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
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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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Approved:

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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