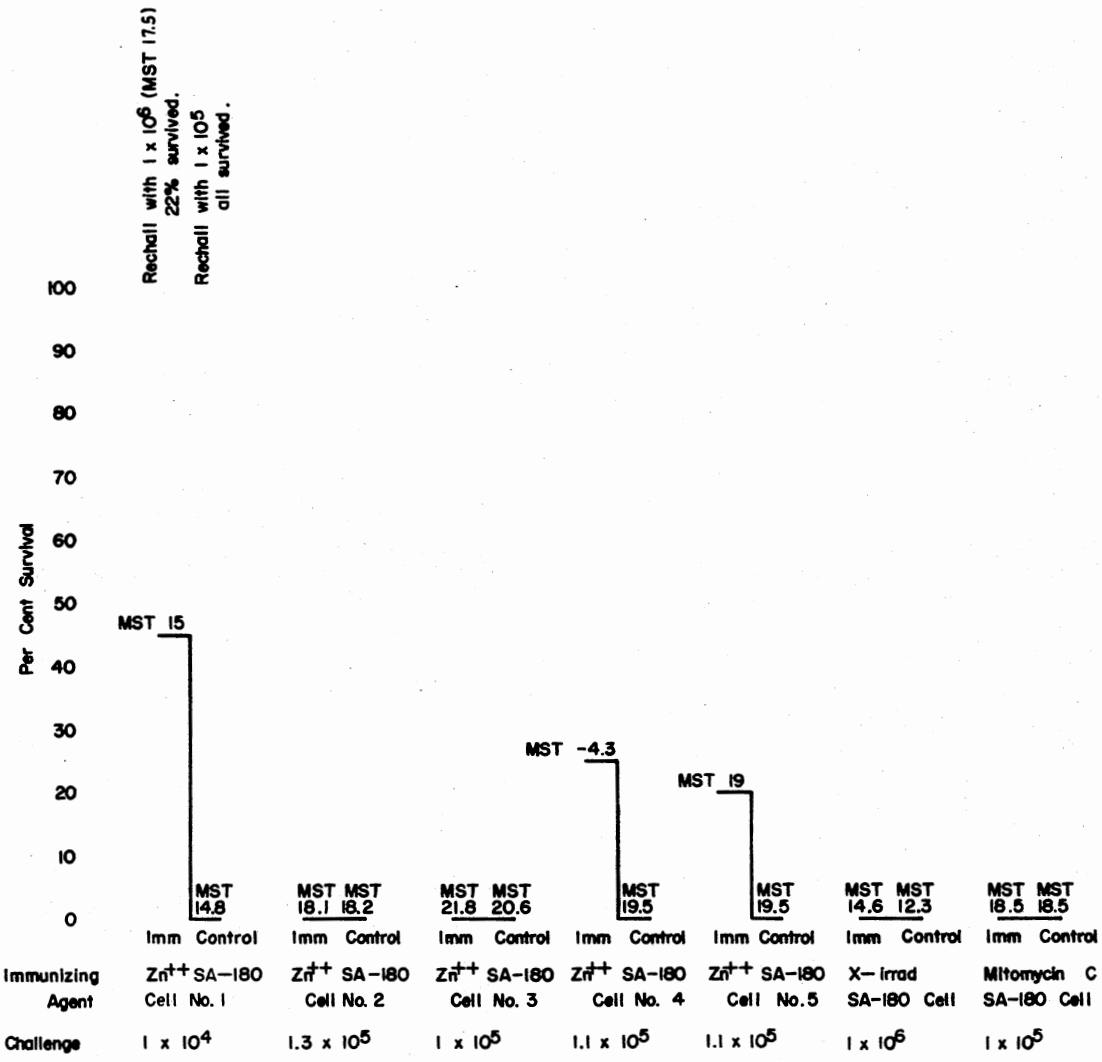
Figure 16. Per Cent of Animals Surviving Following Live Tumor Challenge in Immunizations and Immunotherapy with $ZnCl_2$ SA-180 Membranes

(MST is in days) Control groups received HBS.

PER CENT SURVIVAL
100
90
80
70
60
50
40
30
20
10
0
Rechallenge With 1.25 x 10^5 - 71% sur.(MST 17)
Rechallenge w/ 1.0 x 10^5 43% lived (MST 19.5)
7 mice rechall. with 1 x 10^6 all died. (MST 18.5)
1 rechallenge with 1 x 10^5 lived
Rechall. with 1 x 10^5 All 88% lived
Rechall. with 1 x 10^5 and the one mouse lived.
MST 20.8
MST 19.5
MST 18.5 days
MST 17.7
MST 22 days
MST 20.9
MST 18.5
MST 23.25
MST 22.0
MST 33.1
MST 37.9
Imm. Control
Imm. Control
Imm. Control
Imm. Control
Imm. Control
Imm. Control
Immunizing Agent
BDF1 grown SA-180 Zn++ memb
EDTA-gly extracted SA-180 memb No.1
EDTA-gly extracted SA-180 memb No. 2
EDTA-gly 2, mercapto-ethanol ext. SA-180 memb No.3
Immunotherapy Group 1 With Zn++ SA-180 membranes
Immunotherapy Group 2 With Zn++ SA-180 membranes
Challenge
1.1 x 10^4
1 x 10^4
1 x 10^5
1.25 x 10^5
2.5 x 10^2
2.0 x 10^2

Figure 17. Per Cent of Animals Surviving Following Live Tumor Challenge in Immunizations with Whole SA-180 Modified Cells

(MST is in days) All control mice received HBS.


Rechall with 1 x 10^6 (MST 17.5) 22% survived.
Rechall with 1 x 10^5 all survived.
Per Cent Survival
100
90
80
70
60
50
40
30
20
10
0
MST 15
MST 14.8
MST 18.1 MST 18.2
MST 21.8 MST 20.6
MST -4.3
MST 19.5
MST 19
MST 19.5
MST 14.6 MST 12.3
MST 18.5 MST 18.5
Imm Control
Imm Control
Imm Control
Imm Control
Imm Control
Imm Control
Imm Control
Immunizing Agent
Zn++ SA-180 Cell No. 1
Zn++ SA-180 Cell No. 2
Zn++ SA-180 Cell No. 3
Zn++ SA-180 Cell No. 4
Zn++ SA-180 Cell No. 5
X-irrad SA-180 Cell
Mitomycin C SA-180 Cell
Challenge
1 x 10^4
1.3 x 10^5
1 x 10^5
1.1 x 10^5
1.1 x 10^5
1 x 10^6
1 x 10^5

cells as a check for the specificity of the immune response elicited by immunization with SA-180 membranes. No lengthening of survival time was seen in either group (42).

Preliminary work (Huggins, Mullins, and co-workers) (27, 42) with SA-180 tumor cells was important in interpreting results of the immunization schemes in CD_1 mice. In order to detect any improvement in the survival capabilities of the host CD_1 mice as a result of immunization, it was first important to understand the normal tumor-host interaction (27, 42). Different groups of CD_1 mice were administered doses of 1×10^2 through 1×10^7 viable SA-180 cells per animal by i.p. injections and the survival time of the mice noted to determine the MST of unimmunized mice. From these data, any lengthened survival time due to immunizations with modified tumor material could be recognized.

Figure 18 shows the per cent cumulative mortality for each inoculation group plotted against the time of death after inoculation. Because the host in these studies was from outbred stock, the distribution of deaths within each challenge group is greater than that generally observed for inbred strains (27, 42).

From the cumulative mortality data (27), a diagram plotting the mean survival time as a function of the cell inoculum concentrations was constructed (Figure 19). The results show that the mean survival time for the CD_1 host is a linear function of the inoculation density and, therefore, a good measure of the effects of immunization on tumor survival. The approximately 20 hours deviation in mean survival time resulting from a 10^2 tumor cell inoculation may result from the efforts of the host in combating tumor challenge by a small tumor burden.

Figure 18. 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 1×10^2 through 1×10^8 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.

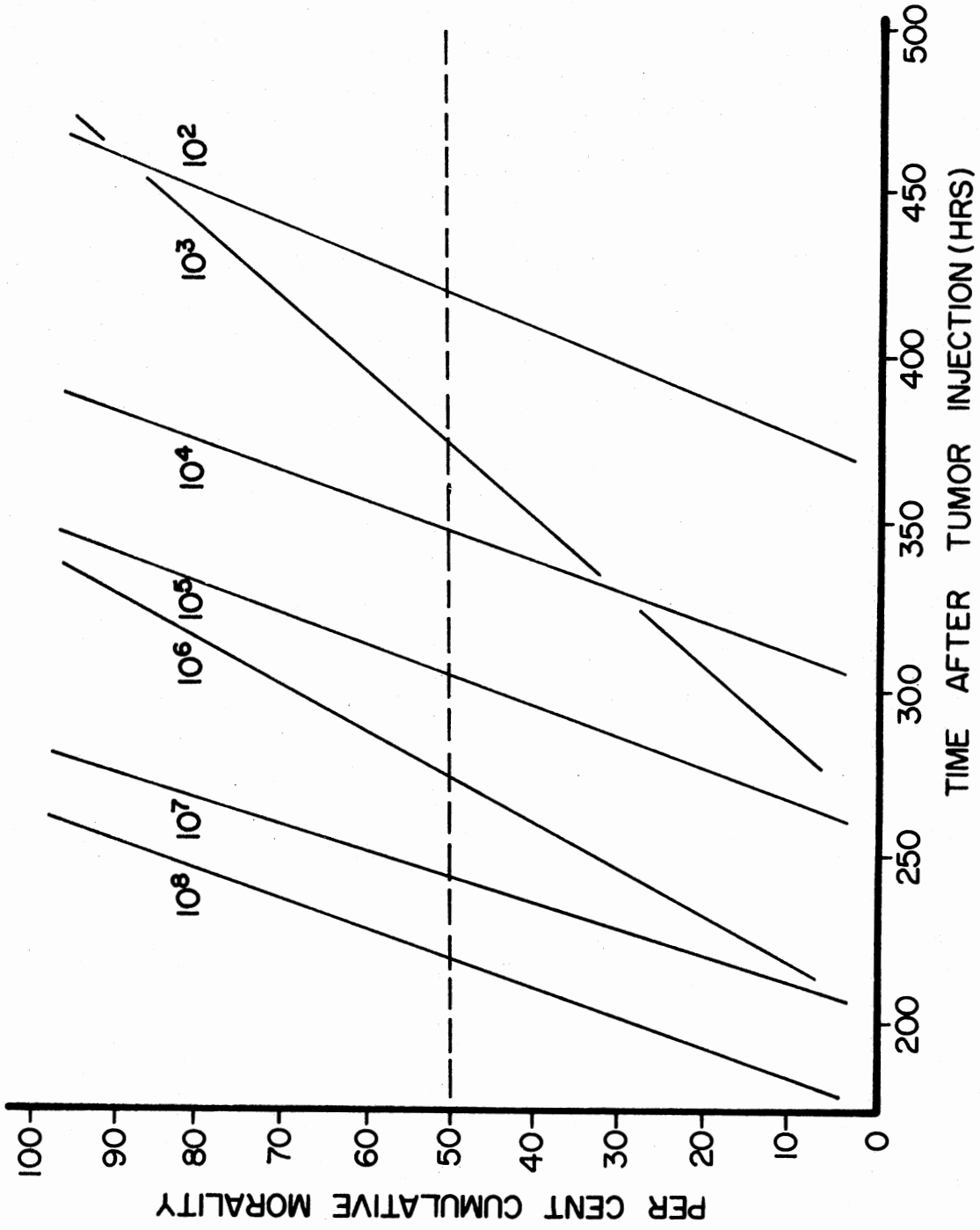
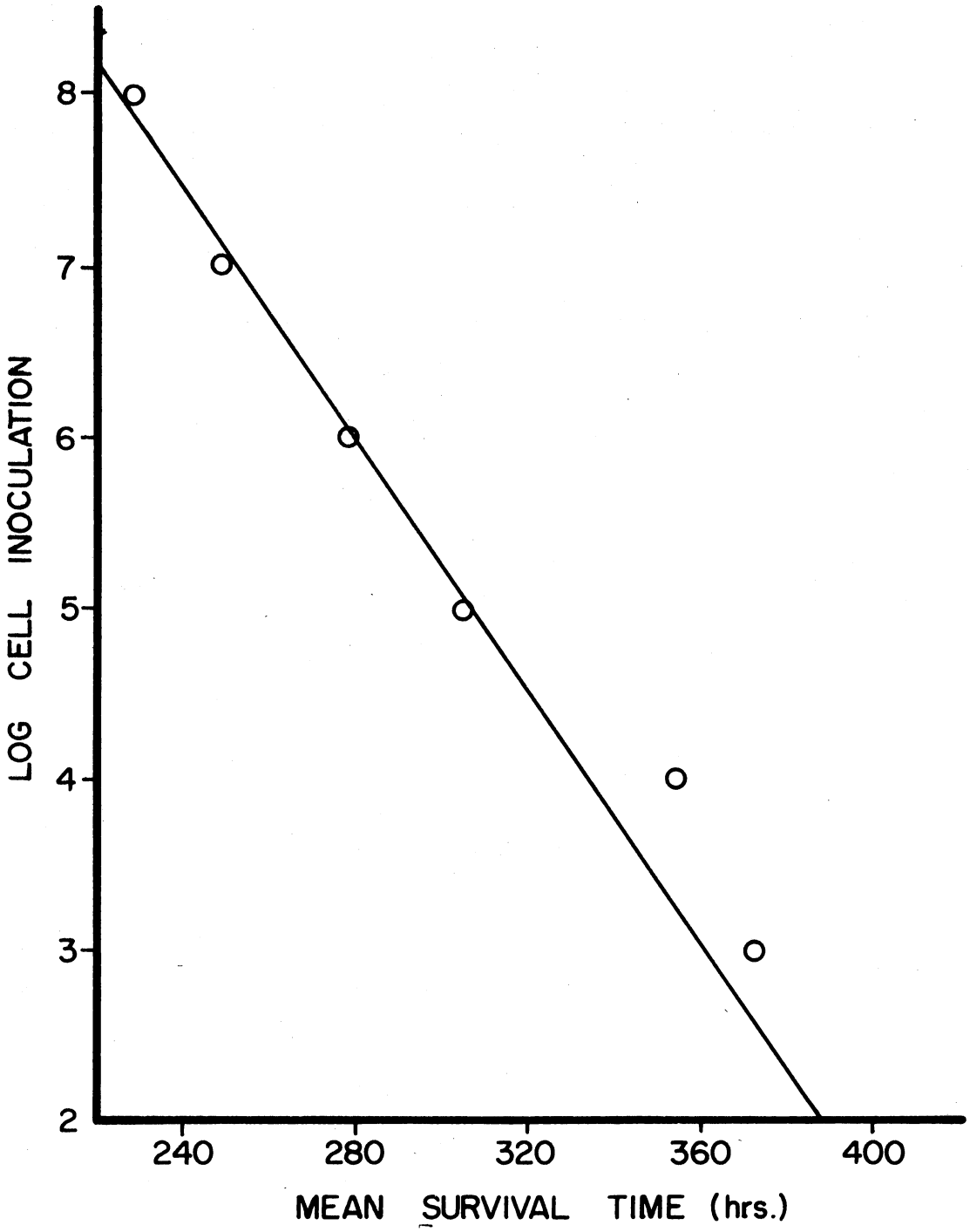

PER CENT CUMULATIVE MORALITY
100
90
80
70
60
50
40
30
20
10
0
200
250
300
350
400
450
500
TIME AFTER TUMOR INJECTION (HRS)
10^8
10^7
10^6
10^5
10^4
10^3
10^2

Figure 19. 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
280
320
360
400
MEAN SURVIVAL TIME (hrs.)

The results of this study agreed with earlier experiments by others that were conducted and confirmed that the immunizing potential, i.e. those tumor-associated antigens which are capable of eliciting an effective anti-SA-180 immune response, were retained in an active conformation on the isolated plasma membrane (27).

To further substantiate the anti-cancer immunizing potential of the isolated SA-180 plasma membranes, other immunization experiments were performed which involved additional controls as well as a test group receiving SA-180 plasma membranes (27). The different control groups received saline, HBS, human erythrocyte membranes from rbc types A, B, or O, HaM/ICR (CD_1) mouse erythrocyte membranes, or washed suspensions of *in vitro* grown fetal mouse cells, with the immunization scheme described in Chapter II. None of the control mice were afforded protection against a live tumor challenge, yet 70% of the SA-180 tumor cell membrane immunized animals survived the challenge.

These findings established that isolated SA-180 plasma membranes can be effective immunizing agents against homologous tumor. The administration of non-tumor membranes or *in vitro* grown fetal mouse cells showed no stimulation of the host's immune system in combating tumor challenge. Tumor cell membranes did enhance the survival time of the host and in many cases caused complete elimination of the challenge dose. This indicates the reaction of the host to the SA-180 tumor material results in a specific immune stimulation and not a non-specific response as with BCG (27).

A titration of antigenic material in relation to the immune response was conducted, in which smaller doses of membrane material were given while trying to maintain maximal results (42). It was found that

comparable results were not obtained when the level of antigenic material was lowered to one-half the standard dose or lower, and for that reason the level used in this study was kept at the standard dose. The standard dose referred to is 0.4 mg for the first injection and 0.8 mg for the 5 remaining injections.

These results strongly suggest that the isolated plasma membrane exhibits antigens accessible to the immune system of the host that are also cross-reactive with the viable tumor cell.

Further studies were conducted to determine the effectiveness of immunizations with SA-180 plasma membranes with injections of membranes given twice a week for 3 weeks with a total of 4.4 mg membrane protein given per mouse (0.4 mg for the first injection and 0.8 mg given for the 5 subsequent injections) against challenges of different numbers of viable SA-180 tumor cells (10^2 through 10^5 (17) and 10^4 through 10^7 (42)).

The results indicate that under these conditions, with the challenge given 1 week after the last immunization, the immune system of the host is relatively successful in eradicating tumor doses of 10^4 cells or smaller, but is less successful when the tumor burden reaches 10^7 cells (27, 42, 13). These findings are in general agreement with experiments using other tumor-host systems which show that the limits of tumor burden for successful immunotherapy are quite low (29, 26). When the challenge was given 3 days after the last immunization (13), there was a 20% lower survival rate at a 10^4 live tumor cell challenge. This could indicate that the immune system requires an extra 4 days to better combat the challenge dose.

In this study, immunization with whole irradiated SA-180 cells, or

SA-180 cells that had been treated with mitomycin C, when administered under conditions given in Chapter II, has shown that the whole SA-180 cell was not immunogenic when rendered non-viable by these agents. However, treatment of whole SA-180 cells with $ZnCl_2$ enhanced the whole cell's immunogenicity, with 45% of the immunized animals able to neutralize all of the 1 x 10^4 viable tumor cell challenge in immunization 1 with whole Zn^{++} treated SA-180 cells. The first injection of the series in this immunization was not frozen before administered. Two of these survivors were able to combat a rechallenge dose of 1 x 10^6 viable SA-180 cells. As mentioned in Chapter III, it is possible that the Zn^{++} treatment helps maintain the antigenic distribution of the cell membrane.

In immunizations 2, 3 and 5 with $ZnCl_2$ treated SA-180 cells, all injections were frozen before administered. In immunization 2, the animals were given a first injection of ten times more whole cell protein than given in the first injection of all other immunizations. In immunization 4, like in immunization 1, the first injection was not frozen before administered, yet all subsequent injections were frozen. Since no mice in the immunized group in immunization 3 with Zn^{++} treated SA-180 cells were able to combat the challenge dose of 1 x 10^5 viable SA-180 cells, a repeat of the procedure (not freezing the Zn^{++} treated cells before giving the first injection) that was used in immunization 1 with Zn^{++} whole cells (in which there was 45% survival) was performed in immunization 4 in an attempt to see if the difference in immunity obtained between the two methods was due to the first injection not being frozen before administered.

In immunization 4 (first injection not frozen before administered),

there were 2 survivors out of the 8 mice (25% survival) after a 1.1 x 10^5 viable SA-180 tumor cell challenge. The 6 immunized mice that died had a MST of -4.3 days since all of the mice that died did so before the challenge was given (day 0) due to proliferation of some of the tumor cells in the first injection which had not been killed by Zn^{++} treatment (and were usually killed by freezing). All control mice died with a MST of 19.5 days. The reason for the lower survival rate in the immunized mice than in immunization 1 can possibly be accounted for by the fact that in immunization 1 a ten times lower live tumor challenge was given (1 x 10^4 SA-180 cells).

In immunization 5 with Zn^{++} treated whole SA-180 cells, there were also 2 survivors (20% survivors, since there were 10 mice in this trial). This scheme was set up so that all injections were frozen before administered, so it appears that there is no difference in immunizing potential between frozen and non-frozen whole $ZnCl_2$ treated SA-180 cells. The immunized mice that did die had a MST of 19 days, whereas all control mice died with a MST of 19.5 days. It appears, then, that the mice in the immunization 3 with Zn^{++} whole cells died possibly because smaller-sized mice were used or to a difference in tumor cell viability rather than to an effect attributable to the cells having been frozen before administered.

Extraction of the high-molecular-weight proteins from the Zn^{++} treated SA-180 membrane sheets with glycine-EDTA did not decrease their immunizing potential against the initial live tumor challenge. When the high-molecular-weight proteins are extracted with EDTA, the SA-180 membrane changes shape, becoming small membrane vesicles. The correlation between the disappearance of the high-molecular-weight proteins

and the shape change of the SA-180 membrane lends support to the possibility that these proteins were involved in maintaining the shape of the cell (27). That it is the high-molecular-weight proteins that are being extracted with EDTA is a problem a colleague is attempting to solve by work with gel electrophoresis. The high-molecular-weight proteins are only found associated with the plasma membrane under certain conditions, which would yield whole envelopes or very large sheets of plasma membranes (27). The use of Zn^{++} allowed isolation of the proteins with the membrane in a form where a large percentage could be extracted by low ionic strength buffers (27).

In one immunization with EDTA-glycine-extracted SA-180 membranes, 80% of the immunized animals were able to combat the initial challenge of viable SA-180 cells. In another group, 88% of the immunized animals were able to combat the initial challenge of live tumor. However, in the third EDTA-glycine extraction, 2, mercaptoethanol was added. If proteins on the membrane are held together by disulfide bonds, the 2, mercaptoethanol will disrupt the bond and allow the protein to open up. Since only 10% (1 mouse) was able to survive a challenge of 1.25×10^5 viable SA-180 cells per animal, it appears the extraction result was not the same immunogenically as in immunizations 1 and 2 with EDTA-glycine-extracted membranes. The 1 survivor was able to combat a re-challenge dose of 1.05×10^5 live SA-180 cells.

The results of both immunotherapy trials (Groups 1 and 2) and of immunizations with both EDTA-extracted SA-180 membranes (Group 1) and Zn^{++} treated whole SA-180 cells (Group 2) show the problem of trying to find and administer the level of antigenic stimulus that provides the host with the best immunologic protection without paralyzing the

host's immune system.

In experiment 2 with Zn^{++} treated whole SA-180 cells, the immunized animals received a first injection of 10 times more protein (4.0 mg) than given in all other immunizations (0.4 mg). The subsequent 5 injections were 0.8 mg protein. Upon a live tumor challenge, the immunized mice died sooner than control animals. The immunized mice had a MST of 18.1 days and control mice had a MST of 18.2 days.

Upon a rechallenge of the first group in the immunization with EDTA-glycine-extracted membranes, all animals injected with 1×10^6 viable cells died. The one mouse that had not been rechallenged was injected with 1×10^5 viable SA-180 cells and survived. The higher number of tumor cells in the rechallenge could have accounted for a higher rate of death. Since all mice in the second immunization with glycine-EDTA-extracted SA-180 membranes were able to survive a rechallenge of 1.0×10^5 viable SA-180 cells, it appears that the higher number of cells in the rechallenge of the first immunization was the cause of the higher rate of death rather than an effect on long-term immunogenicity that removal of the high-molecular-weight proteins had.

In these experiments, as in the immunotherapy trials, the level of antigenic stimulus given seemed to have been too high, and since in most of these cases, the control mice lived longer than the immunized animals, we can infer that the antigenic stimulus in the amount given interferred with any immune reactions that took place when the non-immunized animals were introduced to the tumor dose. In immunotherapy group 1, the immunized group died with a MST of 23.25 days, while control mice died with a MST of 22.0 days. In group 2, the immunized mice died with a MST of 33.1 days and the control mice died with a MST of

37.9 days.

Since in the immunoprophylaxis immunizations, the test animals received amounts of membrane protein identical to the amount received in immunotherapy groups, the only difference noted between the 2 methods is the time of live tumor injection and the size of the challenge dose. In immunoprophylaxis, the challenge of 1×10^5 cells (in most cases) was given 1 week after the last of the 6 immunizations. In both immunotherapy groups, a live tumor challenge of 1×10^2 viable SA-180 cells (Group 2) or 2.5×10^2 cells (Group 1) was given first and immunizations with Zn^{++} treated SA-180 membranes were started 24 hours later.

Perhaps the membrane immunizations coupled with the increasing tumor burden in immunotherapy trials as the tumor cells multiplied overwhelmed the immune system of the host. Since the immunized animals died sooner than the controls, it appears that the immune system in these cases were paralyzed by too much antigen. In all these experiments, it seems the higher level of antigenic stimulus presented in each case went above the narrow zone under which no stimulation occurs and above which paralysis of the immune system develops (40). That the choice of the immunological response between paralysis and immunity can be controlled by antigen dosage was first noted by Glenny and Hopkins (1924) (40).

In summary, work done in this study correlates well to previous work done by Huggins (27), Mullins (42) and co-workers. Once it was verified that intact Zn^{++} treated SA-180 membrane sheets were immunogenic, modifications of these membranes were performed in an attempt to elucidate the effect of the modification on the Zn^{++} treated membrane's

immunogenicity. Although most of the immunizations with modified whole SA-180 cells were unsuccessful, it was found that $ZnCl_2$ treatment of the whole cell enhanced the whole SA-180 cell's immunogenicity, with 45% of the immunized animals able to combat a live SA-180 tumor cell challenge of 1×10^4 cells per animal. It was also found that extraction of the high-molecular-weight proteins with glycine-EDTA did not decrease the membrane's immunizing potential, and that immunizations with SA-180 membranes isolated from tumor cells grown in another strain of mice would successfully immunize CD_1 mice. Furthermore, the resulting immunity has been shown to be the long-term type, lasting in some cases greater than 6 months. The use of combination immunotherapy with modified whole tumor cells or modified tumor cell membranes may facilitate the rejection of even greater numbers of tumor cells. Huggins and Chestnut expressed doubt that all tumor cells or membranes will be immunogenic in their native form (27, 13). Most likely they can be made antigenic and effective immunizing agents by enzymatic and/or chemical modifications (27, 13).

In the isolation of tumor cell membranes for use in immunization for humans it must first be ascertained that the procedure does not concentrate any C-type membrane-associated virus that could infect the host to which it is administered during immunoprophylaxis or immunotherapy. In the case of SA-180 cells, a search of the literature did not turn up any evidence of C-type viruses associated with the SA-180 cell. Although certain findings suggest that an RNA polymerase enzyme is present in SA-180 cells (which could indicate the presence of a virus), no evidence of a virus particle has been found in the literature. The success of the Zn^{++} treated membrane as an effective

immunizing agent in mice with no tumor development, lends support to the theory of the safety of this agent to the host.

The findings in this study verify that certain modifications of the SA-180 tumor cell or it's cell membrane prior to their use in immunoprophylaxis or immunotherapy can make them antigenic and effective immunizing agents. The evidence indicates that these agents may represent an important initial step towards the development of more effective tumor-specific immunotherapy and immunoprophylaxis agents.

CHAPTER V

SUMMARY

It has been shown that plasma membranes isolated from SA-180 tumor cells by the method of Shin and Carraway can be administered to host CD_1 mice on schedules and quantities to induce immunity against a viable tumor challenge. Certain further modifications of these isolated membranes produced an immunogenic agent that allowed immunity to develop against a live tumor challenge following immunization with this agent.

Whole SA-180 tumor cells were treated in fashions similar to procedures used for membrane treatment and these procedures enhanced the whole tumor cell's immunogenicity in some cases, allowing almost 50% of the immunized mice to reject a live tumor challenge following immunization.

It can be concluded from this study that modifications of SA-180 tumor cells and tumor cell membranes can produce immunizing agents capable of inducing immunity against live SA-180 tumor cells. This study also represents a starting point for possible immunotherapy agents, if the times of administration of the tumor and initiation of the immunotherapy agents, as well as the dose of both live tumor and the immunotherapy agents are manipulated in such a way as to allow the host to develop tumor immunity.

It seems plausible that isolated tumor cell membranes and modified

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

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