ABSTRACT

Gail Orr McKenzie. THE EFFECTS OF ETHYL METHANESULFONATE (EMS) ON THE PLAQUE-FORMING CAPACITY OF BALB/cAn MICE. (Under the direction of Dr. Mason Smith) Department of Biology, May 1974.

The effects of ethyl methanesulfonate (EMS) on the immune cytokinetics of mice was studied using the Jerne hemolytic plaque technique in which single antibody-producing cells are detected as clear plaques in a layer of agar. The results showed that under appropriate experimental conditions EMS suppressed both the direct and indirect response of BALB/cAn mice to sheep erythrocytes and that optimum suppression occurred when 200 mg/kg of the drug was injected intraperitoneally eight hours prior to the antigenic stimulation. However, as in many instances of drug-induced suppression, there is a finite duration and by the end of forty-eight hours, EMS-pretreated mice exhibited not only a normal, but enhanced PFC response.

The decrease in plaque-forming cells was attributed to the alkylating properties of EMS which is specific for the N-7 moiety of the DNA base guanine. Thus, when EMS is applied during critical periods of immune induction it prevents lymphocytes from differentiating into mature antibody-producing cells. However, EMS is easily hydrolyzed, and elimination of the drug from the mouse system eliminates the suppressive effects, indicating that the ultimate stem cells involved in antibody production are not damaged.

From these results it was concluded that when the biochemical effects of exogenous EMS are appropriately integrated with the molecular and cellular events of the immune response it does act as an immunosuppressant. Consequently, the drug may be used as a tool to study many aspects of the immune response and learn more about immunosuppression and its clinical possibilities.

THE EFFECT OF ETHYL METHANESULFONATE (EMS) ON THE PLAQUE-FORMING CAPACITY OF BALB/cAn MICE

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INTRODUCTION

Studies on experimental alteration of the immune response were initiated early in this century, especially by those concerned with the effects of X-irradiation (Benjamin, 1908; Lawen, 1909). Approximately fifteen years ago there was increased interest in immunosuppressive agents because of the following discoveries in experimental and clinical immunology: the ability to induce in adults specific unresponsiveness to an otherwise effective antigen (Dresser and Mitchison, 1968; Hasek, Lengerova, and Vojtiskova, 1962); the ability to perform long-term transplantation of organs that normally would be rejected within a short time due to the genetic disparity between donor and host (Merrill, 1967); and the recognition that drugs may be used to treat and combat autoimmune disease (Schwartz, 1965).

The present study was designed to investigate the effects of the mutagen ethyl methanesulfonate (EMS) on the immune response of the mouse, particularly the BALB/cAn strain, and to determine whether or not EMS was an immunosuppressive agent. An assay described by Jerne, Nordin, and Henry (1963) which recognizes the number of specific antibody-forming cells in lymphoid tissue was used to quantitate the results. After giving mice a primary intraperitoneal injection of SRBC, splenic cells from these mice were mixed with sheep red cells in a shallow layer of agar in a Petri dish and incubated at 37°C for one hour. Single anti-SRBC antibody-producing cells form clear plaques

that become visible in the lawn of red cells after the addition of guinea pig complement. By simply counting the plaques and performing statistical tests on the data, any difference between control and experimental animals can be detected.

In such a study there are many variables to be considered including dose of EMS, route of administration, time of administration in relation to antigenic exposure, etc. Therefore, any conclusions drawn here as to the immunosuppressive capacity of EMS must be interpreted in terms of the conditions described in this experiment.

LITERATURE REVIEW

A. The Immune Response

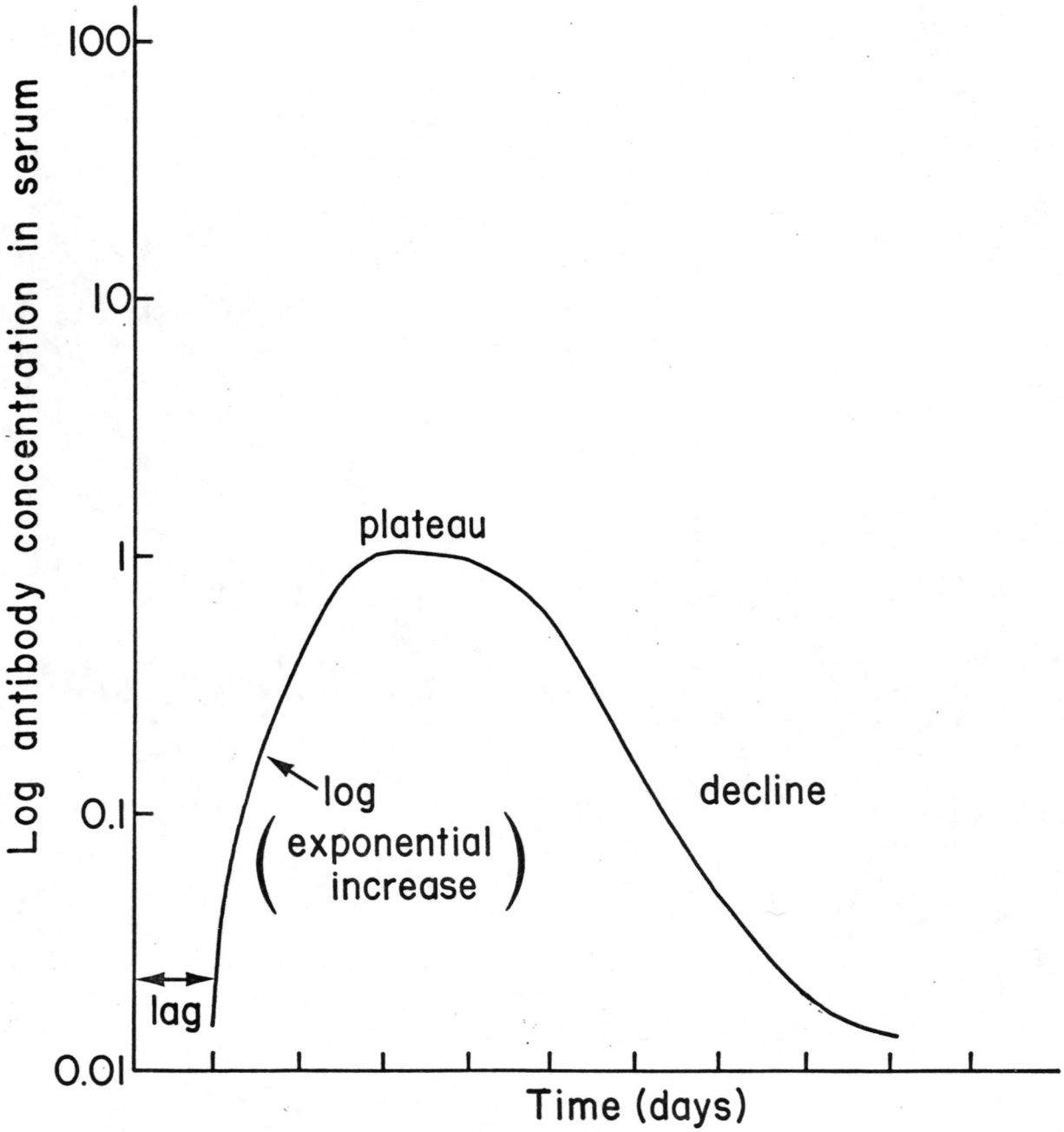
In order to emphasize the cellular events which might be susceptible to immunosuppressive drugs, a summary of the mechanism of the immune response will follow.

Weiser, et al. (1969) defined an antigen as a substance that can stimulate an animal to form proteins capable of reacting specifically with that antigen. The term immunogen is also often used to refer to the substance that stimulated the formation of corresponding antibodies (Davis, Dulbecco, Eisen, Ginsberg, and Wood, 1968). The proteins formed in response to an antigen (immunogen) are called antibodies (Weiser, Myrvik, and Pearsall, 1969). These antibodies belong to a group of serum proteins collectively called immunoglobulins of which there are three: immunoglobulin M (IgM), immunoglobulin G (IgG), and immunoglobulin A (IgA) (Sell, Park, and Nordin, 1970). The unique capacity to produce these immunoglobulins is possessed only by vertebrates (Weiser, et al., 1969).

Cell-free antibodies are commonly called "humoral antibodies" (Weiser, et al., 1969) and in the undisturbed system the production of serum or humoral antibodies can be divided into four distinct sequential phases: lag, log, plateau, and decline (Figure 1) (Makinodan, Santos, and Quinn, 1970). The lag phase comprises the interval between time of antigen exposure and the beginning of the increase in antibody concentration in the blood. During the log phase antibodies are released exponentially into the blood such that the concentration doubles

Figure 1. Idealized representation of the changing levels of serum antibody following injection of antigen

Note the logarithmic scale of antibody concentration
(Davis, et al., 1968)

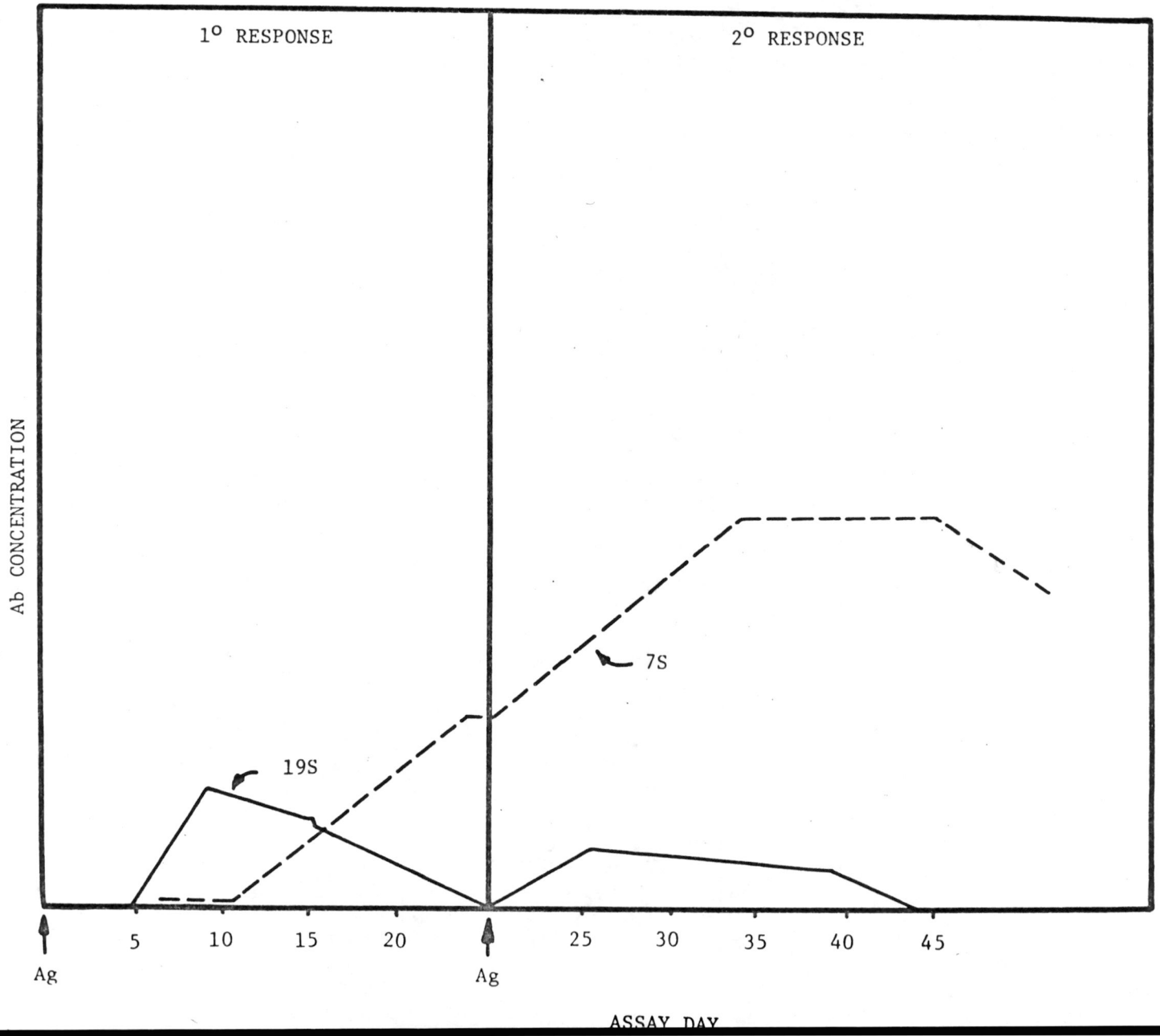

Log antibody concentration in serum
100
10
1
0.1
0.01
plateau
log
exponential
increase
decline
lag
Time (days)

approximately every eight hours. The post-log phases of antibody production vary significantly in the mouse and depend on the type and dose of antigen as well as the quality of antibody produced. During the plateau phase, the rate of antibody production is the same as the rate of elimination from the blood. This phase is followed by a decline that, in some cases, extends from days to months (Makinodan, et al., 1970). While this schematic division is a formal convenience it is actually an oversimplification, for the lymphoid cells do not respond synchronously (Davis, et al., 1968).

When an organism undergoes an immune response for the first time it is called a primary response (Davis, et al., 1968). A primary response generally produces little antibody and is characterized by cells synthesizing 19S immunoglobulin antibody (IgM) followed by the induction of cells synthesizing 7S immunoglobulin antibodies (IgG) (Makinodan, et al., 1970). The terms 19S and 7S refer to the sedimentation constants of the respective immunoglobulins. After a second exposure to the antigen there is an accelerated, intensified, and more prolonged response than in the primary response. This constitutes the secondary, memory, or anamestic response (Weiser, et al., 1969). In the secondary response the 7S antibody-producing cells appear at about the same time as 19S antibody-producing cells, but in much greater magnitude (Figure 2) (Makinodan, et al., 1970).

In addition to primary and secondary responses, there are certain conditions when an organism may not respond to an antigen at all; instead, the antigen is handled as if it were a component of the host

Figure 2. Sequential changes in the type of antibody produced during primary and secondary responses


1° RESPONSE
2° RESPONSE
Ab CONCENTRATION
7S
19S
5
10
15
20
25
30
35
40
45
Ag
Ag
ASSAY DAY

(Makinodan, et al., 1970). This is known as immunological tolerance and according to Makinodan (1970) is the ultimate objective of immunosuppression. Since the production of antibody is a cellular event, it is of interest to discuss the type of cell or cells which produce the immunoglobulins.

Morphologically, antibody-synthesizing cells constitute a heterogenous population (Makinodan, et al., 1970). In the case of certain antigens, including sheep red blood cells (SRBC), the first step in the cellular sequence resulting in antibody formation is macrophage processing (Weiss, 1972). Chen and Hirsh (1972) established the fact that the "in vitro" response of mouse spleen cells to SRBC requires the presence of macrophages.

Although they are not the producers of antibody, various hypotheses for the relation of macrophages to antibody production have been suggested. Weiss (1972) proposes that macrophages engulf particulate or colloidal antigen, process it in some way, and then transmit information to immunologically competent cells which in turn synthesize antibody. Other possibilities include a) antigen-macrophage interaction leading to production of informational RNA, antigen-RNA complex, or some other form of processed antigen (Mosier, 1967); b) solubilization of the particulate antigen with release of immunogenic materials (Shortman and Palmer, 1971); c) "cluster" formation which would facilitate interaction between various lymphoid cells on macrophages (Mosier, 1969).

The location of macrophages also suggests their involvement in antibody production. They lie among lymphocytes and plasma cells and send out processes which follow or indent them; in some states of immunological sensitivity, lymphocytes actually invade the cytoplasm of the macrophages, move about, and then move out (Weiss, 1972).

A second important group of cells in antibody synthesis are lymphocytes. Lymphocytes have essential roles in antibody production (Weiss, 1972). They are small, free cells widely distributed in the blood, lymph, and connective tissue; but especially concentrated in portions of the bone marrow, thymus, spleen and lymph nodes where they are produced and perform many of their functions (Weiss, 1972).

Although they are morphologically indistinguishable, lymphocytes are, in fact, a heterogeneous group of cells. They can be separated into immunologically competent cells, into rapidly dividing and slowly dividing cells, into cells capable of differentiating into erythrocytes and those without that capacity, into cells with antigens on their surfaces and cells without surface-bound antigen, into cells capable of binding complement-antigen-antibody complexes to their surfaces and cells unable to, and into cells with one or another type of immunoglobulin surface receptor (Weiss, 1972).

It is known that multipotential hematopoetic cells or stem cells exist, and there are many indications that these stem cells may be a type of lymphocyte (Weiss, 1972). In young adulthood most of the stem cells reside in the bone marrow. From there some cells migrate directly by way of the blood and lymph to the spleen, lymph nodes, and

peritoneal cavity and are thus called bone marrow-derived cells. Others migrate first to the thymus and from there to the various organs and are thus called thymus-derived cells (Makinodan, et al., 1970).

Claman and coworkers (1966) were the first to demonstrate definitely that the interaction of bone marrow-derived cells and thymus-derived cells was essential for the initiation of certain antibody responses. Later Mosier, et al. (1967) proposed that the events after antigenic stimulation probably occur in the following sequence: the antigen reacts with the thymus-derived cell; the bone marrow-derived cells then make contact with thymus-derived cells that have reacted with the antigen; and then, in response to antigen stimulation, by some unknown mechanism, the bone marrow-derived cells undergo transformation and proliferation and give rise to many antibody-synthesizing cells.

In humoral antibody production, lymphocytes have been accorded the following roles: precursors of the plasma cells which produce antibody (Weiss, 1972); antigen-reactive cells which do not produce antibody but are a prerequisite for antibody production against some antigens (Sell and Asofsky, 1968); as antibody secretors (Weiss, 1972; Sell and Asofsky, 1968; Weiser, 1969).

The cells that are most active in antibody synthesis are the dividing, RNA-rich plasmablasts (Weiser, et al., 1969). The plasmablast is a transitional cell not yet possessed of the differentiated features of a mature plasma cell (Weiss, 1972). It is the mature plasma cell, however, that actually secretes antibody and contains the appropriate morphological structures such as abundant rough endoplasmic reticulum

and well developed Golgi necessary for secretion of the protein (Sell and Asofsky, 1968). The plasma cell is also characterized morphologically by its polar nucleus. These cells are short lived and do not divide (Weiss, 1972). The plasma cells are derived from the multipotential stem cells, or their progeny, but the exact precursor is not known. The relationship of the plasma cell precursors and lymphocytes has already been noted (Weiss, 1972).

The previous discussion of the immune response and antibody-synthesizing cells was designed to highlight the points which might be most susceptible to immunosuppressive agents. It was established that an antigen such as sheep red blood cells must first be processed by macrophages in order to initiate an immune reaction. Therefore, a possible suppressive mechanism could be prevention of antigen-processing. Depending on the antigen, very small quantities are required to induce antibody production; in fact, a state of immunological paralysis may be induced by massive doses of antigen (Weiser, et al., 1970). On the other hand, there is a low-zone tolerance which employs subimmunogenic doses of antigen over a period of time to render the animal tolerant (Nossal, 1969). However, because much more antigen is usually present in an animal than is necessary for antibody production, and because most of this antigen is held in the macrophages, it has been surmised that macrophages may protect animals against immunological paralysis by sequestering excess antigen and permitting only small quantities to reach immunologically competent cells (Weiss, 1972). Thus, a drug or any other agent which interferes with the normal functioning of the macrophage would induce suppression.

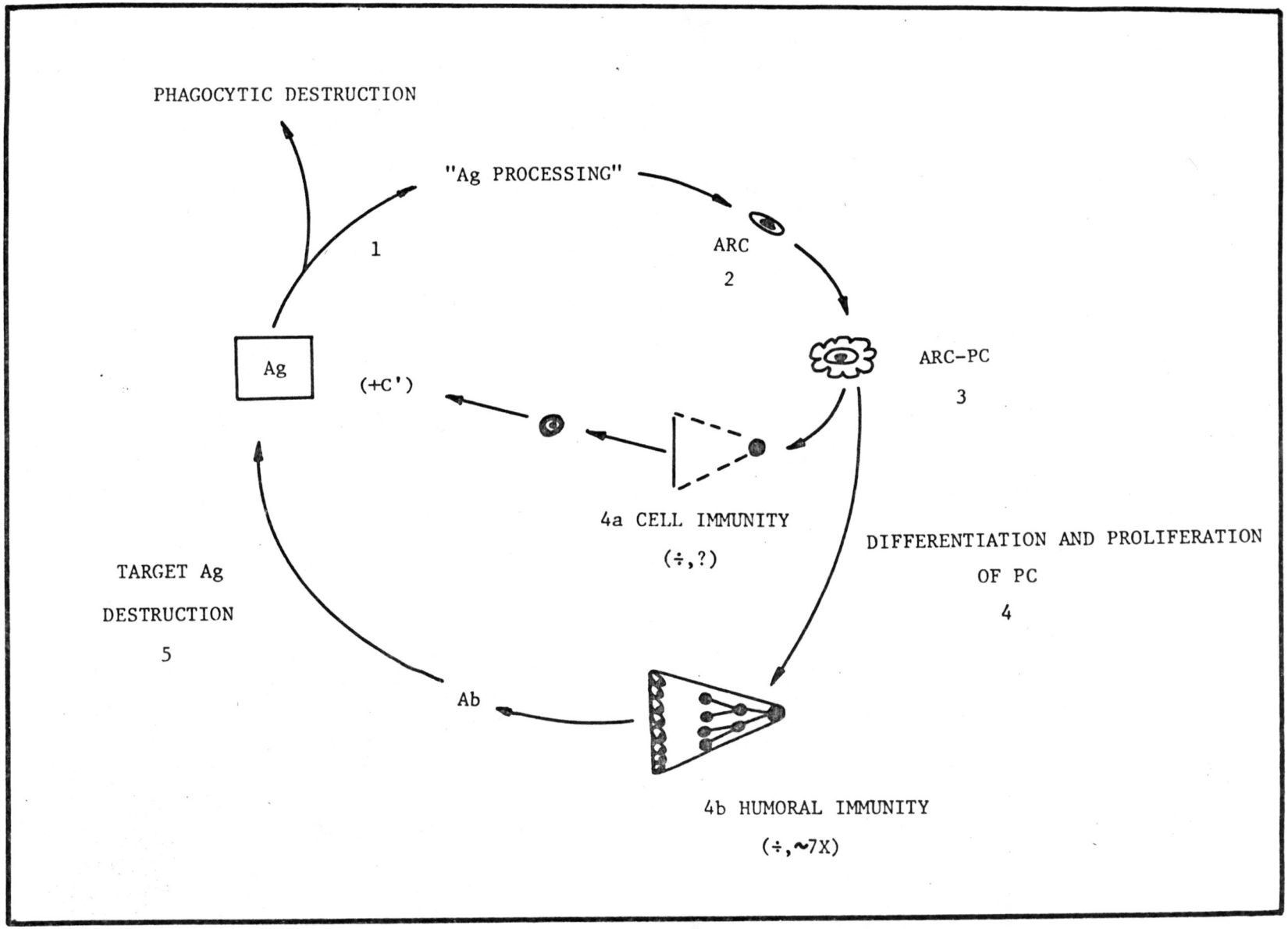
It was also pointed out that at least two cell types, thymus-dependent antigen reactive cells and bone marrow-derived precursor cells, are required for the antigen induced events leading to the generation of antibody synthesizing cells (Makinodan, et al., 1970). The actual mechanism for this interaction is not known, but in both primary and secondary responses precursor cells proliferate and become mature (Makinodan, et al., 1970). This theory reveals several other events in the immune response which might be susceptible to interference by immunosuppressive agents (1 through 5, as illustrated in Figure 3).

Theoretically, events involving the thymus-derived antigen reactive cells can be suppressed by a) destroying the cells, b) neutralizing their ability to react with the antigen, or c) neutralizing their ability to react with the precursor cells. Likewise, events involving the bone marrow-derived precursor cells can be suppressed by a) destroying the cells, b) neutralizing their ability to react with the antigen-reactive cells, c) neutralizing their ability and their progeny's ability to proliferate, d) neutralizing the ability of the progeny to secrete antibody, or e) shortening the life span of the terminal progeny (Makinodan, et al., 1970).

The most difficult phase of the antibody response to suppress is the post-log growth phase involving the terminal antibody-synthesizing cells. These cells are very resistant to injury; thus, in order to develop methods of immunosuppression, emphasis should be placed on interference with the antigen-reactive and precursor cells rather than on the mature plasma cell (Makinodan, et al., 1970).

Figure 3. A model of differentiation within the immune system which shows points possibly vulnerable to the action of immunosuppressive agents. Arrows indicate the direction of differentiation of stem cells.

Ag, antigen
Ab, antibody
PC, precursors of antibody-synthesizing cells
ARC, antigen-reactive cells
Diff., differentiation
Prolif., proliferation
C', complement
$\dot{\div}$, mitotic division


PHAGOCYTIC DESTRUCTION
"Ag PROCESSING"
1
ARC
2
Ag
(+C')
ARC-PC
3
4a CELL IMMUNITY
(÷,?)
DIFFERENTIATION AND PROLIFERATION
OF PC
4
TARGET Ag
DESTRUCTION
5
Ab
4b HUMORAL IMMUNITY
(÷,~7X)

B. Immunosuppression

As was pointed out earlier, exposure to an antigen produces two possible but opposing effects: in moderate doses it can stimulate antibody synthesis; but in very large doses it can inhibit antibody production (Davis, et al., 1968). This inhibitory action is often termed immunological tolerance, paralysis, or immunosuppression (Makinodan, et al., 1970). These terms are used synonymously to describe the observation that exposure to certain antigens, under certain circumstances, leads to a complete absence or great reduction of the expected immunological reactivity toward those antigens instead of eliciting an immune response (Humphrey and White, 1971). The cellular mechanism of induction of immune suppression is not known, but success is greatly dependent upon the dose, route, and time of administration of the antigen; its physical state; and the immunological status of the individual recipient (Makinodan, et al., 1970).

For example, tolerance of an antigen is much more difficult to establish in adults than in newborns (Davis, et al., 1968). This is possibly explained by the observation that once antibody formation has been initiated greater doses of antigen are required to induce tolerance than would normally be required (Davis, et al., 1968). Also, it is difficult to suppress antibody formation in animals that have been previously exposed to antigen (Weiser, et al., 1971). Presumably this is due to the fact that unresponsiveness is not easily induced in memory cells or that the number of inducible cells and/or memory cells is already exceptionally high in the primed animal (Weiser, et al., 1971).

The basis of immunological suppression probably rests on either a destruction of cells or a repressive action of the antigen on a cell type which would otherwise be capable of proliferating, differentiating, and producing antibody (Weiser, et al., 1971). If the latter is the case, it is still not known whether cellular repression involves failure of one or more of the following processes: a) recognition of the "processed" antigen, b) recognition of the antigen-activated antigen-reactive cell previously mentioned, c) differentiation into functional effector cells, or d) proliferation (Makinodan, et al., 1970).

Recent evidence supports the theory that in many cases immunosuppression involves inducible cells and primed cells, but not stem cells (Schwartz, 1966). Therefore, it is possible that natural recovery from the suppression results from new inducible cells arising from non-suppressed stem cells.

Experiments by Nettesheim and Hammons (1970) emphasized the dual effects of immunosuppressive agents on the lymphatic organs. They proposed that, in addition to destruction of the immunocompetent cell population, immunosuppressive agents impaired the capacity of such tissue to capture and retain antigen in their germinal centers. All four immunosuppressants tested (X-ray, actinomycin, cyclophosphoamide, and cortisone acetate), whether given before or after the antigen, impaired the ability of spleen tissue to localize and retain the antigen. It is interesting to note that such damage was more easily induced if the immunosuppressive insult preceded the test antigen. These investigators concluded that the functional significance of antigen depots

in the germinal centers was to provide continuous antigenic stimulation necessary for prolonging antibody production during the late stages of an immune response.

The mechanism by which immunosuppressive agents interfere with antigen trapping and subsequent retention is not known. One possibility is that the antigen-retaining cells themselves are damaged. Another possibility is interference with some auxiliary mechanism such as destruction of lymphocytes producing opsonic factors (Nettesheim and Hammons, 1970).

Chemical agents which act as immunosuppressive agents are quite varied in structure and action; thus, one would expect that different stages of the immune response would show different sensitivities to the action of these agents (Makinodan, et al., 1970). For this reason it has been useful to classify the various immunosuppressive agents according to the stage of the immune response most sensitive to their action. Class I agents are most effective in suppressing an immune response when given just before the antigenic stimulation and are relatively ineffective when given after. The very early processes of the immune response on which these agents are assumed to act include antigen processing and early information transfer (Makinodan, et al., 1970).

Class II agents are most effective as immunosuppressants when given a day or two after the antigenic stimulus. In general, proliferation and differentiation of immunocompetent cells seem to be more sensitive to these agents than other stages. The major action of most of these drugs seems to be killing the cells (Makinodan, et al., 1970).

Class III agents may be immunosuppressive if given either before or after the antigenic stimulus and thus share properties of both Class I and Class II agents. Cyclophosphoamide is the only agent clearly in this class (Makinodan, et al., 1970).

Davis, et al. (1968) proposes two slightly different categories of immunosuppressive agents. They are lympholytic agents and antimetabolites. The lympholytic agents include X-rays, alkylating agents, and eleven-oxycorticosteroids. They damage DNA and their effects are particularly noticeable on the rapidly dividing cells such as the hematopoietic stem cells. Thus, these agents' effectiveness are due not only to reduction in the total population of small lymphocytes, but to interference with the capacity of many surviving cells to undergo multiplication in response to antigenic stimulation. The lympholytic agents are effective immunosuppressors when given before an immunogen is administered.

The antimetabolites, which include purine analogs, pyrimidine analogs, and folic acid antagonists, affect nucleic acid metabolism and damage actively dividing cells. The antimetabolites are probably most effective in the lag phase which occurs between immunogen administration and the appearance of corresponding antibodies in the serum.

C. Ethyl Methanesulfonate

The work reported in the literature to date on ethyl methanesulfonate (EMS) has been confined primarily to its action as a chemical mutagen. It has been shown to be a highly effective mutagen in a wide

variety of organisms. The drug induces high frequencies of sex-linked recessive lethals in Drosophila (Alderson, 1964) and point mutations in barley and maize (Cattanach, Pollard, and Isaacson, 1968). In contrast to its effectiveness in inducing point or gene mutations the ability of EMS to cause chromosome breakage is far less impressive. In Drosophila only a very few translocations occur with the same treatment that causes the high frequency of sex-linked lethals (Lim and Snyder, 1968). In maize and barley chromosome breakage was relatively infrequent (Neuffer and Ficsor, 1963). However, in recent work done in the mammalian system, Cattanach, et al. (1968) showed that EMS did induce high chromosome breakage in the post-meiotic germ cells of the mouse, and that the most sensitive of these cells were the epidymal sperm and late spermatids. The fact that the chemical does produce breakage in the mouse and not Drosophila suggests the possibility of a difference in its mutagenic properties in the two organisms or perhaps the difference is one of comparative doses between the two organisms.

Little or no information has been reported on the drug's effect on other mammalian systems, specifically the immune response. Experiments now underway by Dr. Michael Potter of the National Cancer Institute, Bethesda, Maryland, suggest increases in the incidence of Pristane (2,6,10,14-tetramethylpentadecane) induced plasma cell tumors in BALB/c mice pretreated with EMS (personal communication). A study by Hrushesky, et al. (1972) revealed that EMS is highly carcinogenic in the female rat. Of 66 treated animals, 35 developed histologically confirmed tumors with the highest incidence being adenocarcinomas of the lung and abdominal wall.

The structural formula of EMS is CH_3-SO_3-C_2H_5, and the molecular weight is 124.6. The drug is an alkylating agent which implies the capacity for substitution of its alkyl group (C_nH_{2n+1}) for a hydrogen atom of another molecule. In the case of EMS, *in vitro* studies of the alkylation of the root tip of the bean *Vicia faba* and examination of the alkylation products by paper chromatography showed that EMS reacts specifically with the DNA base guanine, and that 7-ethylguanine is the primary product of the alkylation. The most reactive site of the guanine moiety is the N-7 atom (Scalera and Ward, 1971). This ethylation usually results in a GC-AT transition; however, GC-TA and GC-CG transversions have been shown to occur (Bautz-Freeze, 1961).

As in the case of many drugs, the administered form may not be the effective form. A chemical may be administered, undergo enzymatic or non-enzymatic change in one part of the mammalian body, and then circulate to another anatomical region to react. It also appears that there may be large differences in these variables between one mammalian species and another, and even between two individuals of the same species that have different genetic or environmental backgrounds. Therefore, Cumming and Walton (1970) made a study of the fate and metabolism of EMS at sublethal doses in the mouse in which EMS with a ^{14}C-alkyl label was injected intraperitoneally into the mice and the radioactivity levels measured in various tissues at eight time periods ranging from 15 minutes to 24 hours after injection. Their findings revealed that EMS was rapidly and evenly distributed to all parts of the body and that within 15 minutes most tissues, including the spleen, contained

the total amount of label originally injected. With few exceptions the highest total activity levels were obtained in the first sampling period, 15 minutes after injection. After this the tissues decreased slowly in total activity for the first four hours and more rapidly thereafter. Only 15% of the labeled EMS was excreted in the urine.

The blood level of EMS was substantially lower than tissue levels which implies that the concentration in the various tissues is not passive. According to Cumming and Walton (1970) this suggests that at least part of the concentration in specific tissues is due to the presence of reactive sites susceptible to ethylation by EMS and accumulation of its reaction products. Whether other processes are involved in maintaining a particular level of chemical in a certain tissue is not known.

EMS-induced damage in the mouse is correlated to a great extent with the total alkylation of macromolecules. However, several factors seem to influence the degree of alkylation in tissues. One important factor is the degree to which compounds are hydrolyzed before they have the opportunity to alkylate biologically important molecules. The products of hydrolysis of EMS are ethyl alcohol and methanesulfonic acid. Methanesulfonic acid is excreted rapidly and almost unchanged in the urine; ethanol is largely oxidized to CO_2 and excreted by the lungs. The principal products of alkylation of EMS, on the other hand, are excreted in urinary metabolites with only a small amount being degraded to CO_2. EMS is rapidly hydrolyzed, and hydrolysis competes with the alkylation reaction and may be a limiting factor in the amount of alkylation.

With EMS, as with any other potential suppressant, it is important to know whether effective treatment starts with exposure or some time later, and also to know how long effective treatment lasts.

In attempting to determine if EMS acts as an immunosuppressive agent, the first problem was to determine the normal response of the mouse to an antigen, specifically SRBC, and from this baseline information to determine the effects of ethyl methanesulfonate on the animals' capacity to respond to the same antigen. And, if there are any differences between the two groups, to explain them in terms of what is now known about the kinetics of the immune response and immunosuppression.

MATERIALS AND METHODS

Animals

In vivo experiments utilized three to four month old BALB/cAn mice obtained from the laboratory of Dr. Michael Potter, Laboratory of Biology at the National Cancer Institute, Bethesda, Maryland, and (BALB/cAn X C3H/LW)F_1 hybrids bred at East Carolina University, Greenville, North Carolina.

The animals were housed in clear plastic cages and allowed free access to Wayne mouse chow and tap water. The weight of the mice ranged between 20.0 and 25.0 grams.

Drug

Ethyl methanesulfonate (EMS) was obtained from Eastman Organic Chemicals, Rochester, New York. The EMS was prepared fresh in 0.85% saline at such a concentration that an intraperitoneal (i.p.) injection of 0.2 ml gave a dose of 200 mg per kilogram of body weight to each animal. The drug was administered according to a specific schedule prior to initial exposure to the antigen (see EMS Treatment Schedule of Mice).

Immunization

Sheep erythrocytes (SRBC) suspended in Alseaver's solution were obtained from Scott Laboratories, Chapel Hill, North Carolina. The cells were washed three times in 0.85% saline (w/v) and made up to a final concentration of 10% (v/v) in 0.02M Tris (Hydroxymethyl)amino-methane buffered saline (0.15M) (TBS) (Sigma Chemical, St. Louis,

Missouri). The standard challenge to induce a primary response in the mouse was an i.p. injection of 0.2 ml of the 10% SRBC suspension (4×10^8 cells).

EMS Treatment Schedule of Mice

Five groups of experimental mice with three to four animals per group received 200 mg/kg of EMS according to the following schedule: 1) four hours prior to antigenic stimulation, 2) eight hours prior to antigenic stimulation, 3) twelve hours prior to antigenic stimulation, 4) twenty-four hours prior to antigenic stimulation, and 5) forty-eight hours prior to antigenic stimulation. In all cases the EMS was administered intraperitoneally.

Control mice were divided into two groups: 1) those receiving SRBC, but no ethyl methanesulfonate, and 2) those receiving scheduled doses of EMS but no antigen stimulation.

In an attempt to determine whether or not the drug's effect was species specific BALB/cAn, C3H/LW, and (BALB/cAn X C3H/LW)F_1 hybrids were subjected to multiple injections of EMS, given 24 hours apart, at the following dosages: day 1, 200 mg/kg; day 2, 100 mg/kg; day 3, 50 mg/kg. The SRBC antigen was administered 24 hours after the final injection of EMS.

Assay for Antibody-Forming Cells

Both direct and indirect plaque-forming cells were assayed using the method of Jerne and Nordin (1963) with modifications. Mice were sacrificed five days after a single intraperitoneal injection of sheep

erythrocytes, and the cell contents of each spleen teased out into Eagle's media obtained from Baltimore Biological Laboratories, Cockeysville, Maryland. Day 5 was chosen because plaque-forming cells (PFC) belonging to the 7S immunoglobulin classes do not appear until at least 5 days after immunization while IgM PFC are still at a significantly high level (Sell, Park, and Nordin, 1970). The spleen cells from each mouse were washed in Eagle's media and the pellet reconstituted to a total volume of 2.0 ml in fresh Eagle's media by gently aspirating through 18 gauge and 25 gauge needles, respectively. An aliquot of these immune cells (0.1 ml) was saved for determination of percent viability and cell counts. The remainder of the cells were kept at 4°C for later use.

Cell viability was assessed by trypan blue exclusion according to the method of Kaliss (1969) and the dye-positive cells counted directly with a hemocytometer. Total spleen cell counts were performed on the Fisher Autocytometer II (Fisher Scientific Co., Fairlawn, New Jersey).

For plating, 100 X 20 mm Falcon plastic petri dishes were used. These plates had previously been provided with a bottom layer of ten milliliters of 1.2% Difco agar dissolved in TBS.

Immediately before plating, 0.2 ml of 0.6% SeaPlaque agarose (Marine Colloids, Rockland, Maine), 0.2 ml of 25% SRBC (v/v), and 0.1 ml of spleen cells were added to 12 X 75 mm tubes in a 37°C waterbath. The tubes were inverted and the contents poured into the plates and

gently swirled to form a thin, even overlay of agar. All plates were prepared in duplicate and left at room temperature approximately ten minutes to solidify.

The plates were then incubated in a moisture saturated environment at 37°C for one hour. At the end of one hour complement was added in the form of two milliliters of guinea pig serum (Baltimore Biological Laboratories, Cockeysville, Md.) diluted 1:10 in TBS, and the plates were incubated at 37°C for a further one-half hour. After pouring off the guinea pig complement, the plaques which are circular hemolytic clearances in the sheet of red cells were counted using the 2X magnification of a dissecting scope equipped with diffuse light.

The indirect or facilitated plaque test was carried out according to the method described by Walters and Jackson (1968) by adding 0.1 ml of a 1:50 dilution of rabbit anti-mouse IgG directly to the tubes containing the overlay agar. The plates were then prepared, treated, and counted exactly as described previously. The number of IgG-forming cells were ascertained by subtracting the number of IgM PFC that developed with complement alone from the number of plaques that occurred with complement and rabbit anti-mouse IgG.

Production of Anti-gamma G

In order to apply the plaque technique to the detection of 7S antibody-producing cells, antiserum specific for the 7S antibody was added to the system. The antiserum was raised in a rabbit by three weekly injections of purified mouse IgG. Purified IgG, which was generously supplied by Dr. K. R. McIntire of the National Cancer

Institute, was obtained from the ascites fluid of BALB/cAn mice bearing the plasma cell tumor, MOPC 821. The first injection of 250 μg of IgG was given intramuscularly in complete Freund's adjuvant; the second intramuscular injection consisted of 250 μg of IgG in incomplete Freund's adjuvant; the final injection was 100 μg of IgG dissolved in saline and administered subcutaneously. Complete and incomplete Freund's adjuvant are distinguished by the presence or absence of modified tubercullin baccillus, respectively. Twenty-one days after the initial injection, approximately five milliliters of rabbit blood was obtained from the marginal ear vein. After centrifugation (600xg) for 15 minutes, the serum was separated from the packed cells and stored at -20°C. Later μ and K chain specificity was removed from the antisera by absorption with the purified mouse myeloma proteins MOPC-104E (IgM) for μ and TEPC-15 (IgA) for the K chain. Absorption involved adding 2 to 3 drops of the absorbing protein to 2 ml volumes of anti-IgG and incubating one-half hour at 37°C. This was followed by overnight incubation at 4°C and centrifugation at 600xg for 10 minutes to remove the precipitate.

In addition, the rabbit was given two subcutaneous boosters of 100 μg IgG dissolved in saline at weekly intervals beginning seven days after the first bleeding. The animal was exsanguinated two weeks after the final booster and the serum processed in the aforementioned manner. The Ouchterlony gel double-diffusion technique was used to determine antibody activity (antiserum tested against a solution of purified IgG) and antibody specificity (antiserum tested against whole

rabbit serum (See Double Diffusion in Agar). A single precipitin line between the well with whole rabbit serum and the well with the test serum indicated that the antiserum was monospecific for IgG. Specificity was further verified by performing immunoelectrophoresis on the antiserum (See Immunoelectrophoresis).

The number of plaques formed varies greatly with the concentration of anti-IgG serum (Walters and Jackson, 1968; Sterzl and Riha, 1965; Sell, et al., 1970); therefore, a dilution of antiserum was chosen which produced the maximum of 7S PFC without inhibiting the 19S-producing cells. The 19S-PFC may be inhibited by concentrations above the optimal dilution, presumably because of the presence of antibody directed to the light chains, which are common to both 7S and 19S molecules (Sterzl and Riha, 1965). To determine the most efficient dilution of antiserum, dilutions of anti-IgG from 1:10 to 1:100 were added to a series of plates containing the spleen cells of a mouse immunized five days previously with SRBC and the resulting plaques counted. The "optimal dilution" of 1:50 was chosen for use in the experiment since greater dilutions did not significantly increase the number of indirect PFC.

Double Diffusion in Agar

An agar double-diffusion test, the Ouchterlony, is a simple and reliable test for the detection of antibody. One adaptation of this test employs wells containing the test reagents (i.e. antigen and serum being tested for antibody presence) arranged in a layer of agar

so that antigen and antibody diffuse toward each other simultaneously (Weiser, et al., 1969). As a result of the reactants diffusing from the wells, precipitin bands are formed where they meet in equal proportions. The results are interpreted in terms of the number of precipitin lines, closeness to the wells, curvature of the precipitin lines, and the relationship of the precipitin lines of one well to the precipitin lines of adjoining wells.

To carry out this technique, 3" x 1" glass microscope slides were placed singularly on a warm hot plate and supported by glass rods. They were then painted, using a soft paint brush, with 0.5% Difco-Bacto-Agar dissolved in distilled water. The slides were brushed only in one direction and allowed to dry. After the base agar had dried, three milliliters of a 1% solution of ionagar dissolved in Tris (hydroxymethyl) aminomethane-acetate, 0.05M, pH 8.2 was gently pipetted onto each slide. After the agar solidified, the slides were stored at $4^{o}C$ in moistened chambers until ready for use.

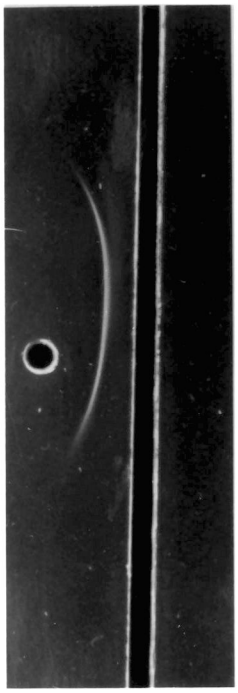
On the day of use an appropriate geometric design was cut into the agar on the slide leaving open wells to be filled with the test reagents. In this case, 0.01 ml of anti-IgG serum was delivered to the center well with an elongated Pasteur pipet. Two opposing wells were filled, one with 0.01 ml of whole rabbit serum and the other with an equal volume of purified IgG. The slide was left in a moistened chamber at room temperature and read approximately four hours later.

The Ouchterlony was then washed overnight in a container of saline and allowed to air dry. This reduced the agar to a thin, dry film that was stained with courcon stain. The slide was totally emersed in the stain for approximately 30 to 45 minutes to obtain the desired depth of staining. Destaining was accomplished by placing the plate into 2% (v/v) acetic acid.

Immunoelectrophoresis

In order to determine the specificity of the anti-IgG serum an immunoelectrophoresis was performed on the antiserum. First, glass lantern slides (3" x 2") are painted with a thin layer of agar as previously described in the section on Ouchterlonys. These slides are then coated with 15 milliliters of a 1% solution of Panagar (Batch #052-7665, Colab Labs, Chicago, Illinois) dissolved in 0.05 M Barbitone Acetate buffer pH 8.6 also obtained from Colab Labs. When the coatings have solidified, an appropriate geometric design is cut into the agar and the wells filled with 0.01 ml rabbit anti-whole BALB/cAn serum. The slide is then placed in the electrophoresis chamber (Colab Labs, Chicago, Illinois) under the following conditions: 150 volts; 45 milliamps; 60 minutes. The test antiserum was then added to the appropriate troughs and precipitin lines allowed to develop overnight at room temperature. Figure 4 was taken in a Cordis immunodiffusion camera (Cordis Labs, Miami, Florida).

Figure 4. An immunoelectrophoresis plate showing the specificity of the anti-IgG antiserum. Whole mouse serum was placed in the well and the anti-IgG reagent in the trough.



Haemagglutination Assay

Before the mice were sacrificed for the PFC assay, the animals were bled by cardiac puncture. The blood was allowed to sit at room temperature for approximately ten minutes and the serum separated as before. Haemagglutination tests were then performed using the Cooke microtiter system (Cooke Engineering Co., Alexandria, Virginia) to determine the anti-SRBC humoral antibody titer of the serum. With micropipets 0.025 ml of modified barbitol buffer containing 1% normal rabbit serum (NRS) was added as a diluent to each well of the microtiter plate. Antisera was added with 25 λ diluting loops and doubling dilutions were completed to 12 dilutions. Finally, one drop (0.025 ml) of 2% (v/v) SRBC was delivered to each well, the plate gently swirled, and then incubated for one hour at 37°C. The endpoint of the titration was the highest dilution showing any agglutination. The antibody titer was expressed as the reciprocal of the greatest dilution that agglutinated. Differences of two or more dilutions were considered to be significant.

Statistical Analysis

A comparison of the number of PFC in each EMS treatment group with those of control animals was made by the two-tailed Student t-test (Weast, 1969). Since the numbers of PFC for the various groups do not show a normal distribution (Sell, et al., 1970), the comparisons were performed using the logarithms of the number of plaques for each

experimental group. The t-tests were performed using the Olivetti Programma 101 desk computer, and p-values obtained from a statistical table of the distribution of t (Weast, 1969). Significance was considered demonstrated with p was <0.05.

RESULTS

A. The effect of a single injection of EMS on the 19S plaque-forming cells of BALB/cAn recipients

Table I summarizes the result of EMS exposure on the 19S plaque-forming capacity of spleen cells at these time intervals. In order to compare the data, results were expressed as the number of plaque-forming cells per 10^6 spleen cells plated.

As seen in Table I, a single intraperitoneal injection of EMS (200 mg/kg) depressed the normal immune response of the mice to sheep erythrocytes when administered 4, 8, and 12 hours prior to antigenic stimulation. The maximal sensitivity to the drug occurred at 8 hours prior to the injection of SRBC. Control animals in the experiment received 0.2 ml of a 10% solution of SRBC (4×10^8 cells). In addition, normal mice were injected with various concentrations of EMS to determine the lethal dose of the drug. Eight hundred mg/kg killed 100% of the animals within 24 hours, and prior to death they all exhibited extremely nervous and agitated behavior.

When given 24 hours prior to immunization with SRBC, the EMS had an intermediate effect on the immune response with the number of PFC increasing, but still significantly different from the control values. When the drug was administered 48 hours before the antigen, there was an apparent enhancement of the PFC response.

Application of the t-test confirmed that there was a significant difference between control data and the 4 hour, 8 hour, 12 hour, and even the 24 hour experimental groups.

TABLE I

THE EFFECT OF EMS ON DIRECT (19S) PLAQUE-FORMING CELLS IN BALB/cAN MICE[1]

Treatment[2]	No. of Cells/Spleen	Avg. #PFC/Plate	PFC/10^6 Spleen Cells
Control	1.60×10^7	814 ± 406	1159 ± 658
4 hour	1.65×10^8	1283 ± 610	226 ± 66
8 hour	1.53×10^8	1227 ± 633	186 ± 89
12 hour	1.18×10^7	178 ± 132	373 ± 334
24 hour	1.48×10^7	606 ± 478	813 ± 647
48 hour	1.45×10^7	1103 ± 362	1431 ± 608

[1] Each experimental point consisted of 6-12 animals.

[2] Control animals received only SRBC; assayed 5 days after immunization.

Both male and female mice were used in these experiments since pre-testing with control animals showed no significant difference in the number of hemolytic plaques formed between the two sexes. Background PFC were detected in non-immunized mice at a rate of 9 PFC/10^6 spleen cells. This spontaneous occurrence of specific antibody-forming cells represents natural occurring antibodies to SRBC in the mouse system, and the number of plaques was subtracted from all experimental results.

B. The effect of a single exposure of EMS on the 7S plaque-forming cells of BALB/cAn recipients

In studying the mode of action of an immunosuppressant, it is important to study the agent's effect on both the 19S and the 7S response. Table II shows the effect of EMS treatment on the 7S antibody-forming cells at 4, 8, 12, 24, and 48 hours before antigen administration. During this phase of the immune response EMS significantly reduced the plaque-forming capacity of BALB/c spleen cells when given to the animal four to eight hours prior to antigen. As in the case of the 19S plaque-forming cells, the maximal sensitivity to the drug occurred at 8 hours. None of the other experimental results varied significantly from controls. Once again the t-test was used to show that the difference between the 4 hour and 8 hour groups was significant when compared to controls. Table II also shows an increase in the number of PFC over controls at 48 hours. This appears to be consistent with the increase in the PFC in the 19S 48 hour group (Table I).

TABLE II

THE EFFECT OF EMS ON INDIRECT (7S) PLAQUE-FORMING CELLS IN BALB/cAN MICE

Treatment	No. of Cells/Spleen	Avg. #PFC/Plate	PFC/10^6 Spleen Cells
Control	6.3×10^7	1381 ± 1255	833 ± 538
4 hour	1.78×10^8	2475 ± 27	281 ± 13
8 hour	1.53×10^8	401 ± 42	57 ± 17
12 hour	1.03×10^7	693 ± 616	1133 ± 899
24 hour	1.27×10^8	1750 ± 740	1011 ± 996
48 hour	2.11×10^7	3070 ± 1185	3300 ± 1827

C. The effect of treatment with EMS on haemagglutination titers of BALB/cAn mice

Table III shows the haemagglutinin responses of EMS-treated and normal mice in relation to the time of the drug treatment before antigen injection. Titers are expressed as the reciprocal of the highest dilution showing agglutination.

When EMS was administered 4 hours before the antigen there was no depression of the HA titer. However, as seen from Table III, a single injection of EMS (200 mg/kg) depressed the haemagglutinin response of BALB/c mice to SRBC most markedly when given 8 to 12 hours prior to SRBC. There was a reduction of three dilutions from control values in both the 8 and 12 hour experimental groups. This decrease was concomitant with the observed decrease in both the 19S and 7S plaque-forming cells.

D. The effect of multiple EMS injections on the plaque-forming capacity of BALB/cAn, C3H, and (BALB/cAn X C3H)F_1 mice

Table IV shows the effect of multiple EMS injections on the plaque-forming capacity of BALB/cAn, C3H/LW, and (BALB/cAn X C3H/LW)F_1 recipients. In all cases, multiple EMS injections depressed the number of plaque-forming cells from that of controls.

The data revealed that in BALB/cAn the degree of suppression due to multiple drug exposure was greater than any of the five experimental points in Table I in which the animals received only a single i.p. injection of EMS. Even the number of plaques in the most sensitive 8 hour group was significantly greater than the multiply injected group.

TABLE III

THE EFFECT OF A SINGLE EMS TREATMENT ON AGGLUTINATION TITERS IN BALB/cAn MICE

Time of Treatment[1]	Mean Titer[2]
Controls:	
No EMS	256
No Immunization	0
4 hours	256
8 hours	16
12 hours	16
24 hours	256
48 hours	128

[1]Time of EMS treatment prior to a single injection of SRBC (0.2 ml of 10%). Titers measured 5 days after immunization with SRBC.

[2]Titer expressed as the reciprocal of the highest dilution showing agglutination.

TABLE IV

THE EFFECT OF MULTIPLE EMS INJECTIONS ON THE PFC RESPONSE OF BALB/cAN, C3H/LW, (BALB/cAN x C3H/LW) F1

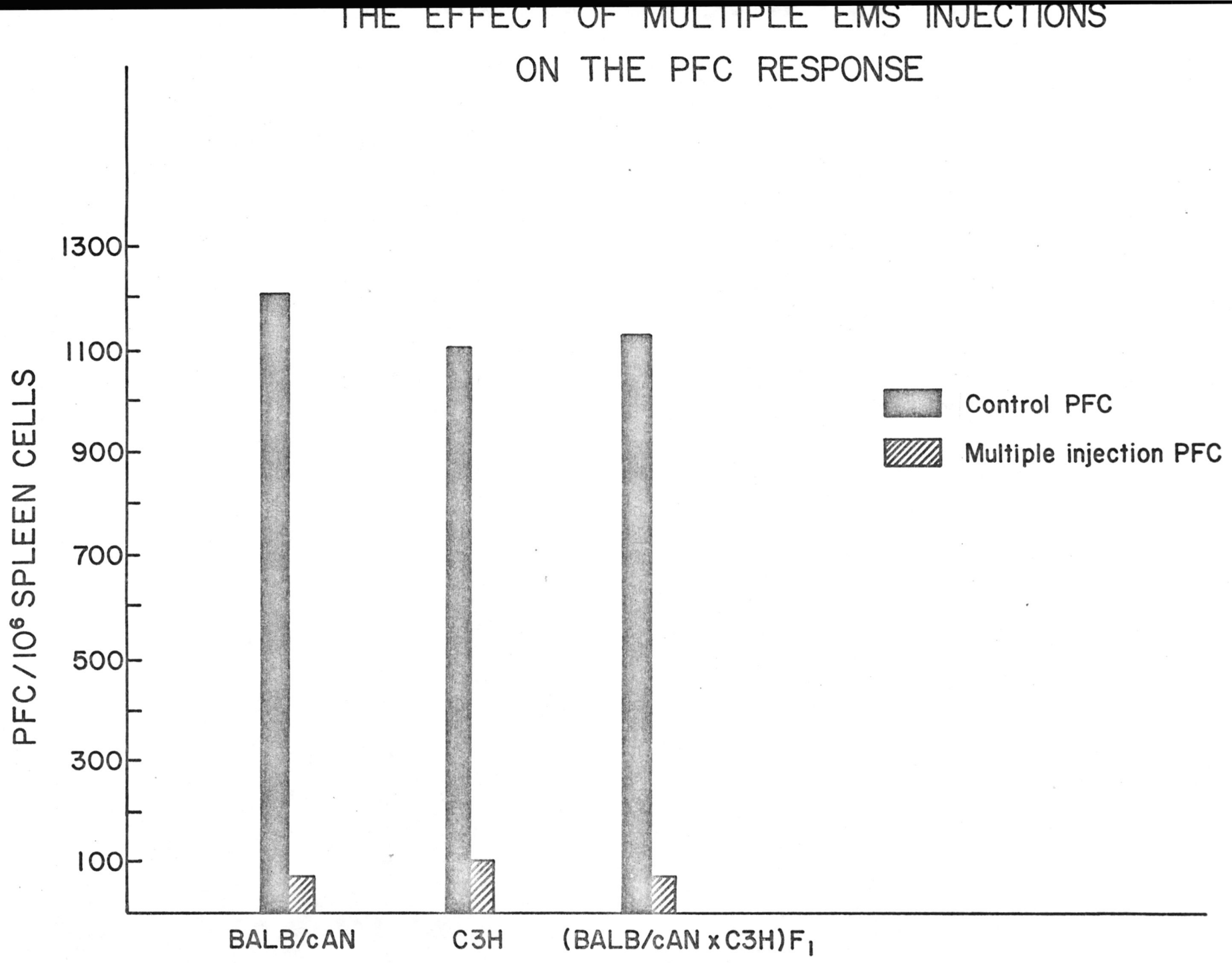
Treatment[1]	Avg. #PFC/Plate	PFC/10^6 Spleen Cells
BALB/cAN		
1. Controls	814 ± 406	1159 ± 658
2. Experimental	63 ± 72	65 ± 70
C3H/LW		
1. Controls	1658 ± 369	1179 ± 362
2. Experimental	88 ± 22	102 ± 38
(BALB/cAN x C3H/LW) F1		
1. Controls	2113 ± 324	1123 ± 144
2. Experimental	38 ± 24	60 ± 37

[1] Experimental animals received three injections of EMS 24 hours apart of 200 mg/kg; 100 mg/kg; 50 mg/kg, respectively.

In terms of the number of PFC/10^6 cells, the BALB/cAn mice exhibited greater suppression than the C3H/LW. But, the 19S PFC response of the (BALB/cAn X C3H/LW)F_1 mice subjected to multiple EMS injections was not intermediate between the two parent strains as might be expected. Instead, the F_1 animals' response was comparable to that of the BALB/cAn.

Using the Student t-test, the differences between experimental and control results proved to be statistically significant.

Figure 5. The effect of multiple EMS injections on the PFC response


THE EFFECT OF MULTIPLE EMS INJECTIONS
ON THE PFC RESPONSE
PFC/10⁶ SPLEEN CELLS
1300
1100
900
700
500
300
100
Control PFC
Multiple injection PFC
BALB/cAN
C3H
(BALB/cAN x C3H)F1

DISCUSSION

Examination of the experimental results revealed that ethyl methanesulfonate (EMS) acted as an immunosuppressant in the mouse, but that its effectiveness is directly related to the time of administration prior to antigenic stimulation by sheep erythrocytes. When given 4, 8, or 12 hours prior to antigen, EMS at a concentration of 200 mg/kg caused significant suppression of the normal immune response. The maximal sensitivity to the drug occurred 8 hours prior to injection of SRBC. There was no suppressive effect when EMS was given after antigenic exposure (Smith, personal communication).

In the murine system the development of an antibody response to SRBC has been shown to be both macrophage and thymus-derived lymphocyte (T-cell) dependent (Chen and Hirsh, 1972; Kappler and Hoffman, 1973). This means that in a normal sequence of events antigen processing by macrophages is a prerequisite for antibody production in response to sheep erythrocytes, and that it is necessary for the T-cell to react with the processed antigen and then upon contact with the bone marrow-derived lymphocyte (B-cell) stimulate the B-cells to divide and eventually produce antibody. This pathway provides several points at which EMS might act. The question is, which of the cell types involved is most susceptible to the drug's action?

The work of Cumming and Walton (1970) provides direct evidence that EMS reaches target organs, including the spleen, unaltered and free to act when injected intraperitoneally; and, it is in the spleen where most, if not all, antibody-forming cells first appear in the

mouse following immunization with SRBC (Sell, Park, and Nordin, 1970). Therefore, one obvious reason leading to the unresponsiveness is that the spleen is depleted of one or more cell populations necessary for the development of the PFC response in the EMS pretreated mouse. It has been established that EMS is an alkylating agent which acts specifically on the N-7 moiety of the deoxyribonucleic acid (DNA)-base guanine (Scalera and Ward, 1971). Thus, direct interference with DNA synthesis of the immunocompetent spleen cells is the probable mode of action of the drug.

It is now considered that most, if not all, of the 19S antibody-forming cells arise as a result of the rapid proliferation of a small number of immunocompetent precursor cells (Sell, Park, and Nordin, 1970). These cells, however, constitute a large number of different classes each capable of synthesizing antibodies of one, or perhaps a few, specificities. The different classes are called clones and this concept is the Clonal Selection Theory of antibody formation (Burnet, 1959). It is possible that EMS suppressed normal 19S antibody production by alkylation of the DNA of these precursor cells thus preventing the rapid proliferation which would normally ensue in response to the antigen. Recent work by Nakamura, et al. (1972) has shown that at least one replication of DNA is critical for the induction of a primary antibody response. They also pointed out that his prerequisite was true for both helper T-cells and the precursors of antibody-producing B-cells. Therefore, the alkylation which occurs due to the presence of EMS in the mouse system could prevent this critical replication, and thus prevent antibody formation.

Since examination of the experimental results has revealed that the unresponsiveness induced by ethyl methanesulfonate is short-termed and that in approximately 24 hours the animal is capable of mounting a normal response, it can be assumed that the ultimate stem cells involved in antibody production, that is those residing in the bone marrow, are not affected by the drug. However, EMS may act specifically on either the bone marrow-derived lymphocytes or the thymus-derived lymphocytes in the spleen. The present study is a broad examination of the immunosuppressive capacity of EMS, but the specific cell line involved could be determined as follows. Administer EMS 8 hours prior to antigen stimulation; instead of sacrificing the animals 5 days after injection of SRBC, wait 14 days, rechallenge with SRBC, and 5 days after the second injection perform the PFC assay. If there is a heightened response, one could assume that at the initial exposure to the antigen, the thymus-derived cells had been primed and on later contact with the same antigen were able to mount an anamnestic response. Such results would imply that EMS was selective for the bone marrow-derived precursor cells and prevented them from producing antibody, but by the time of the second injection of SRBC, the spleen had been repopulated with B-cells and could respond to the information relayed by the memory T-cell. Such results would at the same time virtually eliminate the possibility that EMS interfered with macrophage processing.

On the other hand, if the depression continued after a second injection of SRBC one could conclude that EMS had specifically interfered with the activity of the T-cells by either preventing uptake of

information from the macrophage or inhibiting relay of this information to the antibody-producing B-cell. Continued depression also includes the possibility that EMS is not selective and alkylates all dividing cells indiscriminately.

As shown in Table I, the suppression of 19S PFC at 4, 8, and 12 hours appears to be the result of the inability of the impaired precursor cells to divide and probably represents the degree of alkylation of these cells. The greatest suppression occurred when EMS was given 8 hours before SRBC and this result may be interpreted in several ways. Perhaps, but not likely (Cumming and Walton, 1970), it is 8 hours before the drug reaches the spleen. More plausible is the theory proposed by Cumming and Walton (1970) in their study of the metabolism of EMS. They discovered that there was an increase in the nucleic acids of the testis for the first 8 hours after ^{14}C labeled-EMS injection and interpreted this to mean that the alkylation of or the incorporation of alkylated products into DNA goes on for 8 hours after injection. This hypothesis was collaborated by data obtained from analyzing a nonvolatile sample of various mouse tissues. The nonvolatile fractions represent label transferred by an alkylation reaction to some other molecule, and the results revealed an increase of radioactive counts (i.e. alkylation products) in the spleen up to 8 hours after injection of EMS. This accumulation of alkylation products in the spleen helps to explain the severe depression at eight hours post-injection. Approximately 42% of the injected label is still in the spleen 8 hours after injection (Cumming and Walton, 1970) and

is capable of alkylating the precursor cells which are continually recruited from the supply of stem cells in the bone marrow; the accumulation of alkylation products helps account for the maximum suppression seen when EMS is administered 8 hours before the antigen.

Notice, however, that the immunosuppressive effect of EMS is only temporary. From the 12 hour to the 48 hour experimental points there is a gradual increase in the number of PFC over that of the 8 hour group. This recovery is due to the elimination of EMS from the spleen by hydrolysis. As pointed out earlier, EMS is easily hydrolyzed, and the hydrolysis reaction actively competes with the alkylation process limiting the amount of alkylation (Cumming and Walton, 1970). According to Cumming's data, only 349 radioactive counts per minute (16%) of a total 2200 injected counts remain at the end of twenty-four hours. Thus, all EMS would be expected to have been eliminated from the mouse body within 48 hours. The newly recruited precursor cells which have repopulated the spleen are therefore unaffected and capable of responding to the antigen in a normal fashion. This theory appears to be consistent with what is currently known about the estimated turnover time of antibody-producing cells which is between eight and twelve hours (Makinodan, et al., 1970; Weiss, 1972). The significant increase in PFC at 48 hours from that of controls is suggestive of an overshoot phenomenon which is often exhibited by populations regenerating after severe depletion (Marbrook and Baugley, 1971).

The fact that EMS is not a suppressant when given after the antigen as shown by Smith (personal communication) appears to be

consistent with the kinetics of a normal response. According to Makinodan, et al. (1970) the most difficult phase of the antibody response to suppress is the post-log growth phase. So, once antibody formation has been initiated, it is difficult to induce suppression with the same dose that would normally suppress the system (Davis, et al., 1968).

The possibility that EMS may function as an immunosuppressant by interfering with antigen retention in the spleen may also be considered. Nettesheim and Hammons (1970) experimented with four immunosuppressants (X-ray, actinomycin, cyclophosphoamide, and cortisone acetate) and found that in addition to destruction of immunocompetent cells, all four agents impaired the ability of spleen tissue to localize and retain antigen. The mechanism by which these agents interfere with antigen trapping and subsequent retention is not known. One possibility is that the antigen retaining cells themselves are damaged; another possibility is interference with some ancillary mechanism such as the destruction of lymphocytes elaborating opsonic factors.

The data on 7S plaque-forming cells (Table II) appears to follow the same pattern exhibited by the 19S PRC; that is, early suppression followed by complete recovery from the drug's effect. Once again the most pronounced suppression occurred when EMS was injected eight hours prior to SRBC.

Determining the origin of antibody-forming cells belonging to the 7S immunoglobulin classes is complicated by the fact that IgM antibody

has already appeared (Sell, et al., 1970). Some of the possibilities of their origin include: a) proliferation of a small number of precursor cells each restricted to a given immunoglobulin class (Miller and Mitchell, 1968); b) proliferation of a small number of precursor cells that are not restricted to an immunoglobulin class during proliferation but become restricted during differentiation (Sell, 1967); c) recruitment of a new and separate group of potential antibody-producing cells restricted to each immunoglobulin class; d) a switch of expression of cells restricted to producing 19S immunoglobulin antibody to a given 7S class (Nossal, et al., 1964). Regardless of which of these alternatives is accepted to account for the origin of 7S antibody-forming cells, it appears that EMS does not select one immunoglobulin class over another, but instead inhibits both from secreting their respective antibodies. This infers that the B-cells are the most sensitive to EMS and thus explains the corresponding suppression of both 19S and 7S PFC as the spleen cells are indiscriminately alkylated by EMS.

Again, there is the possibility that the suppression of 7S PFC could be accounted for by the interference with antigen retention as proposed by Nettesheim and Hammons (1970) and alluded to previously. The impairment of their capacity to capture and retain antigen is of particular significance for late, particularly 7S, antibody production.

The haemagglutination assay is a quantitative test for the detection of the amount of antibody in the blood serum. The results of this assay on serum from control and EMS-treated mice verified the

fact that EMS did suppress the immune response of the animals in question and was most effective in suppressing humoral antibody production when administered eight to twelve hours before antigenic stimulation (Table III). This reduction in antibody titer is a reflection of the inability of the alkylated lymphatic cells to produce antibody.

The effect of single intraperitoneal injection of EMS on the immune response is a reflection of the cellular events in the spleen such as cellular differentiation and proliferation. In untreated controls, SRBC-specific precursor cells of the spleen are selectively stimulated by antigen with the help of T-cells; these precursors then rapidly divide and differentiate into cells actively secreting antibody which appears in the spleen with exponential kinetics (Kappler and Hoffman, 1973). However, this pattern was altered when EMS was given to mice at various times prior to immunization with SRBC. The drug is an alkylating agent and is capable of alkylating the chemicals within the spleen cells thus preventing their normal differentiation and proliferation in response to SRBC. On the other hand, EMS is easily hydrolyzed and as it is eliminated, new precursor cells in the bone marrow are able to mount a normal antibody response with no signs of suppression when challenged with sheep erythrocytes.

After examination of the effects of a single injection of EMS, it became of interest to observe the effect of multiple exposures to the drug on the BALB/cAn's response to SRBC. As seen in Table IV, three injections of EMS suppressed the number of 19S PFC significantly.

In the BALB/cAn the number of PFC decreased approximately 20-fold from control mice, and this suppression induced by multiple injections was even more severe than the previously described eight hour low point. In addition to BALB/cAn, C3H/LW and (C3H/LW X BALB/cAn)F_1 were subjected to multiple injections to determine whether or not the severe suppression caused by the drug might be species specific. Once again the multiple exposures produced severe suppression in all three strains. After three injections of EMS the BALB/cAn and the F_1 response was most depressed and the C3H/LW the least. Statistically, the difference between the BALB/cAn and the C3H/LW was significant (N=11, p=2.23, t=5.23); however, the number of (BALB/cAn X C3H/LW)F_1 PFC was not significantly different from the BALB/cAn, but was from the C3H/LW. Knowing that strain differences in the pharmacological response of mice to many drugs do exist (Meier and Fuller, 1966), this data may be suggestive of a species difference in EMS metabolism which has been reported in other, particularly genetic, EMS studies (Generoso and Russell, 1969; Cumming and Walton, 1970).

The severe suppression caused by multiple EMS injections could possibly be due to the depletion of the plaque-forming cell population. This effect may be similar to the phenomenon suggested by DeWys and Knight (1960). They proposed that sublethal damage due to cyclophosphoamide may be accumulated and that the induced damage is cell-cycle dependent. Perhaps EMS damage is accumulated, also, over the three 24 hour intervals of multiple injection treatment. But, when given as a single injection, EMS may be degraded at a rate nearly equaling

activation (i.e. hydrolysis versus alkylation); therefore, toxic levels are not reached with low doses but may be attained with higher doses. On the other hand, the severe suppression due to multiple exposures to the drug may merely be a matter of a greater concentration of EMS being present in the mouse system over a longer period of time. The lack of total suppression is again a matter of competition between hydrolysis and alkylation.

It must be pointed out, however, that any conclusions drawn from these experiments are applicable only under the described conditions. These include dose of EMS, route of administration, time of administration in relation to time of antigen exposure, etc. Under these conditions, the present data indicate that a single intraperitoneal injection of ethyl methanesulfonate induces short term unresponsiveness in BALB/cAn mice subsequently challenged with SRBC. The immunosuppressive capacity of EMS is greatest when it is given eight hours prior to SRBC, and its effectiveness is most likely related directly to its alkylating properties. As a result of the alkylation of the immunocompetent cells in the spleen, further replication and differentiation is inhibited accounting for the lack of antibody response. However, as the EMS is hydrolyzed and eliminated from the mouse, new immunocompetent cells, which are continually being recruited from the bone marrow, are available to respond to the antigen in a normal fashion. This accounts for the observed recovery from the suppressed state.

Multiple injections on the other hand, produce much more drastic results. After three injections of EMS there is a significant

depression of the immune response which may be due to accumulation of sublethal effects of the drug or perhaps merely to its presence in the system for a longer period of time which would allow for continual alkylation of the newly recruited cells.

In summary, this study has shown that under appropriate conditions ethyl methanesulfonate (EMS) does act as an immunosuppressant. However, further application of the use of the drug in clinical immunotherapy would require more extensive investigation. The practical goal of use of these agents in organ transplants and treatment of autoimmune disease is self-evident. But, these agents can cause acute toxicity, possibly death, and many have equally dangerous long-term effects. It becomes a question of their benefits outweighing their hazards. The presently available agents do not represent the ultimate in immunosuppression, and perhaps, EMS in its immunosuppressive capacity may be used to dissect and further examine many aspects of the immune response.

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