MONTANA STATE UNIVERSITY

Alteration of the graft-versus-host reaction by endotoxin
by Bynum McNeil Jackson

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Microbiology
Montana State University
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Abstract:
The ability of an aqueous-ether extract of Salmonella enteritidis (endotoxin) to influence the ability of spleen cells from adult CBA mice to produce graft-versus-host (GVH) disease in neonatal Balb/c animals was investigated. Adult animals were treated in vivo with 7 daily 30 µg injections of endotoxin. Adult spleen cells were treated in vitro with 30 µg of endotoxin for one hour. Doses of either 1 x 107 or 2x10 CBA adult spleen cells produced surprisingly similar findings when injected into Balb/c neonates. The degree of GVH disease produced was not remarkably different when assessed by weight loss plots and the Simonsen assay. In vitro treatment of spleen cells by endotoxin failed to have a demonstrable effect on the GVH reaction as assessed by weight loss and by mortality. Conversely, treatment of donor animals with 30 µg of endotoxin daily for 7 days demonstrated protection to the neonate receiving cells both in regard to weight loss and mortality.

The protective effect was demonstrated to be due to a factor other than immunologic abrogation of the GVH reaction. Animals which demonstrated protection against weight loss and mortality were shown by the Simonsen assay to have the same degree of liver, spleen, and thymic involvement as their littermates which were undergoing the classical GVH reaction. Histologic studies demonstrated no difference from the cellular alteration caused by the injection of allogeneic cells. It is believed that this protective effect is due to "passive" transfer of cells which have been educated to cope with toxic products.

In vitro studies utilizing anti-theta antiserum demonstrated a decrease in the number of theta-positive cells in the spleens of mice treated with endotoxin. The production of changes indistinguishable from those produced by untreated animals in the GVH reaction argued against a dilution explanation. Additionally, animals treated with endotoxin rejected skin grafts as rapidly as the non-treated animals did. Experiments using spleen cells from carrageenan-treated adults to induce GVH indicated an altered macrophage function.

The Mishell-Dutton system of in vitro measurement of cellular ability to respond to antigens was utilized to dissect out the influenced cells. Animals treated in Vivo with endotoxin were immuno-suppressed in respect to their ability to respond to sheep erythrocytes (SE). This immunosuppression seemingly involved all cells in the

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in

Microbiology

Approved:

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MONTANA STATE UNIVERSITY
Bozeman, Montana

March, 1974

ACKNOWLEDGMENTS

I would like to express my appreciation and gratitude to my advisor, Dr. John W. Jutila, for his invaluable assistance and encouragement. I would also like to express my appreciation to Dr. Alvin G. Fiscus and Dr. David G. Stuart for consultation and review of the manuscript. Consultation and suggestions received from Dr. Norman D. Reed and Dr. Dean D. Manning are gratefully acknowledged.

The assistance given by Dr. Stephen R. Chapman in statistical evaluation of data and that given by Dr. William D. Hill in interpretation of histological changes was appreciated.

I would also like to express my appreciation to Dr. Richard H. McBee and Dr. William G. Walter for extending an invitation to pursue an advanced degree at Montana State University.

The assistance of Mr. Philip D. Thomson in photography and preparation of graphs was most helpful. Dr. David P. Aden kindly demonstrated the Mishell-Dutton system. The association with colleagues and fellow graduate students was a constant source of helpful criticism and suggestions.

The encouragement of my wife, Peggy Ann, and her patience in understanding the problems associated and engendered by immunobiological research is gratefully acknowledged.

A portion of the academic and research studies was supported by Public Health Service Grant 5 A02 AH 0034-05. Other support was supplied in part by NIH Grant AI-06552-09.

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ABSTRACT

The ability of an aqueous-ether extract of Salmonella enteritidis (endotoxin) to influence the ability of spleen cells from adult CBA mice to produce graft-versus-host (GVH) disease in neonatal Balb/c animals was investigated. Adult animals were treated in vivo with 7 daily 30 µg injections of endotoxin. Adult spleen cells were treated in vitro with 30 µg of endotoxin for one hour. Doses of either 1×10^7 or 2×10^7 CBA adult spleen cells produced surprisingly similar findings when injected into Balb/c neonates. The degree of GVH disease produced was not remarkably different when assessed by weight loss plots and the Simonsen assay. In vitro treatment of spleen cells by endotoxin failed to have a demonstrable effect on the GVH reaction as assessed by weight loss and by mortality. Conversely, treatment of donor animals with 30 µg of endotoxin daily for 7 days demonstrated protection to the neonate receiving cells both in regard to weight loss and mortality.

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In vitro studies utilizing anti-theta antiserum demonstrated a decrease in the number of theta-positive cells in the spleens of mice treated with endotoxin. The production of changes indistinguishable from those produced by untreated animals in the GVH reaction argued against a dilution explanation. Additionally, animals treated with endotoxin rejected skin grafts as rapidly as the non-treated animals did. Experiments using spleen cells from carrageenan-treated adults to induce GVH indicated an altered macrophage function.

The Mishell-Dutton system of in vitro measurement of cellular ability to respond to antigens was utilized to dissect out the influenced cells. Animals treated in vivo with endotoxin were immunosuppressed in respect to their ability to respond to sheep erythrocytes (SE). This immunosuppression seemingly involved all cells in the

system inasmuch as reconstitution and/or supplementation experiments with adherent cells, B cells from nude mice, and T cells failed to restore immunologic competence. It was felt that the complete immunosuppression in regard to the SE antigen in this system was a result of immunologic precommitment to endotoxin.

INTRODUCTION

Historically, the graft-versus-host (GVH) reaction is rather unique. Murphy (1) of the Rockefeller Institute noticed in 1916 that inoculation of the chorioallantoic membrane of 7-day old chicken embryos with fragments of adult chicken liver, spleen, bone marrow, or kidney caused stimulation of the embryonic spleen which resulted in splenomegaly. He also recognized proliferation of certain leukocytic elements in the mesoderm, subcutaneous tissue, and around the vessels in liver and kidney tissue. He discovered no bacterial etiology for this phenomenon and was unable to create the phenomenon using tissues from adult donors of other species.

Danchakoff (2,3) investigated the splenomegaly resulting from the injection of adult tissue from unrelated chicken donors into the chicken embryo. Her investigations were primarily concerned with discovering underlying aspects of hematopoietic embryogenesis. However, she did postulate two possible mechanisms for the splenomegaly feeling the splenic enlargement was either a result of donor cells being transmitted to the embryonic spleen or that the proliferation was a result of blood-borne metabolic products from the transplanted, growing cells.

Very little research was pursued on these rather intriguing observations until the development of adequate surgical techniques which allowed the replacement by tissue grafting of diseased or non-functioning organs in man. The technological success of grafting was

regretfully handicapped by the immunological rejection of grafts. Investigators were stimulated by the necessity to find the basic mechanisms of graft rejection by the host and modalities by which rejection could be prevented.

The concept that the graft might attempt to reject the host was first suggested independently in 1953 by Simonsen and by Dempster (4). These two researchers each noted large pyroninophilic cells in the renal cortex of grafted dog kidneys. Inasmuch as pyroninophilic cells had been demonstrated to be involved in antibody production, they suggested the possibility that these cells were of graft origin. It was not definitely shown that the cells were actually of host derivation until 1966 (5).

The areas of host-versus-graft (HVG) and GVH interactions rapidly developed into the new field of transplantation immunology. The resultant literature published in this area since 1955 has reached voluminous proportions and excellent reviews on the GVH reaction have been published (5,6,7,8,9,10,11).

GVH reactions are complex immunopathologic syndromes which result when immunocompetent cells are injected into a recipient animal possessing target cells bearing disparate histocompatibility antigens. If the recipient is mature and immunocompetent, the donor cells are destroyed by the classical HVG reaction. If the recipient is

immunodeficient, a GVH reaction ensues which may develop into GVH disease. Recently Elkins (11) has reviewed the cellular immunology and pathogenesis of these syndromes and has defined GVH reactions as the immunologic response of donor lymphoid cells to foreign histocompatibility (H) antigens expressed in the host. He then defines GVH disease as the complex syndrome resulting from the combined effects of the GVH reaction sometimes associated with secondary infectious disease and the response of the host to these immunopathologic and clinicopathologic entities.

Weiser et al. (12) consider the GVH reactions as systemic allogeneic diseases resulting from grafting. In their classification scheme, the syndromes are established as (a) acute allogeneic disease which is produced in experimental animals by the intraperitoneal injection of large numbers of alloimmune peritoneal macrophages and (b) chronic allogeneic disease which is produced by the injection of immunocompetent cells into an immunologically compromised host. Runt disease is considered to represent a special form of chronic allogeneic disease in this classification scheme. It should be noted that runt disease occurs as a result of exposure of the neonate to foreign immunocompetent cells. If an immunologically incompetent animal is exposed to foreign immunocompetent cells, the resulting disease is termed "secondary disease" or "wasting disease". Other forms of GVH

reactions are classified by this group as (c) local allograft reactions (transfer reactions), (d) alloparabiosis reactions, (e) xenoparabiosis reactions, and (f) allogeneic bone marrow grafting reactions.

A specialized form of chronic allogeneic disease, which has been extremely useful in immunogenetic studies in addition to classical immunological research, is demonstrable in the F_1 hybrid derived from inbred parents. The adult F_1 hybrid is incapable of an immunologic attack on parental cells due to the inherited genetic components and yet is susceptible to immunologic attack by lymphoid cells of the parent. This unique attribute has contributed greatly to the analysis of both major and minor histocompatibility antigens inasmuch as the response of the animal to the immunologic challenge is unidirectional. An additional benefit to the researcher using this model is the opportunity to work with adult animals in place of the usual neonatal animal.

At present, research regarding GVH disease appears to be concentrated primarily in five major areas: (a) attempting to understand more completely the immunogenetics and histocompatibility factors involved, (b) improving the methodology for assessment and measurement, (c) dissecting the complex immunopathological elements that cause GVH reactions, (d) suppressing the GVH reactions, and (e) identifying the cellular components involved.

It should be possible to replace diseased organs without fear of graft rejection if the immunocompetent host cells could be removed or neutralized. One possible approach to this problem would be to eliminate the host's marrow and stem cells by total body irradiation and then utilize a bone marrow engraftment from the prospective donor. This technique should also serve as a therapeutic approach for the cure of leukemias and genetically determined immunodeficiency states of man. In order for these techniques to succeed, accurate tissue typing of both donor and host would be essential as well as a more comprehensive understanding of cell-cell interactions in both the HVG and GVH reactions.

The major histocompatibility antigen in the mouse is H-2; at least 20 H-2 alleles have been described. Additional weaker antigens, H-1 to H-14 and a sex-linked H-X have also been described (13). The genetics of the mouse and tissue transplantation in the mouse are relatively well delineated; this is due to the existence of inbred strains and the ability to develop congenic and coisogenic strains (14). For obvious reasons, these techniques are not adaptable to man.

Tissue typing in man has been limited primarily to the ABO and HL-A systems. The major histocompatibility locus in man is HL-A. Currently there are two major methods of tissue typing: serological identification using human alloantisera and the mixed leucoyte reaction.

By these techniques, over 17,500 genotypes have been defined (13). It is a reasonable assumption that the genetics of man will be found to be no less complicated than that of the mouse.

A definitive review of histocompatibility antigens in transplantation is beyond the scope of this introduction. Ceppellini (15) has reviewed the current status of transplantation antigens in a succinct fashion; van Rood (16) likewise has reviewed the role of HL-A antigens in kidney transplants. Two recent reviews (17,18) survey this area in depth.

Two tragic sequelae have accompanied many attempts to graft either bone marrow or thymus glands in man. In spite of careful ABO and HL-A matches, many patients develop GVH disease which is more severe in its course than HVG reactions. In a series of 27 patients with identical ABO and HL-A systems, 11 patients who received bone marrow infusions in reconstitution experiments expired of GVH disease. In 33 patients in which the donor and recipient were nonidentical for ABO and/or HL-A systems, 12 patients developed GVH reactions (19). The problems associated with reconstitution experiments have been recently reviewed by Buckley (20) and the clinical problems of secondary disease which is also closely associated with reconstitution attempts utilizing bone marrow or thymus have been reviewed thoroughly by van Bekkum and DeVries (9). The second major complication of

immunosuppression and tissue grafting in man has been the development of malignancies in greater numbers than that seen in normal unmanipulated populations (21). The basic immunological reason for this remains unexplained.

Control and prevention of GVH disease have been attempted through immunosuppression of the host both prior to and following grafting or by alteration of the material to be grafted. In man, the classical approach has been to render the patient as immunologically nonresponsive as possible by large doses of immunosuppressive and/or gamma irradiation. This is followed by the grafting of marrow, thymus or the desired reconstituent. Prevention of secondary disease is attempted by the administration of either cytotoxic agents, biological immunosuppressives, or combinations of both types of therapy.

Recently Chedid (22) reported that the murine GVH reaction can be inhibited by *in vivo* or *in vitro* pretreatment of donor cells with endotoxins. This observation is rather startling since endotoxin has long been suspected of being one of the basic biochemical complexes involved in the GVH syndrome and indeed is capable of producing runting in neonatal animals if appropriate intestinal flora is present (23,24). Additionally, endotoxin is generally considered to be mitogenic *in vitro* for cells that are not thymus derived (25,26).

The above findings appear paradoxical in view of the additional fact that immunologically both GVH reactions and disease have been

considered as models of cell mediated immunity caused by thymus-derived non-adherent (T-cell) killer cells. This concept is based upon strong experimental evidence. Neonatal thymectomy of several inbred strains of mice eliminates the ability to initiate a GVH reaction (27). Indeed, attempts to obtain GVH reactions utilizing the nude mouse, a congenitally thymusless animal (28), as a source for donor cells have been entirely unsuccessful (29).

The role of various cellular elements in the immune response has been under intense investigation during the last decade. Immunologists are in general concordance that at least three cellular elements are required in most humoral classes of immune responses, namely the T-cell, the B-cell (bone marrow derived non-adherent cell), and the macrophage. Humoral factors may also be involved. The status of current concepts regarding the role of these various elements involved in the immune response is summarized in recent reviews by Talmadge, Radovich and Hemingsen (30), Claman and Mosier (31), Bloom (32), and Miller (33).

The participation of various cells in murine GVH reactions has been intensively investigated by Cantor and Asofsky (34,35,36,37,38,39,40). They have demonstrated that at least two types of cells of thymic derivation are involved in the GVH reaction in the F_1 system. They feel that one cell, present in excess in the thymus, may act as a

precursor of cells which inflict immunologic injury and a second cell, which is present in peripheral tissues and includes blood lymphocytes, is capable of amplifying or enhancing the activity of the first cell.

Assessment of cellular elements in the GVH reaction utilizing an in vitro assay have demonstrated two types of effector cells which react with recipient cells containing alloantigens (41). The two types manifest their activity by plaque formation and by cytotoxicity. Inasmuch as pretreatment of spleen cells with anti-theta antiserum and complement prior to the in vitro assay completely abolished the cytotoxic activity but left the plaque forming cells undamaged, one must assume at least two T-cell populations are involved in the GVH reaction.

The probability of a third T-cell population that is activated in GVH reactions is raised by the research of Elkins (42) who has demonstrated that spleens and lymph nodes of rats, in which a state of transplantation tolerance has been abrogated by adoptive transfer, contain cells which can inhibit syngeneic lymphocytes from normal donors from initiating a GVH response. In mice, Bennett et al. (43) have demonstrated that thymic parental cells administered to irradiated F_1 recipients are capable of reducing the response of the recipient to sheep erythrocytes. They postulate the presence of a thymus suppressor cell.

All of the cellular immunopathological reactions are not entirely due to the donor cells except perhaps in the F_1 system; the host's cellular defense mechanisms respond to the foreign antigenic insult (11). It has generally been assumed that the lymphoid cells of the donor and recipient are primarily involved; however, recently it has been demonstrated that mesothelial cells of the recipient also undergo marked proliferation in the GVH reaction (44).

Inasmuch as current immunological concepts definitely consider the GVH reaction as a form of T-cell mediated cellular immunity, it is difficult to explain why endotoxin would be capable of suppressing the GVH reaction. The more current experimental data based on both *in vivo* and *in vitro* systems strongly suggest that immune responses to endotoxin are thymus-independent (45,46,47,48,49). One recent article does indicate that endotoxin is capable of stimulating T cells (50). These investigators, utilizing simultaneous complement receptor lymphocyte rosette formation in association with radioautographic techniques on murine spleen cells, demonstrated an increase of stimulated cells that was greater than the identifiable B-cell population. Although this evidence was indirect, one must consider the possibility that endotoxin is capable of stimulation of both B- and T-derived lymphocytes. In addition, one must consider the effect of endotoxin on other cellular elements involved in the immune response.

Endotoxin is believed to be processed primarily by the macrophage and many researchers believe that the macrophage, following the ingestion and processing of endotoxin, then presents an antigenic stimulus to the lymphocyte population. This role has recently been reviewed by Bona (51). Other researchers have shown that the macrophage, following contact with endotoxin, become heavily engorged with lysosomes. Thus, they may be considered as "potential enzymatic bombs" capable of releasing increased amounts of destructive enzymes if they sustain an injury (52).

Endotoxin has many variable roles in numerous immunopathological syndromes. Its manifestations vary with the species of animal involved and likewise with the source of the endotoxin itself. The current aspects of endotoxin research and the present interpretations of the various chemical, biological, and immunological aspects of this material can be found in the proceedings of a conference on endotoxins held at Arlie House in 1972 (53).

Introduction to Thesis Problem

Chedid's observation (22) that treatment of donor spleen cells *in vivo* or *in vitro* with endotoxin was capable of suppressing the GVH reaction in mice appeared paradoxical. It was difficult to explain why a compound that is assumed to act primarily on the B-cell

population could affect a biological syndrome that was considered primarily a T-cell reaction.

Therefore, research was implemented to answer several pertinent questions: (a) is the reported phenomenon reproducible in inbred animals? (b) is the phenomenon measurable not only by mortality data but also by the Simonsen assay? (6) (c) which cellular unit(s) is involved in the abrogation of the GVH reaction by endotoxin treatment?

Experimental approaches to the first two questions were planned using standard methodology for the initiation and measurement of GVH reactions. To analyze the third question, an experimental approach was designed to quantify the splenic B- and T-cell components using anti-theta antiserum.

In addition, the role of the macrophage in the GVH reaction was to be measured utilizing carrageenan, a compound reported to be toxic to macrophages in vivo and in vitro (54) and which has been reported to mimic the effects of endotoxin in in vivo systems (55).

Finally, the various cellular aspects of endotoxin treatment in vivo were to be assessed using the in vitro cell culture system of Mishell and Dutton (56) with reconstitution attempts analogous to those utilized by Aden (57). This system was considered especially appropriate inasmuch as one could measure the impact of in vivo endotoxin treatment effects using an in vitro measurement of plaque-forming

cells to sheep erythrocytes, a response which is considered to be dependent on macrophage processing (58).

MATERIALS AND METHODS

Animals

Inbred conventionally reared Balb/c and CBA male and female mice ranging in age from neonates to two months were used. The Balb/c mice were originally obtained either from Baylor University or the National Institute of Health and have been maintained in our laboratory by random mating. The CBA mice were originally obtained from Jackson Memorial Laboratories and have also been maintained by random mating.

Homozygous nude (nu/nu) mice were the offspring of heterozygous (nu/+) animals obtained by crossing nu/nu males with females from either our Balb/c or our CBA colony. These animals were housed in a clean (specific pathogen free) environment.

All animals received sterilized 5010 Purina pellets and acidified chlorinated water (59) ad libitum.

Preparation of Spleen Cells for In Vivo Experiments

Spleen donors were male mice approximately two months of age. The animals were killed by cervical dislocation and the spleens removed utilizing aseptic technique and blunt dissection. Immediately after removal, the spleens were placed in cold Medium 199 (M-199) (Medium-199 with Hanks' Balanced Salt Solution without $NaHCO_3$, Microbiological Associates, Inc., No. 12-120) which had previously been prepared and

chilled. The pH of M-199 was adjusted to a range of 7.15-7.30 utilizing sterile 10% sodium bicarbonate solution and/or 0.1N HCl. M-199 was supplemented with 5% fetal calf serum (FCS)(Grand Island Biological Company). In a limited number of experiments, gamma-globulin free newborn calf serum (Grand Island Biological Company), fetal calf serum supplemented with 1.5 grams percent of bovine gamma globulin (Pentex Biochemicals), or adult CBA mouse serum was substituted for the 5% fetal calf serum. Spleen cells were dissociated by gently abrading the spleen against 60 mesh sterile wire screens. The cells were then washed 2X with cold M-199, diluted 10 fold with cold M-199, and quantitated using standard hematologic techniques. Cell viability was assessed by trypan blue exclusion (60). The concentration of nucleated spleen cells was then adjusted to the desired number of cells per ml.

Endotoxin

Endotoxin, an aqueous-ether preparation (61) from Salmonella enteritidis (Lot 390), was kindly supplied by Dr. K. C. Milner, Rocky Mountain Laboratory, U. S. Public Health Service. It was solubilized in phosphate-buffered saline (PBS), pH 7.2, at a concentration of 300 µg/ml. Solubilization was routinely accomplished by periodic agitation while standing at room temperature over a 24 hour period. For one group of experiments, solubilization was accomplished by

ultrasonication utilizing a Biosonic II (Bronwill Scientific) with a 3/4 inch tip (instrument setting of 90; two 30 second bursts) which produced one minute of exposure to 680 w/in^2. The container was held in ice during the period of exposure to prevent heating.

Experimental Design for In Vivo Assessment of The Effect of Endotoxin on the Graft-versus Host Reaction

GVH disease was produced by the inoculation of male CBA adult spleen cells into neonatal Balb/c animals. The neonates were always used within 24 hours of birth. The effectiveness of various doses of spleen cells in the production of GVH disease was assessed using the Runting Index (R. I.) of Keast (13) and the mortality produced over a 30 day period. The number of cells required to produce approximately 50% mortality was estimated by the Reed-Muench method (62).

Neonatal litters were weighed and reduced in size to no more than 8 animals per litter. Animals were randomly assigned to one of three groups. Group 1 served as controls and received 0.1 ml of M-199. Group 2 received adult spleen cells from CBA animals which had received either parallel doses of pyrogen-free saline to coincide with the treatment of adults or had not been treated. Group 3 received spleen cells from *in vivo* treated endotoxin treated animals or spleen cells which had been exposed *in vitro* to endotoxin. *In vitro*

treatment of cells with endotoxin was accomplished by exposing 1 ml of cells to 30 μg of endotoxin for a period of 1 hour. During this exposure time, the cells were either agitated manually every 5 minutes or else were kept in constant agitation by a mechanical device. Following the exposure to endotoxin, the cells were washed 2x with cold M-199 and then resuspended to the desired concentration.

All neonates were injected intraperitoneally through the thigh to minimize leakage. The cell dose was contained in 0.1 ml M-199.

This experimental design allowed intralitter comparison of the standard GVH reaction against the GVH reaction resulting from cells exposed to endotoxin either in vivo or in vitro. Controls were also performed using Balb/c spleen cells treated with endotoxin in vitro and injected into neonatal Balb/c litters.

Initially the GVH reactions were quantified by the Runting Index of Keast (63). Animals were weighed every 48 hours. All animals expiring prior to 96 hours were considered to have died either as a result of experimental manipulation and handling or as a result of maternal cannibalism and were excluded from the date. All litters were followed over a 30 day period for weight loss and mortality.

After initial experimental data were collected for computation of the R. I. and mortality, the influence of endotoxin treatment methods was investigated by a modification of the Simonsen assay (6). This

assay was applied to the litters at day 10. In brief, surviving animals were sacrificed on the day of assay. The animal was weighed and then killed by cervical dislocation. The spleen, liver, thymus, and right kidney of each animal were removed by blunt dissection and weighed. The spleen, liver, and thymus indices of each animal were computed by dividing the weight of the organ by the weight of the animal. The arithmetic mean of these indexes were then computed for each group of experimental animals and controls in each litter. By this methodology, both intralitter and interlitter variation could be estimated and statistically evaluated. Kidney weights were obtained but were not utilized in analysis of experimental results.

In Vivo Assessment of Macrophage Role in the Graft-versus-Host Reaction as Modified by Carrageenan

Carrageenan, a sulfated polygalactose which is toxic to macrophages *in vivo* (54), was kindly supplied as Sea Kem 9 (Lot RE 6919) by Marine Colloids, Inc., Rockland, Maine. This compound was dissolved in physiological saline by heating in a hot water bath. Adult CBA spleen donors were treated with three consecutive daily intraperitoneal injections of 2 mg carrageenan (0.5 ml of 4 mg/ml solution in saline), and received 2 mg carrageenan intravenously on day 4 (total dosage of 8 mg). The animals were then sacrificed within 48 hours and

spleen cell preparations were prepared and treated with endotoxin *in vitro* as described previously.

In Vivo Assessment of the Role of T cells in the Graft-versus-Host Reaction Modified by Endotoxin

Nude (nu/nu) animals inbred upon a CBA background were utilized in a GVH system to assess the effect of *in vitro* treatment of spleen cells with endotoxin. Additionally, CBA animals which had been treated *in vivo* with 7 daily intraperitoneal injections of 30 μg of endotoxin were skin-grafted utilizing Billingham's technique (64). Anesthesia was accomplished utilizing intraperitoneal pentobarbital as described by Pilgrim (65). (Skin donors were matched to the sex of the recipient). Rejection time was interpreted as the day of complete rejection (100%).

Measurement of Effect of Treatment of Spleen Donors with Endotoxin and Carrageenan

Body weights, mortality, and the weights of the treated animals including spleen, liver, kidney, and thymus weights were obtained. In addition, the yield of nucleated spleen cells from the treated animals was observed.

Histopathology

Random animals were selected for histopathologic studies from the various control and experimental groups. Tissues were obtained,

fixed in buffered 10% formalin, processed and imbedded by standard histological technique. Paraffin sections were prepared and stained with hematoxylin-eosin and examined.

In Vitro Measurement of the Influence of Endotoxin Treatment on T- and B-Cell Concentrations in the Spleen

CBA male mice were divided into two groups, one of which received 210 µg of endotoxin over a 7 day period and the other received matching pyrogen-free saline solution injections. The spleens were harvested and cellular suspensions were prepared in M-199 as previously described. Gamma-globulin free newborn calf serum was used in a 5% concentration in the M-199. High-titered anti-theta serum was supplied by Dr. J. Chiller of the Scripps Foundation. Complement (Colorado Serum Company) was adsorbed with agarose (Sigma Chemical Company, St. Louis) and the number of cells bearing the theta-antigen in the spleen cell preparations were determined by a slight modification of the technique described by Schlesinger (66).

Experimental Design and Methods for In Vitro Assessment of the Cellular Components Involved in Endotoxin Treatment

The technique utilized in this in vitro method of assessment was the dispersed cell culture of Mishell and Dutton (56). The culture system was used to measure the in vitro response of dispersed spleen

cells (harvested from treated and control animals) to sheep erythrocytes. The procedure, equipment, and materials utilized in this technique have been described in detail by Aden (57). In brief, animals were killed by cervical dislocation. Spleens and thymi were removed aseptically and placed in approximately 10-15 ml of sterile balanced salt solution (BSS) in a tissue culture grade plastic 60x15 mm Petri dish (Falcon Plastics). Single cell suspensions of spleen and thymus cells were obtained by gently stripping splenic pulp and thymus cells from their respective capsules. The suspension was allowed to stand until the coarse particles had settled. The supernatant material was then transferred to tissue culture washed 15 ml centrifuge tubes and centrifuged for 5 minutes at 1500 rpm at 6°C.

The cells were then resuspended in Eagle's minimal essential medium (MEM) (Microbiological Associates, No. 12-126), supplemented with L-glutamine (1%, Microbiological Associates, No. 17-605F), nonessential amino acids (1%, Microbiological Associates, No. 13-114), sodium pyruvate (1%, Microbiological Associates, No. 13-115), and 5% fetal bovine serum (Reheis Co;, Inc., Kankakee, Ill., or Grand Island Biological Company) and containing 50 units per ml of penicillin and streptomycin. MEM supplemented as described was termed complete medium.

Adherent cells were removed from the cell suspension for certain reconstitution experiments. This was accomplished by diluting an aliquot of cells 5 times with complete medium and placing them in a 60x15 mm plastic Petri dish. The Petri dish was then placed in a 37° incubator for 15 minutes following which the supernatant cells were transferred to a second similar Petri dish and reincubated for 15 minutes. The adherent cells were collected each time by gentle scraping with a rubber policeman and resuspended in complete medium. Both the adherent cells and the cell population minus the adherent cells were washed one time with complete medium and then resuspended to the desired concentration in complete medium. For some experiments, the adherent cells were harvested by placing them in plastic Petri dishes and incubating them for 2 hours without agitation at 37°C in a gas mixture of 7 percent O_2, 10 percent CO_2, and 83 percent N_2 for two hours. After gentle mixing and careful aspiration, the nonadherent cells were transferred to another dish and incubated under the same conditions for another hour. The cells were then collected, washed, and resuspended in complete medium. This latter technique is basically the method described by Hirsch (67).

In order to assess the effect of endotoxin treatment on the ability of the spleen cells to respond to antigenic stimulation, CBA spleen cells were exposed to antigen and the response assayed by a

slide modification (56) of the localized hemolysis-in-gel technique of Jerne. The effect of the addition of T cells and adherent cells from untreated animals was examined as was the effect of the removal of adherent cells from treated animals in conjunction with reconstitution with adherent cells from normal animals. In some experiments, Balb/c animals were treated with endotoxin as described previously and reconstitution experiments were carried out using nude mice as a source of spleen cells, adherent cells, and non-adherent cells. The ability of thymus cells from 5 week old Balb/c untreated mice to enhance the antigenic response was also examined.

Cultures were established at $2.0 - 2.4 \times 10^7$ spleen cells per ml and thymus cells were plated at 5.0×10^7 per ml in addition to spleen cells. Adherent cells, when used in a supplemental fashion, were plated at a concentration of 1×10^7 per ml in addition to the spleen cells.

A nutritional mixture for daily feeding of the cultures was made as follows: 5 ml essential amino acids (50x concentrated, Eagle, Microbiological Associates, No. 13-606), 25 ml nonessential amino acids (100x concentrated, Eagle, Microbiological Associates, No. 13-114), 2.5 ml L-glutamine, 200mM (Microbiological Associates, No. 17-605F), 500 mg dextrose, and 35 ml MEM, Eagle, modified without $NaHCO_3$ added. The pH was adjusted to 7.2 with 1N NaOH and 7.5 ml of

7.5% $NaHCO_3$ added. The mixture was then sterilized by passage through washed membrane filters (Millipore, 0.22μ pore size). Prior to use, fetal calf serum was added to give a final concentration of 1/3. Each standard culture dish was fed 0.09 ml of this mixture daily after the first day. Cultures which had been supplemented with T cells or adherent cells were fed 0.12 ml daily after the first day.

Sheep Erythrocyte Antigen

Erythrocytes from an individual sheep (No. 1786) had been shown by Aden (57) to give a good response in the culture system. Blood from this animal was obtained from the Colorado Serum Company every three weeks and was used in all experiments. Cells were washed one time in physiological saline followed by a wash in BSS. They were then resuspended in approximately 1% solution in MEM and 3×10^6 were added to each culture dish (30 μl, or one drop from a pasteur pipette) yielded approximately this number.

Hemolytic Plaque Assay

The plaque forming cell (PFC) response of cultured cells was enumerated on day 5 by a slide modification (56) of the localized hemolysis-in-gel assay of Jerne. Agarose (Sigma Chemical Company, St. Louis, Mo.) from a single lot was used in all experiments. Complement was obtained from the Colorado Serum Company and was preadsorbed by packed sheep erythrocytes from the same animal described previously.

RESULTS

Quantitative Cell Requirements for the Production of Fifty Percent Mortality in Graft-versus-Host Disease Produced by CBA Spleen Cells Injected Into Balb/c Neonates

It was desired to utilize cell doses for the production of the GVH reaction that were slightly less and slightly greater than those required to cause 50% mortality in the experimental animals. It was hoped that the selection of these doses would help identify and isolate any subtle effects. Therefore, the number of nucleated CBA spleen cells required to produce LD_{50} when injected intraperitoneally into neonatal Balb/c animals was assessed by the Reed-Muench method. Mortality was observed over a 30 day period coinciding with the period the animals were observed for evidence of runting due to GVH disease. The LD_{50} cell inoculum established by this method was 1.5×10^7 cells (Table I).

Effect of Various Treatments on Cell Viability

Spleen cells from animals treated *in vivo* with 7 daily injections of 30 µg of endotoxin were utilized in the induction of the GVH reaction as well as cells which had been treated *in vitro* with 30 µg of endotoxin for one hour. Additionally, cells were used from animals treated *in vivo* with carrageenan; these cells were also treated

Table I.[a] Reed-Muench computation of LD_{50} for CBA adult spleen cells inoculated into Balb/c neonates.

Cell dose	Mortality ratio	Cumulative dead	Cumulative survival	Ratio	Percent
3×10^7	1/7	18	6	18/24	75
1.9×10^7	7/11	17	10	17/27	63
1×10^7	5/12	10	17	10/27	37
9×10^6	0/6	5	23	5/23	22
7.1×10^6	2/3	5	24	5/24	21
4.75×10^6	2/12	3	34	3/34	9
2.37×10^6	1/1	1	34	1/34	3

Proportional distance: $\frac{.63-.50}{.63-.37} = \frac{.13}{.26} = .50$

Therefore, $0.5 \times 1 \times 10^7 = 5 \times 10^6$

$LD_{50} = 1 \times 10^7 + 5 \times 10^6$ or 1.5×10^7

a. This titration is based upon preliminary experimental findings and does not include all GVH reactions performed.

in vitro with endotoxin as described above. Nude spleen cells were used in some experiments as were cells from Balb/c animals. It was essential that each experimental animal received the same number of viable cells. Therefore, the viability of cell preparations from the various animals (treated and untreated) was assessed by trypan blue exclusion. The viability of all spleen cell preparations exceeded 90%. This finding ruled out any significant effect of cell treatment and manipulation on cell viability.

Effect of Various Protein Supplements in M-199

In a limited number of experiments, the effect of utilizing fetal calf serum supplemented with 1.5 grams percent of bovine gamma globulin or substituting gamma-globulin free newborn calf serum or homologous adult CBA mouse serum in place of the customary fetal calf serum in M-199 was observed. These limited experiments were motivated by a concern that perhaps one of these supplementations would show enhancement of the GVH reactivity. None of these supplementations demonstrated any effect on cell viability. In experiments performed with these various alterations of M-199, no modification of the experimental result was demonstrable.

Morphologic Changes in Adult CBA Animals Receiving Endotoxin Treatment

Treatment of experimental animals with various compounds often produces marked physiological changes which are manifested by gross alterations in the outward appearance of the animal. Observation of physical changes produced by treatment is a basic principle in clinical research and frequently supplies the investigator with insight into underlying changes that otherwise might be overlooked.

Adult CBA mice injected intraperitoneally with 30 µg of endotoxin daily developed symptoms that were variable during the course of the treatment. Within 24 hours the animals showed a watery diarrhea and ruffled fur. They were almost somnolent and moved with a hunched gait. An ocular exudate was frequently present. By 48 hours, the diarrhea cleared and changed to a formed stool. This was followed rapidly by the development of obstipation. On post-mortem, many of the animals would qualify to be included in a megacolon syndrome. After approximately the 5th day, animals which would survive the treatment began to show signs of amelioration of the physical symptomatology. Their fur would become smooth and the gait would return to normal. All physical symptoms would clear and the obstipation would be replaced by normal pelleting.

The effect of daily intraperitoneal endotoxin injections of 30 µg on animal weight and the mortality associated with this

treatment schedule is shown in Table II. It is apparent that the animals suffer rapid weight loss during the initial three days of treatment. The loss of weight reaches a plateau by the 4th day. This time interval, however, is marked by the largest number of animal deaths. Complete daily weight logs (not shown) do not indicate any difference between the weights of survivors and those who expire. Following the 4th day, the animals cease to lose weight and this stability of weight continues throughout the duration of the endotoxin treatment. The mortality also shows a decline with relatively few deaths occurring during the last three days of treatment.

Table II. Weight changes and mortality in 20 adult CBA mice resulting from 7 daily intraperitoneal injections of 30 µg of endotoxin.

Observation	Day 1[a]	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Weight	28.9[b]	26.2	24.6	23.6	23.8	23.4	23.7	N. D.[c]
Deaths	0/20[d]	0/20	1/20	6/20	7/20	8/20	11/20	11/20
Mortality(%)	-	-	5	30	35	40	45	45

a. Weight prior to injection

b. Computed arithmetic mean of body weights in grams

c. N. D.: not done

d. Numerator indicates death; denominator indicates number of animals.

A small group (6) of animals were, after completion of the above treatment schedule, injected every other day with 30 µg of endotoxin for a period of an additional two weeks. These animals regained a weight level compatible with that demonstrated following the initial injection of endotoxin. No deaths occurred in this group.

Additional effects of treatment with endotoxin are presented in Table III. Data from control animals treated with parallel doses of pyrogen-free saline and from unmanipulated animals are also included in this table. It should be noted that the endotoxin treated animals in these data include an additional 5 survivors of endotoxin treatment. Therefore, the data are based upon a different experimental group of animals; some of the animals used in the compilation of Table II were excluded from this group.

Animals receiving endotoxin showed an overall weight loss of approximately 16% by the end of the treatment. This contrasts with the weight loss of less than 2% shown by control animals receiving pyrogen-free saline over the 7 day period. In the course of obtaining these data, 13 animals expired within day 2 and day 6 of treatment (mean = 3.5 days). This correlates with an exposure to endotoxin of 60 to 180 µg; however, the majority of animals expiring had received doses of 60-90 µg of endotoxin.

Table III. Changes in adult CBA mice resulting from 7 daily intraperitoneal injections of 30 µg endotoxin.

Class of animal	# of animals	Spleen[a] weight	Liver weight	Thymus weight	Cell yield from spleen
Untreated	23	0.064 ± 0.003[b]	1.278 ± 0.034	0.031 ± 0.002	1.21×10^8 ± 0.05
Post-endotoxin	20	0.205 ± 0.013 (16)[c]	1.566 ± 0.078 (11)	0.029 ± 0.009 (11)	2.65×10^8 ± 0.19 (16)
Post-saline	20	0.076 ± 0.004 (21)	1.323 ± 0.035 (13)	0.023 ± 0.003 (13)	1.41×10^8 ± 0.11 (30)

Weights of Groups

Untreated	29.9 ± 0.5 gms	(23)
Post-endotoxin	24.5 ± 0.8 gms	(20)
Post-saline	29.7 ± 0.5	(20)

a. Obtained upon sacrifice of animal within 24 to 48 hours following completion of endotoxin treatment

b. Computed arithmetic mean of animals ± standard deviation of the mean

c. Number of animals used in computation of arithmetic mean

The data in Table III demonstrate clearly that endotoxin treatment caused a moderate weight loss associated with approximately a three fold increase in spleen weight. This three fold increase in spleen weight is associated with a two fold increase in the yield of nucleated spleen cells. There is evidence of moderate hepatomegaly.

The mortality in both groups approached 50%. Although not documented statistically, toxicity and mortality were more marked in older animals.

Morphologic Changes in Adult CBA Animals Receiving Carrageenan Treatment

Animals receiving carrageenan showed symptoms that in some respects were similar to those encountered with endotoxin treatment. The animals would show ruffled fur and the high-stepping gait that was present in the endotoxin-treated animals. No diarrhea or obstipation was noted. The animals appeared to have signs of central nervous system involvement which presented itself mainly as lethargy. If held suspended by the tail, they often would go into rapid spinning motion. By the second or third day, evidence of coagulation dysfunction would appear characterized by dry necrosis of the tip of the tail. In a few instances, entire hindquarter areas would be involved in a dry necrotic fashion.

These animals also demonstrated weight loss and occasional deaths. Livers and spleens did not appear to be significantly

increased in weight (C. Sauer, B. Jackson, and J. Jutila: unpublished observations). On sacrifice of treated animals, the liver and spleen were paler than usual and the liver often showed patches of necrosis ranging up to 2 mm in diameter. Thymi were universally atrophied. Yields of nucleated cells from the spleen were in the range of untreated animals.

The injection of endotoxin into animals which had received carrageenan uniformly resulted in death within 24 to 48 hours. No animals were able to survive more than 2 doses of endotoxin (60 µg). Gross post-mortem examination of animals which had received carrageenan and endotoxin showed petechial hemorrhages throughout the viscera and membranous linings. "Flame" hemorrhages were present in the kidneys, and there appeared to be an exacerbation of the liver necrosis. No recovery phase could be demonstrated in animals which had been treated with carrageenan. Even after a two week period of "densitization", the animals would expire within 48 hours after receiving an endotoxin challenge.

Kinetics and Quantitative Cell Requirements for Graft-versus-Host Induction by CBA Spleen Cells Injected into Balb/c Neonates (30 day period of assay)

The influence of cell dosage on the GVH reaction, as assessed by weight loss, is shown in Figure 1. It is interesting to note that

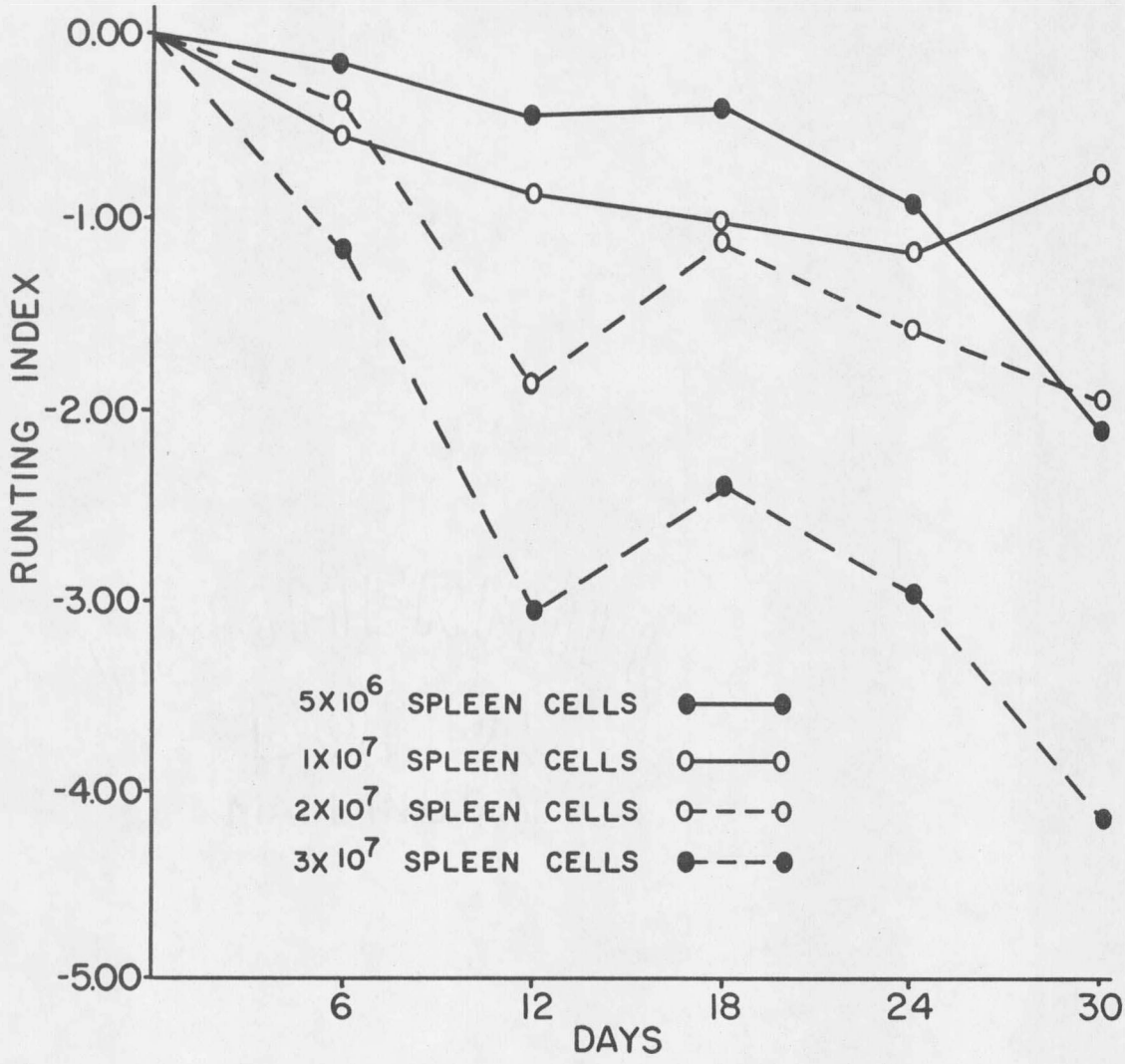


Figure I. Runting Index produced by various doses of CBA spleen cells inoculated into Balb/c neonates.

significant loss of weight (runting) did not occur prior to the 6th day in any test group and did not occur until 20 days in the group inoculated with 5×10^6 cells. One unexpected finding was the extremely low runting index of the animals injected with 1×10^7 cells.

The effect of *in vitro* and *in vivo* treatments of the spleen cells with endotoxin on the R. I. is shown in Figures 2, 3, and 4.

With a dose of 1×10^7 cells, the *in vitro* treatment appeared to enhance the severity of runting. Conversely, *in vivo* treatment maintained the degree of runting at a level similar to that of the GVH reaction resulting from untreated cells. This ameliorated reactivity remained for a period of 24 days. Following this period of time, the degree of runting accelerated and reached the same degree as that produced by *in vitro* treated cells (Figure 2).

With a dose of 2×10^7 cells, the cells treated *in vitro* with endotoxin seemingly had no enhancing effect on the degree of weight loss. The cells from *in vivo* treated animals seemed to maintain the degree of runting at a level not as severe as that encountered in animals which had received untreated cells. This ameliorative effect was again noted until the 24th day. Following day 24, an acceleration of the weight loss of animals receiving cells from *in vivo* treated animals occurred. The runting index reached the same level as that of animals which received untreated cells by the 30th day (Figure 3).

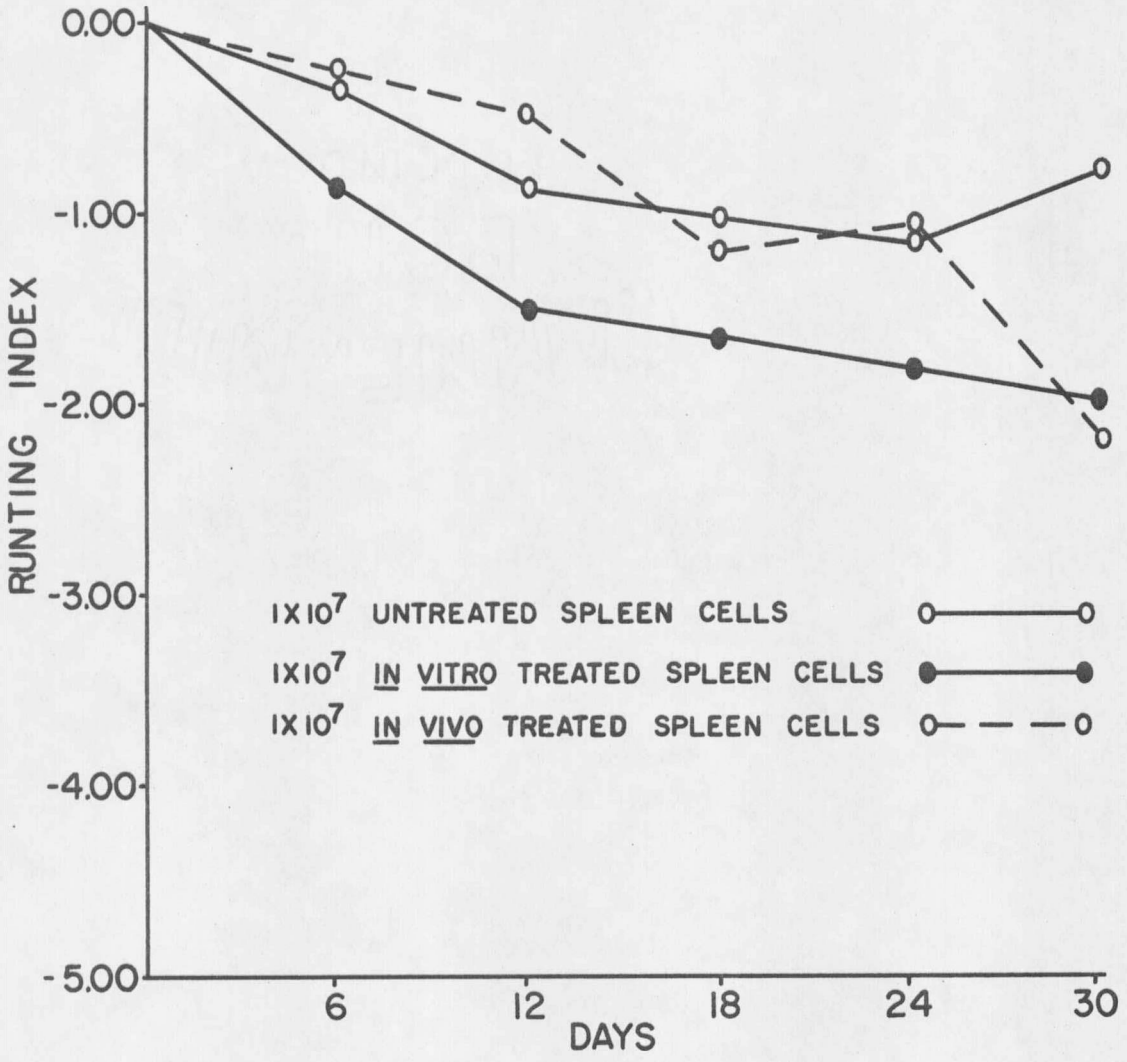


Figure 2. Runting Index produced by inoculation of $1X10^7$ CBA spleen cells (untreated and treated with endotoxin in vivo and in vitro) into Balb/c neonates.

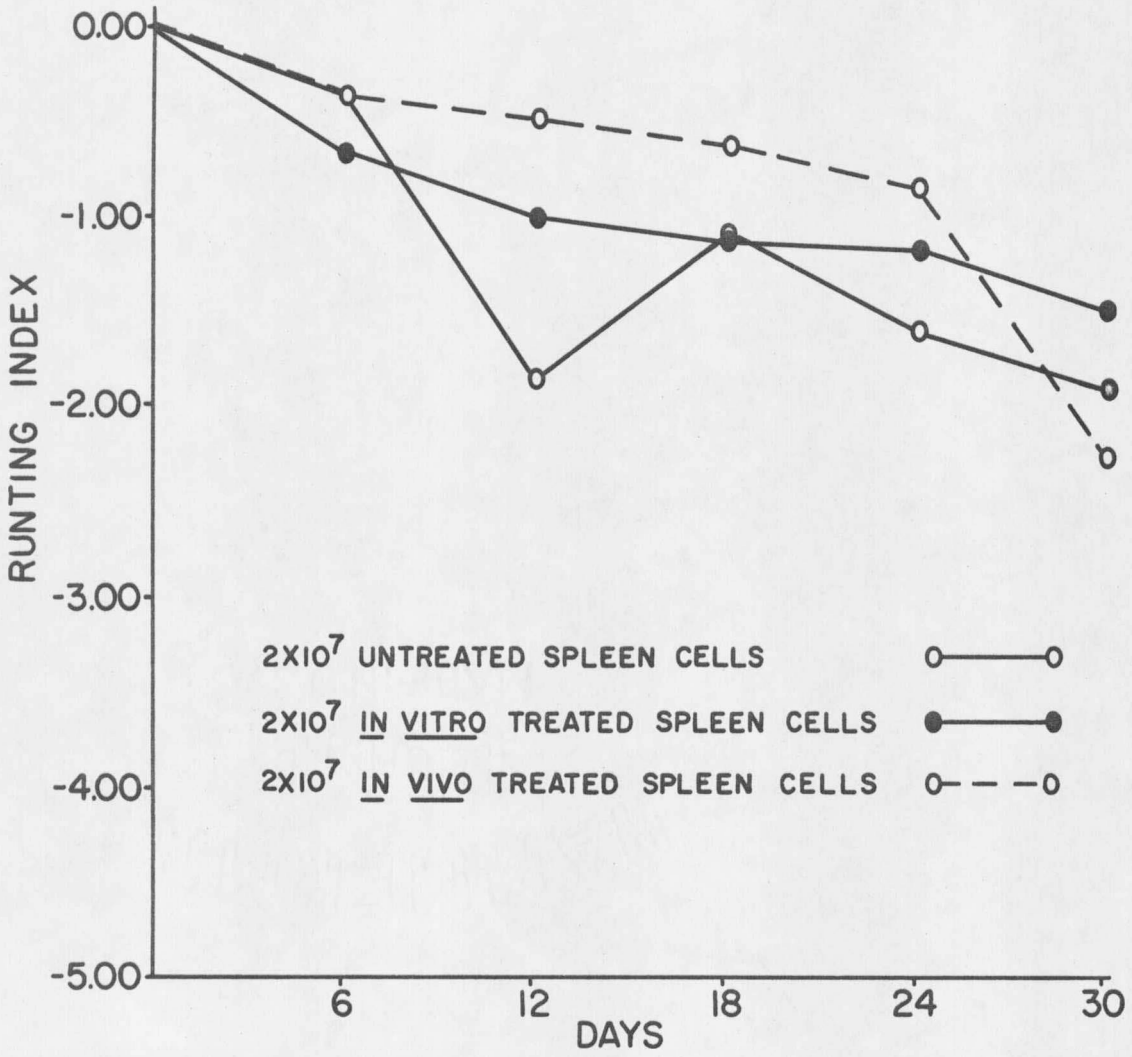


Figure 3. Runting Index produced by inoculation of $2X10^7$ CBA spleen cells (untreated and treated with endotoxin *in vivo* and *in vitro*) into Balb/c neonates.

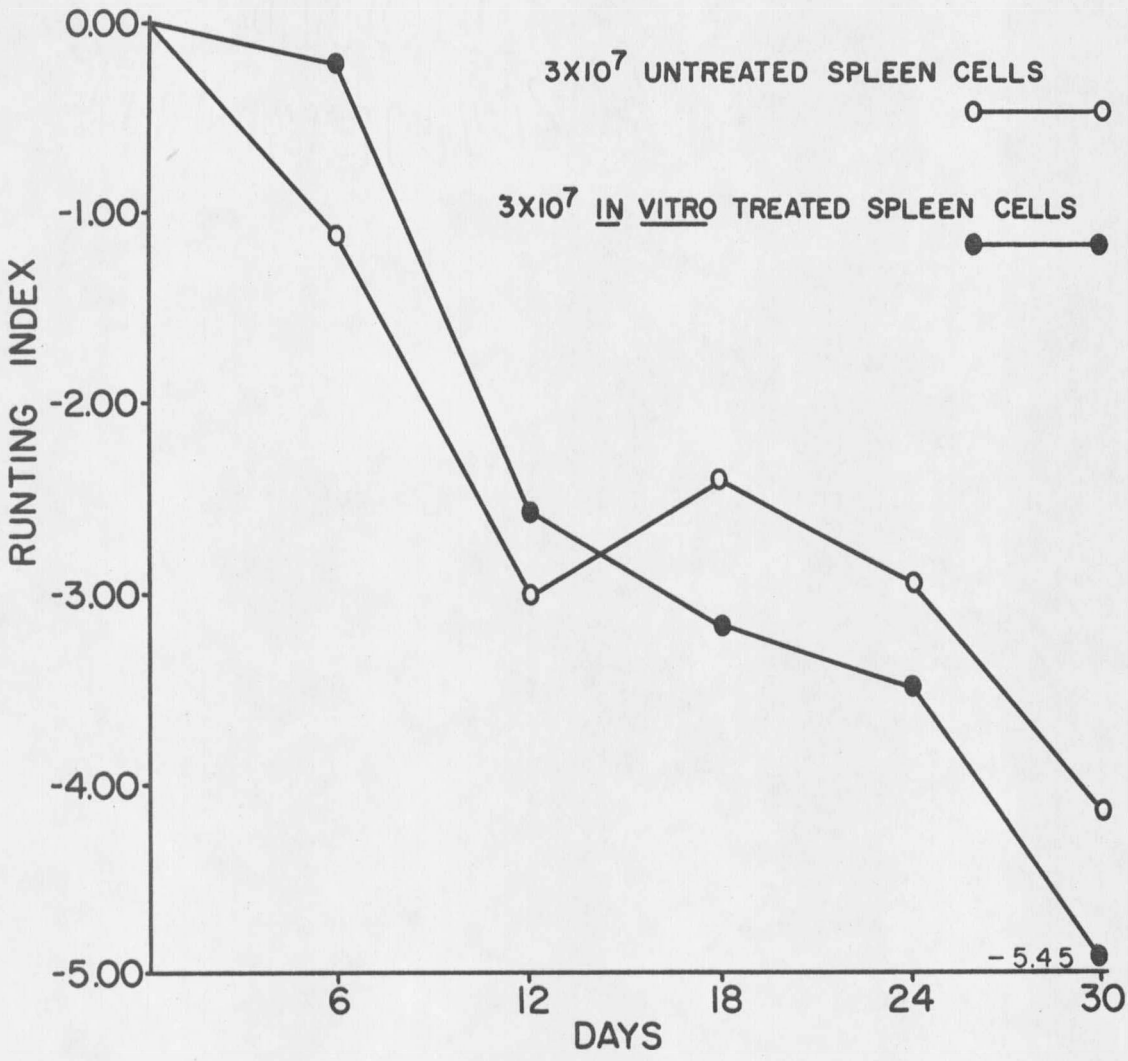


Figure 4. Runting Index produced by inoculation of $3X10^7$ CBA spleen cells (untreated and treated with endotoxin in vitro) into Balb/c neonates.

A cell dose of 3×10^7 cells (Figure 4) produced severe runting disease (GVH disease). The degree of runting produced by *in vitro* treated cells was less severe on the 6th day than that produced by untreated cells. However, this relationship of the runting indices was reversed by 18th day, and the animals injected with the cells treated *in vitro* continued to demonstrate an increased weight loss during the 30 day period of the experiment.

The inoculation of Balb/c cells into Balb/c neonates at a concentration of 1×10^7 cells either untreated or treated *in vitro* with endotoxin did not yield a runting index less than -1 over a 30 day period of observation. In addition, Balb/c neonates inoculated with doses of 1×10^7 or 2×10^7 nucleated spleen cells from nude mice inbred on the CBA background did not produce significant runting. Ten animals inoculated as above with cells which had been treated *in vitro* with endotoxin likewise failed to produce any evidence of weight loss which could be associated with the GVH reaction.

Mortality Resulting from the Inoculation of Spleen Cells from Normal and Endotoxin-Tolerant CBA Donors into Balb/c Neonates (30 day assay)

The mortality produced in neonatal Balb/c mice when inoculated with spleen cells from normal and endotoxin-tolerant CBA mice is shown in Table IV. It should be noted that spleen cells from

endotoxin-tolerant mice produced no mortality when doses of either 1×10^7 or 2×10^7 cells were inoculated into neonatal Balb/c animals. This is in marked contrast to the mortality rate of nearly 50% produced by the inoculation of normal spleen cells.

Table IV. Mortality induced by the inoculation of spleen cells from normal and endotoxin-tolerant CBA mice into neonatal Balb/c mice (30 day period).

Cell Donor	Spleen cell dose	Mortality
Normal CBA mice	5×10^6	3/13[a] (23%)
	1×10^7	7/21 (33%)
	2×10^7	11/16 (69%)
Endotoxin-tolerant mice[b,c]	1×10^7	0/12 -
	2×10^7	0/8 -

a. Numerator represents deaths; denominator represents total number of animals

b. Donor mice had received 7 daily injections of 30 µg of endotoxin

c. Spleens were harvested 24 to 48 hours after the last injection of endotoxin

Mortality Resulting from the Inoculation of Spleen Cells from Normal CBA, Nude, and Balb/c Animals and by the Inoculation of In Vitro Endotoxin Treated Cells from these Animals

The incubation of CBA spleen cells in vitro with 30 µg of endotoxin for 1 hour failed to alter the ability of these cells to produce death in neonatal Balb/c animals (Table V). Although there

Table V. Mortality induced by the inoculation of spleen cells from CBA, nude, and Balb/c animals untreated and treated *in vitro* with 30 μg of endotoxin into Balb/c neonates (30 day period).

Class of cells	Spleen cell dose	Mortality
Treated cells[a]		
CBA	1×10^7	10/19[b] (53%)
	2×10^7	8/20 (40%)
Nude (CBA background)	1×10^7	0/8 -
	2×10^7	0/2 -
Balb/c	1×10^7	0/9 -
Untreated cells		
CBA	1×10^7	7/21 (33%)
	2×10^7	11/16 (69%)
Nude (CBA background)	1×10^7	0/8 -
	2×10^7	1/2[c] -
Balb/c	1×10^7	0/8 -

a. Incubated 1 hour with 30 μg endotoxin

b. Numerator denotes deaths; denominator denotes total number of animals

c. Died on day 13 of experiment; no evidence of weight loss or runting prior to death. Presumed not to represent a death from GVH

appears to be an alteration in the percent of mortality caused by untreated and treated CBA cells, it is felt that this is not significant and probably represents variation in experimental litters.

The treatment of nude spleen cells and syngeneic Balb/c spleen cells with endotoxin did not alter their inability to produce mortality.

Simonsen Assay of the Graft-versus-Host Reaction

The Simonsen assay was utilized to measure the effect of cell dosage and the various treatments of cells on the animals undergoing the GVH reaction. This assay technique is classically applied to spleen weights; in this investigation it was also applied to the weights of liver and thymi.

The effects of cell doses of 1×10^7 and 2×10^7 cells on spleen, liver, and thymic weight as normalized by body weight in the standard GVH reaction are shown in Table VI. There is no significant difference between the influence of these doses of cells on the normalized organ weights.

The mortality resulting from these cell inocula is also presented. When compared to the mortality data presented in Table IV for the same cell doses, it is apparent that there is a lesser mortality in this series with a dose of 2×10^7 cells. This is explained

by the fact that many animals undergoing severe GVH disease expire after the 10th day. The relatively close correlation noted between the two mortality rates obtained with a dose of 1×10^7 cells presumably indicates a milder disease process. This is supported by the relatively minor R. I. obtained with this dose (Figures 1 and 2).

Table VI. Simonsen Index of spleen, liver, and thymus weights and associated mortality resulting from the injection of doses of 1×10^7 and 2×10^7 CBA spleen cells into neonatal Balb/c animals (10 day assay).

Cell Dose	Spleen Index	Liver Index	Thymic Index	Mortality
1×10^7	2.03 ± 0.14[a]	1.63 ± 0.07	0.39 ± 0.06	14/44 (32%)
2×10^7	1.90 ± 0.15	1.68 ± 0.07	0.45 ± 0.07	17/46 (37%)

a. Computed arithmetic index of experimental litters ± standard deviation of the mean.

Inasmuch as a substantial degree of variation was found between litters, the results of various experimental manipulations were compared only within experimental groups in an attempt to partially eliminate large interlitter variables.

The various experimental treatments performed with the 1×10^7 cell dose and their effect on organ weights are summarized in Table VII. In each case, they were compared against the GVH produced by untreated cells in the specific experimental group. No significant

Table VII. Simonsen Index of spleen, liver, and thymus weights and associated mortality resulting from the injection of 1×10^7 CBA spleen cells which had undergone various treatments into Balb/c neonates (10 day assay).

Treatment of cells	Spleen Index	Liver Index	Thymic Index	Mortality
None	2.28 ± 0.18[a]	1.74 ± 0.08	0.31 ± 0.05	8/26[b] (31%)
Treatment A	2.04 ± 0.21	1.56 ± 0.13	0.23 ± 0.07	4/9 (44%)[c]
None	1.39 ± 0.14	1.40 ± 0.16	0.71 ± 0.16	3/10 (33%)
Treatment B	1.26 ± 0.09	1.17 ± 0.04	0.83 ± 0.09	0/12 -
None	1.58 ± 0.24	1.53 ± 0.12	0.71 ± 0.08	0/7 -
Treatment C	1.53 ± 0.18	1.43 ± 0.12	0.74 ± 0.12	0/6 -
None	2.04 ± 0.15	1.91 ± 0.16	0.45 ± 0.09	0/6 -
Treatment D	2.01 ± 0.12	1.74 ± 0.11	0.48 ± 0.13	0/7 -
None	1.60 ± 0.13	1.27 ± 0.18	0.79 ± 0.18	0/5 -
Treatment E	1.60 ± 0.16	1.68 ± 0.40	0.58 ± 0.05	0/5 -
None	1.32 ± 0.06	1.18 ± 0.05	0.94 ± 0.05	0/7 -
Treatment F	1.67 ± 0.18	1.22 ± 0.06	0.86 ± 0.07	0/9 -

Treatments:

A. Donor animals received 7 daily injections of 30 µgm endotoxin
B. One hour treatment of donor cells *in vitro* with 30 µgm endotoxin with agitation every 10 minutes.
C. One hour treatment of donor cells *in vitro* with 30 µgm endotoxin with constant agitation
D. Substitution of homologous adult CBA mouse serum for fetal calf serum in M-199 followed by treatment as in C
E. Treatment as in B except with utilization of endotoxin that had been solubilized with ultrasonication
F. Treatment as in C except with utilization of endotoxin that had been solubilized with ultrasonication

a. Computed arithmetic index of experimental litters ± standard deviation of the mean

b. Numerator denotes deaths; denominator denotes number of animals

c. Mortality may be misleading inasmuch as controls in these litters displayed 25% mortality

difference was found between the spleen, liver, or thymic weights of animals involved in the classical GVH reaction or the GVH reaction induced by cells from endotoxin treated animals. The in vitro treatment of cells with endotoxin likewise produced no significant effect. Additionally, cells treated in vitro with endotoxin solubilized with ultrasonication demonstrated no significant difference in reactivity from the standard GVH. Constant agitation of cells suspended in the ultrasonicated endotoxin solution seemingly had no effect on their reactivity. Replacement of fetal calf serum in M-199 with homologous adult CBA serum failed to alter the cellular reactivity.

The lack of mortality obtained with untreated cells in some groups was unexpected. This probably is due to the "threshold" effect of the 1×10^7 dose which appeared capable of causing the classical splenomegaly associated with GVH disease and yet was capable of producing a R. I. that was extremely low in comparison to those produced by a doubling of the cell dose. This phenomenon is one that deserves to be investigated further.

Results obtained with an inoculum of 2×10^7 cells are summarized in Table VIII. No significant difference was found between the spleen, liver, or thymic indices of animals receiving untreated cells or cells which had been treated in vitro with standard endotoxin solution. Cells from animals treated in vivo with endotoxin

Table VIII. Simonsen Index of spleen, liver, and thymus weights and associated mortality resulting from the injection of 2×10^7 cells treated with endotoxin *in vivo* and *in vitro* into Balb/c neonates (10 day assay).

Treatment of cells	Spleen Index	Liver Index	Thymic Index	Mortality
None	1.87 ± 0.22[a]	1.87 ± 0.12	0.45 ± 0.12	10/24[b] (42%)
In vivo[c]	2.16 ± 0.17	1.48 ± 0.08[d]	0.57 ± 0.07	3/23 (13%)
In vivo[e]	1.31 ± 0.10[f]	1.15 ± 0.07[g]	0.97 ± 0.09[h]	0/6 -
None	1.59 ± 0.25	1.39 ± 0.15	0.55 ± 0.10	4/11 (36%)
In vitro	1.30 ± 0.20	1.29 ± 0.20	0.61 ± 0.14	3/9 (33%)

a. Computed arithmetic mean of experimental litters ± standard deviation of the mean

b. Numerator denotes deaths; denominator denotes number of animals

c. Donor animals received 7 daily injections of 30 µg endotoxin

d. Significant difference ($p<0.05$) when compared to Liver Indices produced by untreated cells and by cells from animals treated with endotoxin solubilized by ultrasonication

e. Donor animals received 7 daily injections of endotoxin solubilized by ultrasonication

f. Significant difference ($p<0.05$) when compared to Spleen Indices produced by untreated cells and by cells from animals treated *in vivo* with endotoxin solubilized by routine procedure

g. Significant difference ($p<0.05$) when compared to Liver Indices produced by untreated cells and by cells from animals treated *in vivo* with endotoxin solubilized by routine procedure

h. Significant difference ($p<0.05$) when compared to Thymus Indices produced by untreated cells and by cells from animals treated *in vivo* with endotoxin solubilized by routine procedure

solubilized by the routine procedure showed a liver index that was significantly smaller ($p<0.05$) than that produced by untreated cells. This index was also significantly larger than that resulting from inoculation of spleen cells from animals receiving ultrasonicated endotoxin. Other indices were not significantly altered.

However, neonates that received cells from donors treated with endotoxin solubilized with ultrasonication demonstrated spleen and liver indices significantly smaller ($p<0.05$) than those produced by either untreated cells or cells from donors which had been treated *in vivo* with the routine endotoxin preparation. The thymic index was significantly larger ($p<0.05$) than the thymic indices produced either by untreated cells or cells from donors which had been treated *in vivo* with the routine endotoxin preparation.

The survival of animals receiving cells from donors treated *in vivo* with endotoxin was considerably enhanced when compared to those receiving untreated cells (26/29 vs. 10/24).

Data obtained from Balb/c neonates receiving untreated Balb/c spleen cells and Balb/c spleen cells treated *in vitro* is collated in Table IX. Experimental design was analogous to that previously utilized. These data definitely show that the inoculum of 2×10^7 syngeneic cells whether treated with endotoxin or not has no effect on the spleen, liver, or thymic indices.

Table IX. Simonsen Index of spleen, liver, and thymus weights and associated mortality resulting from injection of 2×10^7 Balb/c cells untreated and treated with endotoxin into Balb/c neonates (10 day assay).

Type of cell	Spleen Index	Liver Index	Thymic Index	Mortality
Normal Balb/c	0.89 ± 0.22[a]	0.92 ± 0.07	1.08 ± 0.07	1/16[b] (7%)
Treated with endotoxin[c]	0.90 ± 0.06	1.08 ± 0.07	1.00 ± 0.09	0/7 -

a. Computed arithmetic mean of experimental litters ± standard deviation of the mean

b. Numerator denotes deaths; denominator denotes total number of animals

c. Treated for 1 hour *in vitro* with 30 µg endotoxin

The Influence of Carrageenan Treatment of Donor Mice on the Graft-versus-Host Reaction

Carrageenan is a compound which has been shown to be toxic to the macrophage population when animals are treated with 3 daily intraperitoneal injections followed by an intravenous injection on the 4th day (total dose of 8 mg). Initial experiments performed with this compound showed that CBA mice undergoing treatment demonstrated a weight loss analogous to that shown with endotoxin treatment and the phagocytic ability of these animals to clear carbon was impaired (C. Sauer, B. M. Jackson, and J. Jutila; unpublished observations). It was hoped that use of this compound would be of assistance in determining whether or not the effect of *in vivo* treatment of spleen

cells with endotoxin on the GVH reaction was due to macrophage involvement.

Cells from adult CBA donor mice which had been treated with carrageenan were inoculated into neonatal Balb/c animals at a dose of 2×10^7. Cells from untreated animals were used to produce a parallel GVH reaction in littermates. Table X depicts the results of these experiments. Further studies compared the GVH produced by donor cells from carrageenan treated mice following *in vitro* treatment with endotoxin.

No significant difference was found when the degree of runting was assessed by spleen, liver, or thymic indexes between the reaction produced by untreated CBA cells, cells from animals treated with carrageenan, or cells from carrageenan-treated animals exposed to endotoxin *in vitro*.

However, the cells from carrageenan-treated animals showed a definite protective effect in relation to the production of mortality over a 10 day period period. Only one animal out of 22 (1/22) receiving cells from carrageenan animals died. Animals receiving cells from carrageenan-treated animals which had been exposed to endotoxin *in vitro* showed no deaths (0/13). This, when compared to deaths in the group receiving cells from untreated CBA animals (3/11), shows a protective effect similar to that shown by the *in vivo* treatment

with endotoxin (Tables IV and VIII). These data strongly suggest the involvement of the macrophage in the phenomenon under investigation.

Table X. Simonsen Index of spleen, liver, and thymus weights and associated mortality resulting from injection of 2 x 10^7 cells from CBA donors treated with carrageenan and *in vitro* endotoxin treated cells from carrageenan-treated donors when injected into Balb/c neonates (10 day assay).

Treatment of cells	Spleen Index	Liver Index	Thymic Index	Mortality
None	2.31 ± 0.32[a]	1.64 ± 0.14	0.40 ± 0.11	3/11[b] (27%)
Carrageenan *in vivo*[c]	1.76 ± 0.18	1.36 ± 0.11	0.63 ± 0.08	1/22 (4%)
Carrageenan *in vivo* + endotoxin *in vitro*[d]	1.71 ± 0.13	1.38 ± 0.09	0.79 ± 0.06	0/13 -

a. Computed arithmetic mean of experimental litters ± standard deviation of the mean

b. Numerator denotes deaths; denominator denotes total number of animals

c. Donor animals received 3 intraperitoneal injections of 2 mg carrageenan followed by 2 mg of carrageenan intravenously. They were sacrificed within 48 hours of the above treatment.

d. Cells from animals treated as in c treated *in vitro* with 30 µg of endotoxin for one hour

In Vivo Assessment of T-Cell Function

A limited number of nude mice inbred on the CBA background were available. These animals were utilized in a 30 day runting

index study. Eight neonatal Balb/c mice were injected with 1×10^7 spleen cells from nude donors and an additional 7 were injected with 1×10^7 nude spleen cells treated *in vitro* with endotoxin. No evidence of runting was obtained and no deaths occurred during the 30 day observation period.

An additional group of neonates (4 animals) were treated in a similar fashion except with a cell inoculum of 2×10^7. One out of two animals inoculated with the endotoxin-treated cells died; however, it was felt that death was not due to GVH or an endotoxin effect inasmuch as the R. I. of either group did not drop below -.25 during the 30 day period of observation.

A series of experiments were attempted with nudes inbred on the Balb/c background with injection of the nude spleen cells into neonatal CBA animals. The cell inocula were administered without the incorporation of antibiotics and the neonates were not administered antibiotics prophylactically. These experiments resulted in the death of the neonates. Death generally occurred within a 6 day interval. These deaths were attributed to an infectious process inasmuch as post-mortem examination of the dead animals showed gross evidence of peritonitis associated with liver abscesses. Occasional fistula formation was observed. Regrettably, bacterial cultures were not obtained.

Skin grafts utilizing skin from Balb/c donors were performed on adult CBA animals which had been treated *in vivo* with 7 daily intraperitoneal injections of endotoxin. Control animals which had received parallel injections of pyrogen-free saline received similar grafts. The mean rejection time of grafts by 13 control animals was 12.7 days. Rejection time for 10 animals which had been treated with endotoxin was 12.9 days. There was no significant difference between the rejection time of these two groups (Table XI). This finding strongly indicates that the endotoxin treatment has not impaired the function of the T-cell population responsible for the rejection of allogeneic skin. It, however, does not rule out impairment of other T-cell populations in regard to function.

Histopathology

Ten day old animals that had undergone the GVH reaction resulting from either endotoxin-treated or untreated cells often showed histologic evidence of marked areas of liver necrosis (These necrotic areas were generally obvious macroscopically). Sections of the livers showed the presence of increased hematopoietic activity and extensive focal necrosis. The spleens also showed increased hematopoietic activity and marked hyperplasia of the reticuloendothelial cells. Control animals demonstrated moderate residual hematopoietic

activity in the liver. Some sections showed an increase of megakaryocytes in the spleen.

Table XI. Rejection time[a] of Balb/c skin grafts by CBA adult mice after treatment for 7 days with 30 µg of endotoxin daily.

Treatment of CBA	Number of days ensuing before 100% rejection										Mean
In vivo endotoxin[c]	12	13	12	18	12	12	13	15	10	12	12.9 ± 0.69[b]
In vivo pyrogen-free saline[d]	12	15	18	12	13	13	15	10	10	10	12.7 ± 0.65
			11	12	14						

a. Rejection time was considered to be the day of 100% rejection of the graft

b. Standard deviation of the mean

c. Recipient animals were treated daily with 30 µg endotoxin intraperitoneally for 7 days prior to grafting

d. Received 7 daily injections of pyrogen-free saline prior to being grafted

Animals surviving the GVH reaction over a 30 day period had occasional small organized thrombotic areas in the liver sections. Nearly all livers examined histologically showed areas of focal calcification. Occasional sections of liver demonstrated areas of mild hematopoietic activity associated with foci of mild necrosis. Sections of the spleen had changes interpreted as diffuse lymphoid hyperplasia.

Adult animals treated with endotoxin showed decreased lymphocytes in the thymus-dependent areas of lymph nodes. Spleen sections depicted changes compatible with diffuse lymphoid hyperplasia. Liver sections showed cloudy swelling of hepatocytes associated with focal mild polymorphonuclear infiltration throughout the lobules. Occasional cellular alteration suggestive of early fatty changes were frequently noted in the central lobular area. Histologic changes noted in control animals were unremarkable with the exception of minimal cloudy swelling of hepatocytes in occasional areas of the liver.

In Vitro Determination of Ratio of Cells Bearing the Theta-Antigen Prior to and Following Endotoxin Treatment of Adult CBA Mice

An extremely limited amount of high-titered anti-theta antiserum was available. Three animals were treated with endotoxin and three control animals were treated in parallel with pyrogen-free saline. Their spleens were examined for the concentration of cells bearing the theta antigen (Table XII). Nucleated spleen cells from the endotoxin treated animals showed a theta-antigen positive cell concentration of 12.9% (range 6.5-19.3%). Spleens from untreated animals had a concentration of theta-antigen positive cells of 22% associated with a standard deviation of the mean of ± 4% (range of

18-30%). This was considered to represent a definite decrease in the percent of theta-antigen positive cells in spleens of animals treated with endotoxin.

Table XII. Effect of treatment of adult CBA endotoxin-tolerant and normal spleen cells with anti-theta antiserum.

Spleen donor	Expt. animal #	Original cell count[a]	First viability count[b]	Second viability count[c]	% difference between viability counts
Endotoxin-tolerant[d]	1	2.52×10^8	1.86×10^8	1.62×10^8	13
	2	3.88×10^8	2.60×10^8	2.10×10^8	19.3
	3	3.75×10^8	1.72×10^8	1.61×10^8	6.5
	$\bar{x}$	3.38×10^8	2.06×10^8	1.78×10^8	12.9
Normal[e]	1	1.38×10^8	1.34×10^8	1.11×10^8	18
	2	1.15×10^8	0.96×10^8	0.67×10^8	30
	3	1.26×10^8	1.07×10^8	0.88×10^8	18
	$\bar{x}$	1.26×10^{88}	1.12×10^8	0.89×10^8	22

a. Total nucleated cell count of the spleen

b. Cell count obtained following dilution to ca 2×10^8 followed by addition of anti-theta antiserum and incubation for 30 minutes at 37°C.

c. Cell count obtained following the addition of C′ to cells from b and additional incubation for 30 minutes at 37°C

d. Donors had received 7 daily injections of 30 µg of endotoxin

e. Donors had received 7 daily injections of pyrogen-free saline

In Vitro Evaluation of the Ability of Spleen Cells from Endotoxin Treated Animals to Respond to Sheep Erythrocytes (SE)

The Mishell-Dutton technique was used in a series of experiments designed to ascertain which cells were involved in the alteration of the GVH reaction by endotoxin. The first experiments were performed to assess the ability of CBA mice to respond to sheep erythrocytes and to evaluate the influence of addition of thymus cells and adherent cells to the cultures. As seen in Table XIII, the addition of 5×10^7 thymus cells created some suppression of plaque-forming cells (PFC) when calculated as the number per 10^6 surviving cells. However, the number of PFC per culture was not diminished. The addition of 1×10^7 adherent cells caused a moderate reduction of PFC per 10^6 cells and a slight decrease in the number of PFC per culture.

After the above parameters were established, cells from animals that had been treated with endotoxin were plated and their ability to respond to sheep erythrocytes was assessed. Surprisingly, the cells from endotoxin treated animals failed to yield a response to SE (Table XIV). The addition of T cells, adherent cells, and T cells plus adherent cells failed to restore the response above background levels.

Table XIII. Effect of the addition of thymus cells and adherent cells on the immune response of CBA mice to SE in the Mishell-Dutton *in vitro* assay.

Cell System	-SE[a]; PFC per[b] Culture	-SE[a]; PFC per[b] 10^6	+SE[a]; PFC per[b] Culture	+SE[a]; PFC per[b] 10^6	No. of Expt.[c]	No. of Mice[d]
CBA spleen[e]	29	3	295	70	3	8
CBA spleen + T cells[f]			359	16	3	8
CBA spleen + adherent cells[g]			231	41	3	8
T cells			0	0	3	5
Adherent cells			0	0	3	5

a. -SE, sheep erythrocytes not added to cultures; +SE, 3×10^6 sheep erythrocytes added to culture

b. Mean number of direct plaque-forming cells per culture or per 10^6 nucleated cells recovered on day 5

c. Number of experiments

d. Total number of animals used in experiments

e. $2.0\text{-}2.4 \times 10^7$ cells/ml/culture

f. 5.0×10^7 thymus cells/ml/culture

g. 1×10^7 adherent cells/ml/culture

Table XIV. Effect of endotoxin treatment on the *in vitro* response of CBA mice and the effect of the addition of T cells and/or adherent cells.

Cell System	-SE[a]; PFC per[b] Culture	-SE[a]; PFC per[b] 10^6	+SE[a]; PFC per[b] Culture	+SE[a]; PFC per[b] 10^6	No. of Expt.[c]	No. of Mice[d]
CBA untreated	10	2	858	88	4	5
CBA treated[e,f]	9	2	5	3	3	9
CBA treated 2x[g]			6	3	2	9
CBA treated + T cells[h]			4	1	3	14
CBA treated + adherent cells[i]			7	2	4	12
CBA treated + adherent cells + T cells[j]			7	2	3	17
Thymus cells[h]	0	0	9	4	4	5
Adherent cells[i]	0	0	5	2	4	3

a. -SE, sheep erythrocytes not added to cultures; +SE, 3×10^6 sheep erythrocytes added to culture

b. Mean number of direct-plaque forming cells per culture or per 10^6 nucleated cells recovered on day 5

c. Number of experiments

d. Total number of animals used in experiments

e. Treated with 30 µg endotoxin daily for 7 days

f. Plated at $2.0\text{-}2.8 \times 10^7$ cells/ml/culture

g. Plated at $4.0\text{-}4.8 \times 10^7$ cells/ml/culture

h. 5.0×10^7 thymus cells/ml/culture

i. 1×10^7 adherent cells/ml/culture

j. $1.0\text{-}1.2 \times 10^7$ spleen cells + 5×10^7 T cells + 1×10^7 adherent cells

In experiments designed to identify the cell responsible for the immunosuppression to SE, reconstitution experiments were performed in which the adherent cells present in the normal and endotoxin treated animals were removed and then replaced with adherent cells from normal animals. When normal cells were plated after the adherent cells had been removed and then replaced in the culture system a response equal to approximately one-third of the non-manipulated cultural response was obtained. Similar manipulation of spleen cells from endotoxin treated animals failed to yield evidence for any improvement in the immunologic response to SE (Table XV).

The above experimental data indicate that the adherent cell was not the cell responsible for the failure to respond to SE. It therefore seemed possible that the non-adherent (B-cell) might be the affected cell. With the nude mouse serving as a source of B cells, reconstitution experiments were performed to see if the addition of nude spleen cells would abrogate the immunosuppression resulting from endotoxin treatment.

The nude mouse colony, which had been inbred on the CBA background, had been discontinued. This therefore required the utilization of nudes inbred on the Balb/c background. Thus, the ability of Balb/c mice to respond to SE was measured as was the degree of immunosuppression resulting from treatment of adult Balb/c mice. The effect of removal of adherent cells from endotoxin treated Balb/c spleens

Table XV. Effect of removal and/or replacement of adherent cells on the *in vitro* response to SE in endotoxin treated CBA mice.

Cell System	-SE[a]; PFC per[b] Culture	-SE[a]; PFC per[b] 10^6	+SE[a]; PFC per[b] Culture	+SE[a]; PFC per[b] 10^6	No. of Expt.[c]	No. of Mice[d]
Normal CBA	21	2	304	50	4	12
Normal spleen - adherent cell	0	0	0	0	4	12
Normal spleen - adherent cell + adherent cell	0	0	88	15	4	16
Normal spleen + normal adherent	0	0	171	12	3	12
Treated CBA[e]	0	0	14	3	4	8
Treated CBA - adherent cell	0	0	0	0	4	8
Treated - adherent cell + adherent cell from:						
1. Normal	0	0	0	0	4	8
2. Treated	0	1	7	1	4	8
Adherent cell from:						
1. Normal	0	0	0	0	4	12
2. Treated	7	1	0	0	4	8

a. -SE, sheep erythrocytes not added to cultures; +SE, 3×10^6 sheep erythrocytes added to culture

b. Mean number of direct plaque-forming cells per culture or per 10^6 nucleated cells recovered on day 5

c. Number of experiments

d. Total number of animals used in experiments

e. Donor animals received 7 daily injections of 30 µg endotoxin

as well as the ability of adherent cells or non-adherent cells from the nude mouse to restore the response to SE was also analyzed. No significant reconstituion was observed when adherent cells from nudes were added to spleen cells from endotoxin treated animals to replace adherent cells which had been removed.

The ability of non-manipulated nude cells and nude cells in conjunction with T cells was also assessed in this in vitro culture system (Table XVI). These experiments were difficult to interpret. There appeared to be a slight reconstitution effect with the nude adherent cell and a similar effect when nude cells were added to endotoxin-treated cells. The addition of nude plus T cells gave a slightly greater increase. When these results were considered in depth, it was felt that they were extremely inconclusive inasmuch as the nude cells in these experiments were giving a background reading higher than customary.

Therefore, additional experiments were performed using endotoxin-treated Balb/c animals in association with nudes. The number of experimental manipulations was held to a minimum in order that plating might be accomplished as rapidly as possible. In this experiment (Table XVII) it was clearly shown that nude cells plus T cells were incapable of reconstituting immunosuppressed cells resulting from endotoxin-treatment. The plating of 1.2×10^7 nude cells plus

Table XVI. The effect of adherent and non-adherent spleen cells from nude mice on the immune response of endotoxin-treated Balb/c mice.

Cell System	-SE[a];PFC per[b] Culture	-SE[a];PFC per[b] 10^6	+SE[a];PFC per[b] Culture	+SE[a];PFC per[b] 10^6	No. of Expt.[c]	No. of Mice[d]
Normal Balb/c[e]	10	1	106	34	4	12
Treated Balb/c[f]	5	1	3	1	3	9
Treated - adherent cell			3	2	2	
Treated - adherent cell + nude adherent cell			10	5	3	13
Treated - adherent cell + nude non-adherent cell			0	0	2	13
Treated + nude[g]			39	10	3	13
Treated + nude + T cells[h]			125	27	3	17
Nude + T cells			140	8	1	8
Treated + T cells			3	1	2	13
Nude cells	0	0	14	4	2	4
T cells	0	0	5	1	2	4
Adherent cells (nude)	0	0	0	0	2	4

a. -SE, sheep erythrocytes not added to cultures; +SE, 3×10^6 sheep erythrocytes added to cultures

b. Mean number of plaque-forming cells per culture or per 10^6 nucleated cells recovered on day 5

c. Number of experiments

d. Total number of animals used in experiments

e. Plated at $2.0\text{-}2.4 \times 10^7$ cells/ml/culture

f. Treated with 30 µgm endotoxin daily for 7 days

g. Plated at $1.0\text{-}1.2 \times 10^7$ treated + $1.0\text{-}1.2 \times 10^7$ nude cells

h. 5.0×10^7 T cells added

Table XVII. The effect of nude spleen cells and nude spleen cells plus T cells on the *in vitro* response of endotoxin-treated Balb/c mice.

Cell System	-SE[a]; PFC per[b] Culture	-SE[a]; PFC per[b] 10^6	+SE[a]; PFC per[b] Culture	+SE[a]; PFC per[b] 10^6	No. of Expt.[c]	No. of Mice[d]
Normal Balb/c[e]	14	2	139	45	2	6
Treated Balb/c[f]	0	0	0	0	2	5
Treated + nude[g]	0	0	21	0	2	11
Treated + nude + T cells			51	3	2	15
Nude + T			140	8	2	10
Nude	0	0	0	0	2	6
T cells	0	0	0	0	2	4

a. -SE, sheep erythrocytes not added to cultures; +SE, 3×10^6 sheep erythrocytes added to cultures

b. Mean number of plaque-forming cells per culture or 10^6 nucleated cells recovered on day 5

c. Number of experiments

d. Total number of animals used in experiments

e. Plated at $2.0\text{-}2.4 \times 10^7$ cells/ml/culture

f. Treated with 30 µgm endotoxin daily for 7 days

g. Plated at $1.0\text{-}1.2 \times 10^7$ treated + $1.0\text{-}1.2 \times 10^7$ nude cells

h. 5.0×10^7 T cells added

1.2×10^7 endotoxin-treated cells plus T cells yielded a mean plaque-forming cell count which was about 50% of that resulting when 2.4×10^7 nude cells plus T cells were plated. The response in the combined system of endotoxin immunosuppressed cells supplemented with nude cells was due entirely to the nude cell response as aided with thymus cells. It therefore was impossible to restore immunocompetence to these immunosuppressed cells with respect to immunological response to sheep erythrocytes.

DISCUSSION

The initial studies using a 30 day assay disclosed two surprising findings. The first unexpected observation was that 1×10^7 adult CBA spleen cells did not produce a marked runting index (Figures 1 and 2) in Balb/c neonates. Inasmuch as the CBA mouse is $H\text{-}2^k$ and the Balb/c mouse is $H\text{-}2^d$, there exists a strong genetic disparity between these two mouse strains. However, all histocompatibility antigens do not produce the same degree of severity in immunologic reactions as evidenced by differences in the length of time required for skin graft rejection (14). This difference between immunologic strength of reactivity undoubtedly extends to the GVH reaction.

The second unexpected finding was the observation that cells from animals treated in vivo with endotoxin produced only a mild (ameliorated) GVH through a period of approximately 24 days. Following this period, there seemingly is an acceleration of the degree of weight loss with the animals reaching the same level of runting by 30 days. This finding is in contrast to the observation by Chedid (22) who found that animals receiving spleen cells from endotoxin-treated mice had a normal morphology when compared to controls undergoing the classical GVH reaction produced by untreated cells. He found no difference through a 40 day period of observation.

The treatment of spleen cells *in vitro* with endotoxin failed to accomplish any amelioration of death resulting from GVH disease (Table V). This finding is also in contrast with Chedid's observation that preincubation of splenocytes with endotoxin was capable of reducing mortality from 64% to approximately 27%. The daily treatment of donor mice with 30 µgm of endotoxin for 7 days did significantly reduce the mortality of neonates receiving spleen cells (Table IV). No animals receiving *in vivo* endotoxin-treated spleen cells expired from GVH disease during the 30 day period.

This latter observation is in complete concordance with the observation reported by Chedid that pretreatment of spleen cells donors *in vivo* with endotoxin reduced the mortality rate in neonates receiving spleen cells to nearly 0%.

It is difficult to reconcile the disparity between the findings in the research presented in this dissertation and those reported by Chedid. Although Chedid does not identify the endotoxin preparation used in his article, it is believed to be very similar if not identical to the preparation used in these investigations (29). Methods of preparation and utilization of endotoxin solutions presumably would not vary to any great extent. The most plausible explanation for the difference in results is the utilization in this research of inbred animals as opposed to the utilization of neonatal Swiss and F_1 (CBA T_6T_6/AKR) hybrids in Chedid's investigation.

When neonates undergoing the GVH reaction were investigated using the Simonsen method (6), it was found that no real difference existed in the involvement of the various organs examined in neonates receiving a dose of 1×10^7 or 2×10^7 allogeneic cells (Table VI). The mortality observed in various groups over this 10 day period was considerably smaller than that in the 30 day studies, indicating that most deaths occurred following 10 days.

With a dose of 2×10^7 cells (Table VIII), it was noted that the *in vitro* treatment of spleen cells with endotoxin resulted in a mortality rate approaching that obtained with untreated cells. Conversely, cells from animals that had been treated *in vivo* with endotoxin produced a mortality rate (10%) that was markedly decreased from the rate produced by untreated cells (40%). The observation in this experimental group that spleen cells from animals treated *in vivo* with endotoxin solubilized by ultrasonication produced a normal range of spleen, liver, and thymic indices.

Endotoxin is characteristically composed of relatively long polymeric units of Lipid A and polysaccharide (68). Two models have been proposed for the physical conformation of endotoxin. Ribi and others (69) have suggested that it is an aggregation of linear units which form a micellar structure. Others (70,71) have proposed

an ordered bileaflet, membrane-like structure consisting of a bilayer of polysaccharide and lipid with the non-polar lipids occupying the interior of the bilayer. Ultrasonication would be capable of altering either type of structure to produce shorter fragmented units. It is suggested that the normal indices produced by spleen cells from animals treated with ultrasonicated endotoxin may represent an induction of tolerance in the adult animal to endotoxin. This phenomenon is considered analogous to the production of the unresponsive state in mice with deaggregated human globulin (72). Cells so tolerized should demonstrate a lower reactivity pattern. These findings should be investigated in greater detail before any definite conclusions are drawn.

In order to establish that the increases in liver and spleen weight and the decrease in thymus weight were not due to residual endotoxin in or on cells or experimental manipulation of the neonates, Balb/c neonates were injected with spleen cells from syngeneic adult donors. As shown in Table IX, spleen, liver, and thymic indexes were not altered from the normalized value of "1" by either experimental methodology or endotoxin.

The treatment of spleen cell donors with endotoxin had demonstrated a protective effect when based on mortality and weight loss over a 24 day period. In sharp contrast to these findings was the

fact that the disease process was present as measured by the degree of damage to the spleen and liver. Therefore, the question as to which cell was involved in this "physiological protection" was investigated.

Carrageenan is a compound that has been shown to be toxic to macrophages in vivo (54). Additionally, it has been shown to possess the capacity to cause physiological changes in animals that are comparable to those produced by endotoxin (55). Neonates injected with spleen cells from carrageenan-treated adults demonstrated findings entirely comparable to those resulting from injection with in vivo endotoxin-treated cells (Table X). These data strongly suggest the possibility that the macrophage is involved.

The fact that no carrageenan-treated animals could tolerate more than two doses of endotoxin strongly indicates a depleted (or defective) macrophage or sensitization of the macrophage population. The parallel experimental results obtained in the induction of GVH with spleen cells from animals treated in vivo with either carrageenan or endotoxin therefore is considered a strong argument for the consideration of an alteration in the macrophage population as one basis for the amelioration of the GVH reaction.

The degree of involvement of the T-cell in this phenomenon was assessed using several different experimental approaches. The inability of spleen cells from nude mice to produce GVH even when

treated *in vitro* with endotoxin speaks strongly for the necessity of the presence of T cells. *In vitro* studies of the T-cell concentration in spleens of animals treated with endotoxin demonstrated a decrease in the number of T cells. Untreated CBA spleens had a concentration of theta-positive cells of approximately 22%. This concentration is in the same range as that found by Diener (73) in adult CBA mice. Endotoxin treatment *in vivo* resulted in a reduction of the percentage of theta-positive cells in the spleen to 13%; this was associated with a doubling of the yield of nucleated cells. Therefore, an inoculum of spleen cells from endotoxin-treated animals would contain one-half the amount of T cells normally administered. The utilization of this 50% dilution as a premise the explain the phenomenon under investigation is ruled out by two experimental findings. First, the Simonsen data strongly indicate that morphologic changes identical to those of the standard GVH are present. Certainly the evidence is present both grossly and histologically to confirm the presence of a GVH reaction occurring in the liver and spleen. Secondly, the ability of endotoxin-treated mice to reject skin grafts as rapidly as control mice would indicate a T-cell population responsible for graft rejection that is functionally unimpaired.

The skin graft data also would suggest a functional macrophage population. Although the function and necessity of macrophages in skin graft rejection remains controversial (74), it is difficult not

to concede a definite contributory role of the macrophage in skin graft rejection. The possibility remains, however, that various macrophage populations may be involved.

The in vitro system of Mishell and Dutton (56) was utilized to investigate further the effect of endotoxin-treatment on the various cellular elements. Surprisingly, it was found that spleen cells from the in vivo treated animals were entirely incapable of producing an immunologic response to SE (Table XIV). The response to SE has been demonstrated to be macrophage dependent (58). Replacement of the adherent cells from endotoxin-treated spleen cells with adherent cells from untreated mice or nude mice failed to restore an immunologic response (Tables XV, XVI). The addition of T cells in the culture system likewise failed to reconstitute the immune reactivity of endotoxin-treated cells (Table XIV). Supplementation of cultures with non-adherent nude cells (B cells) failed to yield a response.

The addition of nude spleen cells plus T cells did allow a response to SE. This response was not greater than that contributed by the nude cell reconstituted with T cells (Table XVII).

The above data suggest a complete unresponsiveness of cells from in vivo treated animals with respect to SE. The cellular components involved appear to include the macrophage, B-cell, and T-cell inasmuch as replacement and/or supplementation of these cells failed to restore immunologic competence to the system. However, it must be

emphasized that the PFC response in these experiments is below that which could be regarded as significant. During this period of experimentation, serum capable of supporting the cell culture system to the degree established by other investigators was not available. Therefore, the data obtained in the Mishell-Dutton system is considered only as suggestive and must be investigated further.

On the basis of the experimental observations presented in this dissertation, it is proposed that the amelioration of the GVH reaction noted over the 24 day period is due to the injection of macrophages and B cells which have encountered endotoxin in a dose level that could be described as a "physiological tolerant" dose. These cells have the ability and capability to process and neutralize the endotoxins resulting from the gut flora of the animal undergoing the GVH reaction. Presumably these cells are also able to cope with the "autotoxins" resulting from tissue damage and necrosis. The "breaking of tolerance" in respect to the symptomatology is believed due to the exhaustion and destruction of these protective allogeneic cells.

This theory is in accord with the proposal by Reed and Jutila (75) that most wasting diseases possess an infectious or toxemic process engendered by an impaired immune response of the host.

The fact that GVH disease can be produced in germ-free animals does not detract from the above hypothesis. Although germ-free

animals do not possess the intestinal flora prerequisite for the contribution of bacterial endotoxins, the cell damage resulting from the GVH reaction in germ-free animals would produce the "autotoxins" capable of contributing to toxemia. In the system under investigation both "autotoxins" and endotoxins would be present. The "educated" cells from animals treated *in vivo* are postulated to be able to cope with this toxemic insult and give protection.

It is further proposed that the degree of immunosuppression demonstrated in the *in vitro* system is the consequence of immunologic commitment of cells responsible for antibody response to endotoxin. It is theorized that precommitment is so complete that the cells cannot produce a response to SE in the 5 day assay period. This concept of immunologic commitment has also been proposed by Liacopoulus and Merchant (76) to explain their findings that spleen cells from adult C57Bl mice rendered tolerant to endotoxin failed to produce GVH when injected into adult irradiated C_3H recipients.

The models discussed in the preceding paragraphs have their basis on the experimental observations that *in vivo* treatment of spleen cell donors with endotoxin ameliorates the gross physical findings of the GVH reaction while allowing the underlying pathological changes to occur. The immune response to SE is virtually eliminated when assessed by *in vitro* assays. Both of these findings

are strong evidence for the impairment of B-, T-cell, and/or macrophage functions.

Failure of attempts to reconstitue the *in vitro* response to SE is considered evidence for involvement of the three cell types. The fact that spleen cells from carrageenan-treated mice shown the same effect as the cells from *in vivo* endotoxin treated mice in a protective effect against mortality in GVH is considered evidence for the dysfunction of macrophages. Additionally, the failure of the response to SE, a macrophage-dependent function, is strong supportive evidence.

The skin grafting data demonstrate functional T-cell (killer cell) activity. However, different T-cell populations have different degrees of reactivity in the induction of GVH (37,77), and it is entirely probable that the T-cell population responsible for the experimental GVH findings is substantially different from that causing the rejection of allogeneic skin grafts. Also indicative of an altered T-cell population in animals rendered tolerant to endotoxin is the fact that supplementation of T cells does not allow an immune response to SE in the Mishell-Dutton system. The addition of nude cells plus T cells to cells from endotoxin tolerant animals allowed a reaction only proportional to that produced by nude and T cells. This indicates a probable alteration in the reactivity of the T-cell population in the endotoxin tolerant mice.

The B-cell defect is likewise demonstrated by the failure of nude non-adherent cells to reconstitute the *in vitro* response to SE.

The above data strongly suggest the probability of an involvement of all three cells. It is believed that the macrophage is the central cell in this involvement. It is assumed that either membrane changes have occurred that preclude recognition and phagocytosis of other antigens or that the available cells are so completely committed by the long-term exposure to endotoxin that they are no longer capable of processing a different antigen. In this physiological state they are no longer capable of releasing a signal to the B- and T-cell populations.

The B-cell population may have undergone a period of expansion during the endotoxin treatment (Tables III, XII). Due to the mitogenic properties of endotoxin, this cell population must have undergone a period of stress mitogenic activity. In association with this period of stress, most of these cells would have received signals precommiting their available response to endotoxin.

The T-cell is perhaps in the most unique position. One must consider that a portion of T cells are still present functionally. The population responsible for GVH reaction could be diminished to 50% or less of that of untreated animals (Table XII) in the amount of cells injected. Depending on the ratio of T-cell sub-populations, the

number of cells capable of producing GVH could be quite small. In addition, the macrophage may be incapable of sending the "signal" required for T-cell activation. The B-cell also may be completely committed, and thus we have an immunological situation which precludes the ability of the cells to function to an immune challenge--basically the efferent limb of the immune reaction is presumably totally precommited to a recognition and reaction to one antigen only. This defect is believed due mainly to the precommitment of the macrophage and its inability to produce and send out the prerequisite signal.

It was found in the course of this experimental investigation that Chedid's observations could not be reproduced in the CBA vs. Balb/c system. A portion of the irreproducibility can be attributed to the use of inbred strains in this investigation. No protective effect could be found using in vitro treatment of spleen cells with endotoxin. A definite protective effect in regard to mortality was shown by in vivo treatment of donors with endotoxin. The anatomic changes of the classical GVH were present in this system; however, protection, albeit temporary, was found in respect to weight loss. Mortality was definitely altered.

Evidence was strongly suggestive of an involvement of B and T cells as well as the macrophage system in this immunological investigation.

Theoretical models are proposed to explain the experimental findings.

SUMMARY

The ability of endotoxin treatment of spleen cells to influence the graft-versus-host (GVH) reaction was investigated utilizing CBA mice to produce GVH disease in neonatal Balb/c animals. It was found that a dose of 1×10^7 or 2×10^7 CBA spleen cells from adults did not demonstrate a marked difference on the GVH reaction in neonatal Balb/c animals when assessed either by weight loss plots or the Simonsen assay. Treatment of spleen cells in vitro with endotoxin failed to have a demonstrable effect on the GVH reaction either when measured by weight loss or mortality. Conversely, treatment of donor animals with 30 μgm of endotoxin over a period of 7 days demonstrated a protective effect in regard to weight loss that lasted for approximately 24 days and a definite protective effect on the mortality of the animals undergoing the GVH reaction.

The protective effect was demonstrated to be due to a factor other than immunologic abrogation of the GVH reaction. Animals which demonstrated both a protective effect against weight loss and mortality were shown in a Simonsen assay to have the same morphological and microscopic evidence of disease as their runting littermates.

Attempts were made to isolate the cellular alteration responsible for the above findings. Injection of nude cells treated with endotoxin failed to produce a GVH reaction. It was found in in vitro studies that animals which had been treated with endotoxin had a

decrease of cells bearing the theta antigen that approached a 50% decrease of the cell concentration in the non-treated animal. However, the production of changes in animals injected with treated cells which were indistinguishable from those produced by the untreated animal in the GVH reaction argued against a dilution effect of T cells. The ability of animals treated *in vitro* with endotoxin to reject allogeneic skin grafts in the same interval of time as that demonstrated by control animals also argued against an impairment of T-cell function. Evidence indicating that the alteration was due to a macrophage depletion was supplied by experiments using carrageenan, a compound recognized as being toxic *in vivo* to macrophages.

In vitro experiments utilizing the Mishell-Dutton system demonstrated that cells from animals which had been treated *in vivo* with endotoxin were incapable of responding to sheep erythrocytes (SE). Reconstitution experiments showed that this response was incapable of being restored by restoration and/or supplementation by adherent cells or non-adherent cells from nude mice. Attempts to restore the response by thymus cells were also nonproductive.

The above results are felt to be accounted for by a model explaining the protective effect as an enhancement of the neonates' capacity to handle endotoxins resulting from gut flora and to cope with the toxic products resulting from cellular destruction. This

enhancement is created by the passive transfer of macrophages and spleen cells that have become highly proficient in coping with such products. This proficiency or "education" results from the continuous exposure to endotoxin.

The degree of immunosuppression found in the Mishell-Dutton system is explained by a marked immunologic commitment of the capable antibody-producing cells to endotoxin. This commitment would not necessarily preclude their ability to respond to allogeneic challenge; indeed, it is felt that stem cells could develop in an environment not as foreign as the artificial culture system and develop into fully competent cells from an immunologic standpoint.

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