A mechanism for the suppression of the graft-versus-host reaction with endotoxin
by Philip DePoyster Thomson

A thesis submitted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Microbiology
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Abstract:
It had been previously shown that the graft-versus-host reaction is inhibited by the pretreatment of donor splenocytes with endotoxin.

The experiments performed in this study were designed to examine the role of cells and humoral factors involved in the suppression of GVH disease with endotoxin.

It was shown that as few as 5 X 106 adherent spleen cells from CBA mice pretreated with endotoxin would suppress the GVH reactivity of 2 X 10' whole normal spleen cells from CBA mice when given to Balb/c neonates. This suppressive effect was also observed after the incubation of these two spleen cell populations in vitro for two hours.

The adherent spleen cell population of mice treated with endotoxin was composed of macrophages, lymphocytes, and plasma cells; cell-cell contact was observed between macrophages and lymphocytes after in vitro incubation. Although no cell product was detected in the in vitro incubation environment which impaired the GVH reactivity of 2 X 107 CBA whole normal spleen cells, as little as 0.1 ml of serum from endotoxin-treated mice, rich in IgM antibody, would suppress the GVH reactivity of 2 X 107 CBA whole normal spleen cells when given to Balb/c neonates.

Endotoxin treatment of CBA mice also prevented normal T-cell functions in the rejection of allogeneic skin and tumors, but did not depress helper cell function associated with the plaque-forming response to a thymus-dependent antigen (SRBC).

A mechanism was proposed in which adherent spleen cells and humoral factors from mice treated with endotoxin interact with normal spleen cells from CBA mice to abrogate the GVH disease in Balb/c neonates.

A MECHANISM FOR THE SUPPRESSION OF THE GRAFT-VERSUS-HOST REACTION WITH ENDOTOXIN

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ABSTRACT

It had been previously shown that the graft-versus-host reaction is inhibited by the pretreatment of donor splenocytes with endotoxin. The experiments performed in this study were designed to examine the role of cells and humoral factors involved in the suppression of GVH disease with endotoxin.

It was shown that as few as 5×10^6 adherent spleen cells from CBA mice pretreated with endotoxin would suppress the GVH reactivity of 2×10^7 whole normal spleen cells from CBA mice when given to Balb/c neonates. This suppressive effect was also observed after the incubation of these two spleen cell populations *in vitro* for two hours.

The adherent spleen cell population of mice treated with endotoxin was composed of macrophages, lymphocytes, and plasma cells; cell-cell contact was observed between macrophages and lymphocytes after *in vitro* incubation. Although no cell product was detected in the *in vitro* incubation environment which impaired the GVH reactivity of 2×10^7 CBA whole normal spleen cells, as little as 0.1 ml of serum from endotoxin-treated mice, rich in IgM antibody, would suppress the GVH reactivity of 2×10^7 CBA whole normal spleen cells when given to Balb/c neonates.

Endotoxin treatment of CBA mice also prevented normal T-cell functions in the rejection of allogeneic skin and tumors, but did not depress helper cell function associated with the plaque-forming response to a thymus-dependent antigen (SRBC).

A mechanism was proposed in which adherent spleen cells and humoral factors from mice treated with endotoxin interact with normal spleen cells from CBA mice to abrogate the GVH disease in Balb/c neonates.

INTRODUCTION

Tissues grafted into a foreign host may have either of two fates: the grafted tissue may be rejected or accepted. If the graft and the host are sufficiently disparate, the graft evokes an immunologic response within the host and is rejected by the cellular arm of the immune response (1). If, however, the graft contains cells which are immunocompetent and the host is immunoincompetent, the result is a phenomenon which is known as the graft-versus-host (GVH) reaction.

The GVH reaction was originally observed by Murphy (2) when he inoculated the chorioallantoic membranes of young chick embryos with adult chicken spleen cells. The reality that the graft actually mounted an immunologic reaction against the host was expressed some 37 years later by Dempster and by Simonsen (3).

Another form of the GVH reaction termed GVH disease or runt disease was originally observed in a neonatal murine system by Billingham and Brent (4). Since their original observation, other workers have more fully characterized the phenomenon and several excellent reviews have been published (1,5-9).

GVH disease has recently proved a menace to those patients with no immune response who must be reconstituted with lymphoid or lymphoid precursoral tissue. Thus it became vital to attempt to abrogate GVH disease. These attempts have included impairment of viability and function of donor lymphoid cells my neonatal thymectomy (10), anti-thymocyte serum (11), irradiation (12), immunosuppressive drugs (13),

and treatment with chalones from spleen (14) and thymus (15). Chedid has reported that the GVH reaction may be suppressed by the pretreatment of donor splenocytes in vivo and in vitro with endotoxin (16). In most instances, a mechanism describing an impaired function or destruction of a central lymphoid cell which mediates cellular immunity has been elucidated for the immunosuppressive agent. On the other hand, the abrogation of GVH reactivity of donor spleen cells with endotoxin remains to be explained. Keast (17) has presented evidence that bacterial endotoxins may play a major role in the sequence of events and eventual death seen in GVH disease. Thus the suppression of T-cell mediated GVH disease with endotoxin, which influences primarily B-cells, was unexpected.

Endotoxin, a lipopolysaccharide component of Gram negative bacterial cell walls, has been shown to be mitogenic for B-cells (18), an adjuvant of antibody formation (19), and capable of producing a specific antibody response in experimental animals (20). Although one article indicates that endotoxin is capable of stimulating T-cells (21), the ability of endotoxin to circumvent the requirement for T-cells in immune responses to thymus-dependent antigens has been well documented (22-24). Bona (25) has shown endotoxin to be taken up by macrophages within a few hours of exposure and to be passed within 48 hours to autologous lymphocytes which adhere to the macrophage. This two-cell

processing of endotoxin may be significant in the abrogation of GVH disease.

With this information in mind, experiments were designed to investigate the role of cellular and humoral factors involved in the abrogation of GVH disease by endotoxin.

MATERIALS AND METHODS

MICE-- The mice used in these experiments were neonatal and adult Balb/c mice reared in our colony from stock originally obtained from the National Institutes of Health (Bethesda, Md.) or were adult CBA/J (CBA) mice originally purchased from the Jackson Laboratory (Bar Harbor, Maine) and maintained in our colony. All mice received autoclaved Purina 5010 feed and acidified-chlorinated water (26).

ENDOTOXIN-- Endotoxin (ET) was extracted by the hot phenol-water method (27) from a bovine strain of Escherichia coli (B-44). The ET was lyophilized and stored at 10C until used. ET was rehydrated in M/100 phosphate buffered saline pH 7.2 (PBS) to a concentration of 600 µg/ml.

NORMAL AND ET-TREATED SPLEEN CELLS-- Adult CBA mice were given 7 daily intraperitoneal (IP) injections of 60 µg of ET contained in 0.1 ml of PBS, or were left untreated. Spleens were harvested from mice within 48 hrs after the final injection of ET and were pressed through 80-mesh stainless steel screens into Earl's BSS (Grand Island Biological Co., Grand Island, N. Y.) containing 5 % fetal calf serum (BSS). The preparations were used as whole spleen suspensions or separated into adherent and non-adherent cell suspensions using the technique described below. Spleen cells from mice treated with ET may be hereafter referred to as either whole ET-treated, ET-treated adherent, or ET-treated non-adherent spleen cells.

ADHERENT AND NON-ADHERENT SPLEEN CELLS-- Adherent cells were harvested by placing whole spleen cell suspensions into 60 cm plastic Petri dishes (Falcon, Oxnard, Ca.) for 1 hr at 37C. Non-adherent cells were decanted, washed once, and collected in BSS for injection. The attached cells were washed 3 times with BSS and collected for injection.

KILLED ET-TREATED ADHERENT SPLEEN CELLS-- Adherent spleen cells were killed by allowing them to stand at room temperature in a solution of 3% formalin for 30 min. The killed cells were then washed 3 times in BSS prior to use.

VIABILITY AND ENUMERATION OF SPLEEN CELLS-- All spleen cells were checked for viability using the trypan-blue exclusion method and were counted using a hemocytometer.

IN VITRO INCUBATION OF ET ADHERENT SPLEEN CELLS WITH WHOLE NORMAL SPLEEN CELLS-- Adherent spleen cells were allowed to re-adhere to plastic Petri dishes for 30 min, using 5×10^6 cells per dish. To each of these dishes, 2×10^7 whole normal spleen cells were added and allowed to incubate in BSS at 37C for either 1 or 2 hrs. After incubation, all non-adherent cells were washed once with BSS and resuspended in a volume of 0.1 ml. Control cells were prepared using normal adherent cells incubated with whole normal spleen cells in the same manner described above.

DIFFERENTIATION OF ADHERENT SPLEEN CELL POPULATIONS-- Adherent spleen cells from normal and ET-treated CBA mice were collected from

plastic Petri dishes in BSS and transferred to clean glass slides, allowed to air dry, and stained with Wright's staining solution (Fisher Scientific, Fair Lawn, N. J.). Differential counts were then made, and photographs were taken from these preparations. Comparisons were made to whole normal and whole ET-treated spleen cell preparations.

FREEZE-THAW EXTRACT FROM ET-TREATED ADHERENT SPLEEN CELLS-- Adherent spleen cells from ET-treated CBA mice were collected in BSS and placed in a sterile, conical centrifuge tube in a concentration of 5×10^7 cells per ml. These cells were frozen at -70C and thawed at 37C three times to insure disruption of the cell membranes. Cell debris was removed by centrifugation at 3000 X G for 10 min at 4C.

INCUBATION SUPERNATE FROM NORMAL AND ET-TREATED ADHERENT SPLEEN CELLS-- Adherent spleen cells from ET-treated CBA mice were collected in BSS at a concentration of 5×10^7 cells per ml and re-incubated in plastic Petri dishes for 1 hr at 37C, or were placed in a Mishell-Dutton chamber in an atmosphere of 10% CO_2, 7% O_2, and 83% N_2 for 3½ hrs. The BSS, or incubation supernate, was collected by removing the adherent cells by centrifugation at 500 X G for 10 min at 4C. Normal CBA adherent cells were treated in the same manner and the incubation supernate was collected by the same means.

COLLECTION OF SERUM FROM ET-TREATED CBA MICE-- Adult CBA mice treated with ET for 7 days were bled from the tail vein 24 hrs after the last injection of ET. The blood was allowed to clot at room

temperature for 1 hr and then held at 10C overnight to further retract the clot. Serum was then harvested with a Pasteur pipette, pooled, and stored frozen until used. Serum from mice treated with ET may be hereafter referred to as immune serum. Normal serum was collected, pooled, and stored in the same manner. Half of the sera from test and from control mice was absorbed 3 times with ET conjugated to CBA erythrocytes (28).

HEMAGGLUTINATION TITER OF SERUM FROM ET-TREATED CBA MICE-- The sera from normal and from ET-treated CBA mice were two-fold serially diluted with PBS and standard hemagglutination titers were determined using sheep erythrocytes (SRBC) coated with the specific ET. Aliquots of normal and test sera were also subjected to treatment with 0.1M 2-mercaptoethanol (2-ME) for 1 hr at 37C and titered against SRBC coated with the specific ET.

PRODUCTION OF RABBIT ANTI-ET-- An outbred adult rabbit was given 4 weekly injections of 1.2 mg of ET in 3 ml of PBS mixed with 3 ml of Freunds incomplete adjuvant. These injections were given subcutaneously at 3 sites with 2 ml of the suspension injected at each site. Five days after the last injection, the rabbit was bled from the heart, and serum was collected by the same techniques described for mouse serum above. This antiserum was absorbed 3 times with packed CBA erythrocytes for 1 hr at each absorption and stored frozen until used. Normal rabbit serum (NRS) was collected, absorbed, and stored in the same manner.

Both hemagglutination and hemolysin titrations were performed using these sera in combination with ET-coated SRBC.

SRBC PLAQUE-FORMING CELL ASSAY-- The spleens of normal and ET-treated CBA mice were assayed for their plaque-forming cell response to SRBC by a slide modification (29) of the Jerne plaque assay.

SKIN GRAFTING-- The skin grafting method was a modification of the technique employed by Rygaard (30). The modification involved the use of a different cyanoacrylate adhesive (Permabond, Pearl Chem. Co., Tokyo). Rejection was read as the day of graft separation.

TUMOR CELLS-- The tumor used in these experiments was the Balb/c strain specific MOPC-406 myeloma.

STATISTICAL ANALYSIS-- All of the mortality data were subjected to analysis by the contingency chi-square test, and standard deviations were estimated from the data of skin grafting and Jerne plaque assays.

Initial analysis tested independence of mortality/viability versus treatments. Where the model of independence was accepted (ie. P greater than 0.05) treatments were deemed not to differ significantly. In some cases which are noted in the text, groups of treatments were also compared to determine independence.

RESULTS

SUPPRESSION OR MORTALITY DUE TO GRAFT-VERSUS-HOST DISEASE BY ET-TREATED ADHERENT AND ET-TREATED WHOLE CBA SPLEEN CELL PREPARATIONS--Neonatal Balb/c mice less than 24 hrs old were injected IP with various combinations of ET-treated and whole normal spleen cells, adherent and non-adherent spleen cells, or BSS only. All whole spleen cell suspensions were given in a volume of 0.1 ml containing 2×10^7 cells, while all adherent, non-adherent, and formalin-killed adherent cells were given in a volume of 0.1 ml containing 5×10^6 cells.

As shown in Table I, whole spleen cells from mice treated with 60 µg of ET exhibited a pronounced impairment of GVH reactivity (15% mortality) as compared to a high mortality (77%) exhibited by whole normal spleen cells at day 25. The GVH reactivity of non-adherent cells from ET-treated mice was at least partially restored when the adherent cells were removed, whereas, ET-treated adherent spleen cells alone failed to produce GVH disease.

A marked reduction in mortality (77 to 14%) was observed when 5×10^6 adherent spleen cells from ET-treated mice were given together with 2×10^7 whole normal spleen cells (Table II). On the other hand, normal adherent or ET-treated non-adherent spleen cells, when separated and added to 2×10^7 whole normal spleen cells, failed to protect recipient mice from GVH disease. Similarly, formalin-killed adherent cells from ET-treated mice failed to suppress the GVH reaction when combined

TABLE I

MORTALITY OF BALB/C NEONATES RECEIVING NORMAL ADULT CBA/J SPLEEN CELLS AND/OR SPLEEN CELLS FROM ADULT CBA/J MICE TREATED WITH 60 μg[a] ENDOTOXIN[b]

Nature of CBA Donor Spleen Cell Suspension	Cell Dose	Day 25 Mortality Assay of Balb/c Neonatal Recipients	
		Dead/Total	% Mortality
Whole Endotoxin-Treated	2×10^7	4/27	15
Whole Normal	2×10^7	24/31	77
Endotoxin-Treated Non-adherent	5×10^6	5/9	56
Endotoxin-Treated Adherent	5×10^6	1/17	6
Earl's BSS Control	-	1/21	5

[a] 60 μg daily for 7 days

[b] Bovine Strain *E. coli* (B-44)

Goodness of fit based on independence is P less than 0.01

TABLE II

ABROGATION OF MORTALITY DUE TO GRAFT-VERSUS-HOST REACTIVITY OF NORMAL CBA/J SPLEEN CELLS BY CBA/J ADHERENT SPLEEN CELLS FROM ENDOTOXIN[a] TREATED[b] MICE

Nature of CBA Donor Spleen Cell Suspension	Cell Dose	Day 25 Mortality Assay of Balb/c Neonatal Recipients	
		Dead/Total	% Mortality
Whole Normal + Endotoxin-Treated Adherent	2×10^7 5×10^6	4/29	14
Whole Normal	2×10^7	24/31	77
Whole Normal Normal Adherent	2×10^7 5×10^6	18/26	69
Whole Normal + Endotoxin-Treated Non-adherent	2×10^7 5×10^6	13/20	65
Whole Normal + Formalin Killed Endotoxin-Treated Adherent	2×10^7 5×10^6	18/24	75
Formalin Killed Endotoxin-Treated Adherent	5×10^6	2/8	25

[a] Bovine Strain *E. coli* (B-44)

[b] 60μg daily for 7 days

Goodness of fit based on independence is P less than 0.01

with whole normal spleen cells. Killed ET-treated adherent cells alone, however, failed to increase mortality above control values.

GVH REACTIVITY OF NORMAL NON-ADHERENT CBA SPLEEN CELLS INCUBATED WITH ET-TREATED ADHERENT SPLEEN CELLS-- Since ET-treated adherent spleen cells affected the GVH reactivity of whole normal spleen cells *in vivo*, it became of interest to see if the effect was manifest *in vitro*. Therefore, 2×10^7 whole normal spleen cells were incubated with either 5×10^6 ET-treated adherent or normal adherent spleen cells for either 1 or 2 hrs. The resulting non-adherent cells were given IP to Balb/c neonates. Table III shows that non-adherent cells from the 2 hr incubation of whole normal cells with ET-treated adherent cells produced a low mortality (8%). In contrast, high mortality resulted from the injection of whole normal spleen cells incubated for 1 hr with ET-treated adherent cells (83%), or for 2 hrs with normal adherent cells (75%), or whole normal spleen cells incubated alone for 2 hrs (60%). No mortality was seen in neonates given BSS incubated for 2 hrs. It should be noted that only as many as 7×10^6 or as few as 5×10^6 non-adherent spleen cells could ever be recovered after incubation with 5×10^6 adherent cells from ET-treated mice.

DIFFERENTIAL COUNTS OF SPLEEN CELL POPULATIONS-- The cellular components of the ET-treated adherent spleen cell population, whole spleen cells from ET-treated mice, whole normal spleen, and adherent cells from normal spleen were differentiated by cell morphology to

TABLE III

MORTALITY OF BALB/C NEONATES RECEIVING NON-ADHERENT CELLS RESULTING FROM THE IN VITRO INCUBATION OF NORMAL AND ET[a] ADHERENT CBA SPLEEN CELLS WITH WHOLE NORMAL SPLEEN CELLS

CBA NON-ADHERENT CELL SOURCE	No. Cells Incubated	Day 25 Mortality Assay of Balb/c Neonatal Recipients	
		Dead/Total	% Mortality
Whole Normal Spleen + ET-Treated Adherent - 1 hr	2×10^7 / 5×10^6	10/12	83
Whole Normal Spleen + ET-Treated Adherent - 2 hr	2×10^7 / 5×10^6	1/12	8
Whole Normal Spleen + Normal Adherent - 2 hr	2×10^7 / 5×10^6	6/8	75
Whole Normal Spleen - 2 hr	2×10^7	3/5	60
BSS Control - 2 hr	-	0/3	0

[a]ET = endotoxin

Goodness of fit based on independence is P less than 0.01

determine the predominate cell or cells characterizing that cell suspension. Four cell populations were easily identifiable in all of the preparations, and the results of the differential counts appear in Table IV. It should be noted that over 50% of the lymphocytes in the ET-treated adherent population were seen surrounding and seemingly attached to macrophages, Figure 1. Also, there was a high percentage of spent leukocytes (smudge cells) in both the ET-treated adherent and whole ET-treated spleen cell populations which were not included in the computation of Table IV.

MORTALITY OF BALB/C NEONATES GIVEN FREEZE-THAW EXTRACT OR INCUBATION SUPERNATE FROM ADHERENT SPLEEN CELLS OF ET-TREATED CBA MICE-- In order to determine if ET-treated adherent cells contained or were elaborating a product which would suppress the GVH reactivity of whole normal spleen cells, two experiments were designed. In the first experiment, 5×10^7 ET-treated adherent and normal adherent spleen cells were disrupted by freeze-thawing, and 0.1 ml of either extract was given IP in combination with 2×10^7 whole normal spleen cells to Balb/c neonates. Table V shows that neither normal extract nor extract from ET-treated adherent spleen cells was effective in suppressing the GVH reactivity of whole normal spleen cells. Also, neither extract alone produced mortality in Balb/c neonates. In the second experiment, 5×10^7 ET-treated adherent or normal adherent spleen cells were allowed to incubate in BSS for either 1 or 3½ hours. After incubation, 0.1 ml

TABLE IV

DIFFERENTIAL COUNTS OF SPLEEN CELLS FROM NORMAL AND ENDOTOXIN-TREATED[a] CBA MICE

	Spleen Cell Suspension			
	Whole Normal	Normal Adherent	Whole ET[b] Treated	ET-Adherent
Cell Type	%	%	%	%
Lymphocyte	85	39	80	61
Macrophage	4	46	7	30
Plasma Cell	6	8	10	7
Granulocyte	5	7	3	2

[a] 60 µg of E. coli endotoxin daily for 7 days

[b] ET = Endotoxin

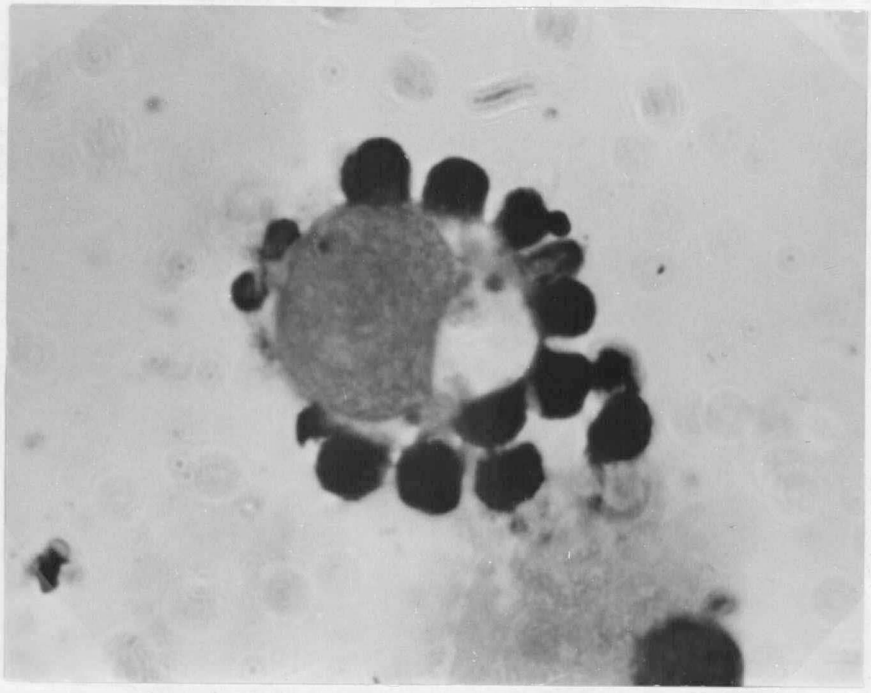

Figure 1. A Macrophage with Closely Associated Lymphocytes from the Adherent cell Fraction of Spleens from Endotoxin-Treated CBA Mouse.

TABLE V

THE EFFECT OF FREEZE-THAW EXTRACTS OF ADHERENT SPLEEN CELLS FROM ENDOTOXIN-TREATED[a] AND NORMAL CBA MICE ON THE GVH REACTIVITY OF WHOLE NORMAL SPLEEN CELLS

Nature of CBA Donor Material	Day 25 Mortality Assay of Balb/c Neonatal Recipients	
	Dead/Total	% Mortality
Whole Normal Spleen[b] + ET Freeze-Thaw Extract[c]	5/10	50
Whole Normal Spleen + Normal Freeze-Thaw Extract	8/11	73
ET Freeze-Thaw Extract	0/4	0
Normal Freeze-Thaw Extract	0/3	0
Whole Normal Spleen	8/10	80

[a] 60μg of E. coli B-44 endotoxin daily for 7 days

[b] Spleen cells given in a dose of 2×10^7

[c] Thrice frozen-thawed extract of 5×10^7 adherent cells from endotoxin-treated CBA mice given in a volume of 0.1 ml

Goodness of fit based on independence is P less than 0.01

of supernatant fluid from normal or from ET-treated adherent cells was given IP in combination with 2 X 10^7 whole normal spleen cells to Balb/c neonates. Table VI shows that neither normal supernate nor supernate from ET-treated adherent cells, whether incubated for 1 or for 3½ hours, was effective in suppressing the GVH reactivity of whole normal spleen cells. Again, neither supernate alone produced mortality in Balb/c neonates.

MORTALITY OF BALB/C NEONATES GIVEN WHOLE NORMAL SPLEEN CELLS IN COMBINATION WITH SERUM FROM ET-TREATED OR NORMAL CBA MICE-- In an effort to further detect the presence of a humoral factor produced by ET-treated CBA spleen cells, 0.1 ml of serum from ET-treated or from normal mice was given IP to Balb/c neonates in combination with 2 X 10^7 whole normal spleen cells. An aliquot of the immune serum was absorbed with ET before injection to determine if the removal of anti-ET activity would influence the GVH reactivity of whole normal spleen cells. Table VII shows that immune serum does impair the GVH reactivity of spleen cells as evidenced by the reduced mortality in neonates (83 to 16%). Whole normal spleen cells alone and whole normal spleen cells in combination with normal serum produced higher mortality in neonates than did whole normal spleen cells in combination with immune serum. Also, when the anti-ET activity was removed from immune serum, the GVH reactivity was restored to whole normal spleen cells. It should be noted that aliquots of normal and of immune sera were titered to

TABLE VI

MORTALITY OF BALB/C NEONATES RECEIVING INCUBATION SUPERNATE FROM ADHERENT SPLEEN CELLS OF ET-TREATED[a] AND NORMAL CBA MICE IN COMBINATION WITH WHOLE NORMAL SPLEEN CELLS

Nature of CBA Donor Material	Day 25 Mortality Assay of Balb/c Neonatal Recipients	
	Dead/Total	% Mortality
Whole Normal Spleen[b] + ET supernate[c] - 1 hr incubation	6/11	54
Whole Normal Spleen + Normal supernate - 1 hr incubation	10/12	83
Whole Normal Spleen + ET supernate - 3½ hr incubation	7/9	77
Whole Normal Spleen + Normal supernate - 3½ hr incubation	8/9	88
ET supernate - 3½ hr incubation	0/4	0
Normal supernate - 3½ hr incubation	0/5	0
Whole Normal Spleen	8/10	80

[a] 60 µg of E. coli B-44 given daily for 7 days

[b] Spleen cells given in a dose of 2×10^7

[c] Balanced salt solution remaining after the removal of cells by centrifugation given in a dose of 0.1 ml

Goodness of fit based on independence is P less than 0.01

TABLE VII

MORTALITY OF BALB/C NEONATES GIVEN WHOLE NORMAL SPLEEN CELLS IN COMBINATION WITH SERUM FROM ENDOTOXIN-TREATED[a] OR NORMAL CBA MICE

CBA Donor Material	Dose	Day 25 Mortality Assay of Balb/c Neonatal Recipients	
		Dead/Total	% Mortality
Whole Normal Spleen + Immune Serum[b]	2×10^7 0.1 ml	3/18	16
Whole Normal Spleen + Normal Serum[c]	2×10^7 0.1 ml	10/12	83
Whole Normal Spleen + ET[d] Absorbed Immune Serum	2×10^7 0.1 ml	8/10	80
Whole Normal Spleen + ET Absorbed Normal Serum	2×10^7 0.1 ml	4/6	75
Whole Normal Spleen	2×10^7	6/8	75

[a] 60 µg of *E. coli* B-44 endotoxin daily for 7 days

[b] Hemagglutination titer = 1/64

[c] Hemagglutination titer = < 1/4

[d] ET = Endotoxin

Goodness of fit based on independence is P less than 0.01

detect hemagglutinating antibody before and after treatment with 2-ME. Normal serum had a hemagglutinating antibody titer of less than 1:4 before and after 2-ME treatment, whereas, immune serum had a titer of 1:64 before 2-ME treatment which fell below 1:4 after 2-ME treatment.

MORTALITY OF BALB/C NEONATES GIVEN CBA SPLEEN CELLS IN COMBINATION WITH EITHER RABBIT ANTI-ET SERUM OR NORMAL RABBIT SERUM-- Inasmuch as serum from ET-treated mice would suppress the GVH reactivity of whole normal spleen cells, the question arose whether heterologous immune serum would also impair the GVH reactivity of whole normal spleen cells. In answer to this question, 0.1 ml of either normal rabbit serum or immune rabbit serum exhibiting an anti-ET hemagglutination titer of 1:160 and a hemolysin titer of 1:320 was given IP to Balb/c neonates in combination with 2×10^7 whole normal CBA spleen cells. The results of this experiment are presented in Table VIII which shows that heterologous immune serum reduces the mortality due to GVH in Balb/c neonates to 9% as compared to the mortality seen in the case of either normal rabbit serum in combination with whole normal spleen cells (71%) or whole normal CBA spleen cells alone (83%). Neither normal rabbit serum alone nor immune rabbit serum alone produced a striking mortality in Balb/c neonates.

GROWTH OF STRAIN SPECIFIC BALB/C TUMOR IN ET-TREATED CBA AND BALB/C MICE-- To provide evidence that endotoxin treatments would suppress cell-mediated immune phenomena other than GVH disease, both

TABLE VIII

MORTALITY OF BALB/C NEONATES GIVEN CBA SPLEEN CELLS IN COMBINATION WITH EITHER RABBIT ANTI-ENDOTOXIN SERUM OR NORMAL RABBIT SERUM

Nature of Donor Material	Dose	Day 25 Mortality Assay of Balb/c Neonatal Recipients	
		Dead/Total	% Mortality
Whole Normal Spleen + Rabbit anti-endotoxin[a] serum	2×10^7 0.1 ml	1/11	9
Whole Normal Spleen + Normal Rabbit Serum	2×10^7 0.1 ml	5/7	71
Rabbit anti-endotoxin serum	0.1 ml	1/8	12
Normal Rabbit Serum	0.1 ml	0/6	0
Whole Normal Spleen	2×10^7	5/6	83

[a]Titer, see text

Goodness of fit based on independence is P less than 0.01

adult Balb/c and CBA mice were either treated with ET for 7 days or were left untreated, and then given a subcutaneous injection of either 5×10^6, 1×10^7, or 2×10^7 Balb/c specific MOPC-406 myeloma cells. All mice receiving initial ET treatment were continued on ET until the day of death or tumor rejection. Table IX shows that a dose of 5×10^6 tumor cells would not produce tumors in either ET-treated or normal CBA mice, whereas, the same dose of tumor cells produced lethal tumor growth in normal Balb/c mice. A dose of 1×10^7 tumor cells, however, did produce tumors in ET-treated CBA mice although the tumors were eventually rejected. As shown with the lower dose of tumor cells, 1×10^7 tumor cells again produced lethal tumors in both normal and ET-treated Balb/c mice, but produced no tumors in normal CBA mice. In the case of 2×10^7 tumor cells, all Balb/c mice developed lethal tumors in 3 days, normal CBA mice developed tumors in 8 days with one mouse surviving to reject the tumor on day 17, and ET-treated CBA mice developed tumors in 6 days with one mouse surviving to reject the tumor on day 23.

REJECTION OF BALB/C SKIN GRAFTS BY CBA MICE GIVEN ENDOTOXIN--Because the rejection of allogeneic skin has been shown to be cell-mediated, an experiment was designed to determine the effect of ET-treatment on CBA mice and their ability to reject skin from adult Balb/c mice. Adult CBA mice received IP either 60 µg of ET daily for 4 days prior to grafting and continuing until the day of graft separation, or were left untreated. Mice treated with ET had a mean

TABLE IX

GROWTH OF STRAIN SPECIFIC BALB/C TUMOR[a] IN ENDOTOXIN-TREATED[b] CBA AND BALB/C MICE

Tumor Cell Dose	Recipient	No. Mice	Results	Death
5×10^6	ET[c]-CBA	4	No tumor	-
	N[d] - CBA	4	No tumor	-
	N - Balb/c	4	Tumor in 6 days	4/4
	ET - Balb/c	-	Not done	-
1×10^7	ET - CBA	4	All had tumor in 10 days, all rejected by day 17	0/4
	N - CBA	4	No tumor	-
	N - Balb/c	4	Tumor in 5 days	4/4
	ET - Balb/c	4	Tumor in 5 days	4/4
2×10^7	ET - CBA	3	Tumor in 6 days, 1 survived to reject tumor on day 23	2/3
	N - CBA	3	Tumor in 8 days, 1 survived to reject tumor on day 17	2/3
	N - Balb/c	4	Tumor in 3 days, all dead by day 14	4/4
	ET - Balb/c	4	Tumor in 3 days, all dead by day 14	4/4

[a] MOPC 406 myeloma

[b] 60 µg of *E. coli* endotoxin daily for 7 days prior to tumor injection and continuing until rejection or death

[c] ET = endotoxin-treated

[d] N = normal

rejection time of 14.9 days which was significantly different from untreated CBA mice which showed a mean rejection time of 10.2 days for Balb/c skin (Table X).

PLAQUE-FORMING CELL RESPONSE OF CBA MICE TO SHEEP ERYTHROCYTES-- To determine the effect of ET on the plaque-forming response of CBA mice to a thymus-dependent antigen such as SRBC (31), adult CBA mice were treated with ET daily for 7 days before receiving IP 5 X 10^8 SRBC and continued to receive ET until the day of plaquing (5 days), or received SRBC only 5 days prior to plaquing, or received ET only each day until plaqued, or were not treated. Table XI shows that CBA mice have a background response to sheep erythrocytes of 3 plaque-forming cells per million spleen cells or 40 plaque-forming cells per spleen. ET treatment elevates that response to 9 plaque-forming cells per million spleen cells or 5,000 plaque-forming cells per spleen. Due to the high standard deviations, mice receiving SRBC alone could not be shown to be different in their plaque-forming cell response to SRBC from mice pretreated with ET and treated with ET after SRBC treatment.

TABLE X

REJECTION OF BALB/C SKIN GRAFTS BY CBA MICE GIVEN ENDOTOXIN

Treatment[a] of CBA Recipients	Number of days ensuing before 100% rejection										Mean ± $S_{\bar{x}}$
Endotoxin-Treated	11	13	14	14	14	15	16	16	18	18	14.9 ± 0.69
Untreated Controls	8	10	10	10	10	11	11	11			10.2 ± 0.35

[a]Each mouse received either 60μg of E. coli endotoxin daily 4 days prior to grafting and continuing until day of 100% graft rejection, or were untreated

TABLE XI

PLAQUE-FORMING CELL RESPONSE OF CBA MICE TO SHEEP ERYTHROCYTES

Treatment[a] of CBA Mice	No. of Mice	PFC/10^6 ± $S_{\bar{x}}$	PFC/Spleen ± $S_{\bar{x}}$
Endotoxin + SRBC	10	98 ± 27.5	47,300 ± 7,197
SRBC only	10	70 ± 16.4	15,400 ± 3,930
Endotoxin only	2	9 ± 0.31	5,000 ± 22
Untreated Control	2	3 ± 0.31	40 ± 2

[a]Mice received 60 µg of E. coli endotoxin daily for 7 days, 5 X 10^8 SRBC on day 8, and then endotoxin daily for 5 days; or SRBC only on day 8, or endotoxin only for 12 days, or were left untreated

DISCUSSION

It appears from these results that a cell capable of suppressing the GVH reaction is present in the adherent cell population of spleens from mice treated with endotoxin. There is evidence that this cell may interfere with activities of the non-adherent cell population, and must be alive to exert its suppressor function. Although both normal and ET adherent spleen cell populations are composed of macrophages, lymphocytes, and plasma cells, it is important to note that suppressor activity is not found in normal adherent spleen cells when used in the numbers employed in this study.

Adherent spleen cells from ET-treated mice will suppress the GVH reactivity of whole normal spleen cells when these two cell populations are incubated together *in vitro* for at least 2 hours. This 2 hour time period of interaction may be critical for cell-cell interaction or for the elaboration of a cell product which impairs the GVH reactivity of whole normal spleen cells. There is, however, no detectable cell product released into the BSS environment during *in vitro* incubation which when given to neonatal mice will impair the GVH reactivity of whole normal spleen cells. On the other hand, small doses of serum from ET-treated mice exhibiting a moderate titer of 2-ME sensitive anti-ET activity are capable of suppressing the GVH reactivity of whole normal spleen cells. One explanation for the absence of humoral factor *in vitro* and the presence of a moderate amount of antibody in the serum may be that 19S anti-endotoxin antibody is cytophilic for one or more

of the cells in the adherent population from ET-treated mice, and although removed from the serum, may still be present on cells. There is evidence that macrophages acquire antibody cytophilicly, although most antibodies cytophilic for macrophages have been reported to be of the 7S class (32). There is, however, evidence that mercaptoethanol-sensitive IgM antibody to Salmonella typhimurium is cytophilic for macrophages in the mouse (33).

Bona (25) has shown that macrophages are capable of pinocytosing ET and passing that ET directly to lymphocytes which may be associated with the production of antibody. This intimate interaction between the macrophage and the lymphocyte has been observed in these experiments as rapidly as 1 hour after disruption and washing of ET-treated spleen cells. Evidence has been presented that ET may be capable of stimulating T-cells mitogenically (21). This mitogenic stimulation may effectively commit T-cells to functions other than GVH.

It may be possible that macrophages from ET-treated animals in the presence of, or armed by, 19S anti-ET antibody are altered in such a fashion that they act as immune adsorbers which remove sufficient numbers of lymphocytes needed to produce GVH disease. Another explanation may be that armed macrophages are capable of lymphocyte destruction as evidenced by the reduction of lymphocytes during incubation with macrophages from ET-treated mice, and by the high number of smudge cells observed in vitro. One other possibility that merits discussion

is lymphotoxin, a substance released by lymphocytes which kills target cells indiscriminately (34). Not only has lymphotoxin been shown to kill following specific and nonspecific stimulation of lymphocytes, but also the substance has been linked to the stimulation of macrophages with the subsequent production of activated macrophages which themselves may destroy target cells (35).

Any of the above explanations would suffice to explain why allogenic skin grafts are slow to reject and why allogeneic tumors are allowed to grow in ET-treated mice. There is however, another applicable reason why the two above mentioned observations may occur. Following ET-treatment, the general health of treated mice may be so poor that these mice may not be capable of mustering a cellular response in a normal fashion.

None of these mechanisms seems to explain the observed increase in plaque-forming cells seen by other investigators (36-37) and reported here. Although the plaque-forming response reported here is inconclusive due to the large standard deviations, no decrease in plaque-forming cells to sheep erythrocytes, a thymus-dependent antigen (31), was observed. This evidence may indicate that only one population of thymus-derived lymphocytes is involved in the abrogation of GVH disease by endotoxin.

SUMMARY

Adult CBA mice treated with a bovine strain Escherichia coli (B-44) endotoxin had an adherent spleen cell capable of suppressing the GVH reaction resulting from the treatment of Balb/c neonates with whole normal CBA spleen cells. Evidence was presented that this adherent cell may interfere with activities of the non-adherent cell population and must be alive to exert its suppressor function. Spleen cells from both normal and endotoxin-treated mice had adherent spleen cell populations composed of macrophages, lymphocytes, and plasma cells. Normal adherent spleen cells, however, would not abrogate the GVH reaction. Cell-cell contact was observed between macrophages and lymphocytes of the adherent spleen cells of endotoxin-treated mice incubated in vitro.

The adherent spleen cell population from endotoxin-treated mice also suppressed the GVH reactivity of whole normal spleen cells when these two cell populations were incubated together in vitro for at least two hours. Although no cell product was detected in the incubation environment which would impair the GVH reactivity of whole normal spleen cells, serum from endotoxin-treated mice, high in IgM antibody, would suppress the GVH reactivity of whole normal spleen cells. It was also observed that heterologous immune serum suppressed the GVH reactivity of CBA whole normal spleen cells given to Balb/c neonates.

It appreared from these results that in endotoxin-treated, adult CBA mice, the ability to carry out cell-mediated immune functions other

than GVH was impaired. Endotoxin-treated CBA mice had a reduced ability to reject Balb/c skin grafts and had the ability to grow Balb/c strain-specific myeloma tumors. On the other hand, their helper cell function associated with the plaque-forming cell response to thymus-dependent antigens was not depressed and may even have been enhanced by the mitogenicity of endotoxin.

Mechanisms by which adherent spleen cells and humoral factors from mice treated with endotoxin interact with normal spleen cells in GVH disease are discussed.

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