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This report covers the progress for the first two years of the active grant. During this time we have developed methods to purify the exfoliative toxins A and B from culture supernatants. These toxins have been used for polyclonal and monoclonal antibody production. We have identified proteins on the cell surface of thymocytes that bind to exfoliative Toxin A. This protein is similar to the protein that is precipitated with antibody preparation to class II molecules. We have shown that several exotoxin of S. aureus stimulate tumoricidal activity in peritoneal macrophages. Tumor necrosi factor ą was also produced in response to the toxins as was interleukin-6. The data suggest each toxin activates macrophages differently.

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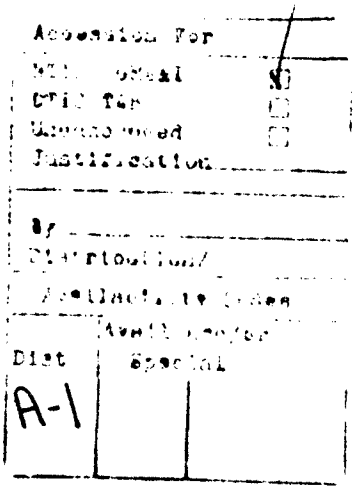

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John J. Daudolo 9/13/91

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Table of Contents

Anti-idiotype probes for toxin detection

by
J.J. Iandolo and S.K. Chapes

INTRODUCTION

Biological agents used as offensive weapons present a covert and insidious danger to the survival of troops on the battlefield. Rapid, sensitive and uncomplicated methods of detection are therefore necessary to preserve the battle potential of our troops and to maintain surveillance on areas once hostilities have ceased. In this project, we proposed to develop a suitable assay that fits the criteria listed. We proposed to identify and isolate the receptor for several staphylococcal exotoxins (in particular the enterotoxins and the exfoliative toxins) from the surface of T-lymphocytes, macrophages and neutrophils. These receptor molecules are to be compared for activity. commonality, antigenicity and specificity. Standard immunological techniques were proposed for assay of receptor binding.

The assay procedure devised for detection of toxins represents the novel aspect of this proposal. It will rely upon the production of anti-idiotypic antibodies to the receptor molecules. Such anti-idiotypes mimic the epitope mosaic of the original antigen (i.e. the receptor) and can be produced in large quantity, thus eliminating the need for expensive purification from lymphocytes or prolonged attempts at genetic manipulation of cloned genes. Receptor anti-idiotypic antibody will specifically bind the toxin or several toxins if common receptors are found. The antibody will be immobilized upon solid supports. Binding to the immobilized antibody can then be assayed by standard ELISA or radioimmune assay.

BODY

Toxin Purification

The grant was activated several months after the proposed start date. After a search for professional and technical help to carry out the goals of the project, we began to develop methods to purify the exfoliative toxins of *Staphylococcus aureus*. Initial attempts centered on high pressure liquid chromatography (HPLC) of ammonium sulfate precipitates. Cultures were grown in 4L quantities and assayed for exfoliative toxin by Western immunoblot and neonatal mouse bioassay of exfoliation. The cells were removed by centrifugation and the cleared medium was filtered through cellulose paper filters to remove particulates. The spent culture fluids were then sterilized by filtration through 0.45μ membrane filters for sterilization. Following this treatment, the supernatants were made to 70% with ammonium sulfate and stirred in the cold overnight. The next morning, the precipitate was collected by centrifugation, dissolved in phosphate buffered saline (PBS) and dialyzed against several changes of PBS to remove residual salt. The dialysate was collected and subjected to HPLC. We used a variety of chromatographic strategies that included molecular size exclusion, hydrophobic interaction, reverse phase and finally ion exchange chromatography. Although we were able to resolve analytical amounts of toxin for mouse bioassay, we were unable to purify significant quantities of either exfoliative toxin A or B (Eta and Etb) by these techniques.

We encountered problems that related to the stability of the proteins in these preparations. Foremost was the loss of toxin by proteolytic activity. If clarified, sterile spent culture medium was stored without further treatment, rapid loss of toxin was observed. Etb

was especially sensitive to protealysis in this melieu. To prevent loss by this means, we examined a number of pretease inhibitors that included phenyl methyl sulfonyl fluoride, ethylene-diamine tetracetic acid, diisopropylfluorophosphate and leupeptin. The most effective agent was leupeptin. This peptide is added after concentration of the medium to a level of 1 μg/ml. Leupeptin binds irreversibly to the active site of proteases and hence a single treatment appears sufficient to prolong toxin activity throughout purification.

The arrival of the preparative isoelectrofocusing device requested in the proposal (BioRad Rotofor) allowed us to finally develop a reliable method to purify large quantities of toxin to apparent homogeniety. A single pass of concentrated culture supernatant through the device results in material that produces 2 to 3 bands on SDS-PAGE gels followed by silver staining. We are able to load approximately 20% of the total volume of the focusing chamber as concentrated culture fluid. The crude toxin is added to 2% ampholytes (pH 3 - 10) in water to make a total volume of 60ml. The unit is run approximately 2 hours at constant power of 12 watts. Fractions are removed with the vacuum apparatus, their pH is measured and they are then analyzed by SDS-PAGE and silver staining and by Western immunoblot against the antiserum to either Eta or Etb. Appropriate samples are pooled and diluted to 60 ml with water. These samples are refocused over the pH range representing the extremes of the pooled samples. The fractions are then re-analyzed and the samples containing the purified toxins are pooled. The pooled toxin preparations are then lyophilized to dryness and stored at -70C until needed.

Recently, we have been optimizing the prefocusing steps of the purification. Initial concentration of the culture is no longer carried out by salt precipitation. We now

concentrate the sterilized culture medium by tangential flow filtration. We typically reduce 4 L of clarified culture liquor to about 200 ml which is then directly subjected to preparative isoelectric focusing.

Using these techniques, we are able to isolate several millegrams of either Eta or Etb from a liter of culture fluid. The material is purified sufficiently to produce a single band on SDS-PAGE gels that have been silver stained. However, the problem of proteolytic loss of toxin remains even after purification and that has baffeled us. Recently, however, the finding that regions of Eta and Etb are similar to the active site of staphylococcal V8 protease by Dancer et al. in 1990 (FEBS Letters 268:129-132) and the recent publication by Redpath et al. in 1991 (FEMS Microbiol. Lett. 81: 151-156) and Prevost et al. in 1991 (Infect. and Immun. 59:3337-3339) that site directed mutagenesis of this region and specifically Ser-197 results in loss of biological activity suggests that the toxins may autodigest. This has yet to be proven.

Antibody Production

At about the same time this grant was activated, the landmark publications of the Marrack group appeared that indicated that the initial interaction of the staphylococcal enterotoxins and the exfoliative toxins were with class II molecules and did not have to be processed by antigen presenting cells. This finding, although startling, did not change the primary hypothesis of our proposal, but added another layer of complexity to the receptor-ligand interactions we proposed to utilize: therefore we also proceeded to immunize animals with class II molecules. This second class of antibody is essential to determine if the toxins interact with known class II molecules.

Mice and rabbits were immunized with purified Eta and Etb and with murine H-2^k class II molecules for production of monoclonal antibodies. Two fusions have resulted in the growth of more than 200 clones of hybridomas. These have been screened and 2 clones have been identified to date that react with Eta. In addition, 1 monoclonal antibody (TIB94), specific for murine H-2^k has been identified that inhibits Eta and Etb induction of TNF by macrophages from C3HeB/FeJ mice, but does not inhibit TNF induction in these same macrophages by Sea or other enterotoxins.

These data suggest that the binding site on murine class II molecules for exfoliative toxins may be distinct from the binding site for the enterotoxins. A number of other hybridoma clones have been obtained. Monoclonal IgG from these clones will be used to identify additional toxin binding sites on class II molecules.

One of the more suprising findings to come out of this work has been the result of our first attempts to produce anti-idiotypic antibody. We immunized mice with murine classII-specific monoclonal antibodies. We hypotheıszed that these antibodies, which inhibit TNF induction by the exfoliative toxins, may be able to induce anti-idiotypic antibodies that would resemble toxin epitopes. The serum of the immunized has recently been tested and shown to produce antibody that reacts with the toxins. As yet we have not produced hybridomas, but these experiments are in progress. In conjunction with this finding, we have also used TIB94 as a probe to identify exfoliative toxin membrane receptors on the surface of spleen cells. Radioiodinated membranes were allowed to bind Eta and then solubilized. These preparations were then immunoprecipitated with antibodies to Eta, and with TIB94. In addition, membranes without Eta treatment were also solubilized and immunoprecipitated

with TIB94. The precipitates were analyzed by SDS-PAGE/Western blot and show a band that appeats to be identical for all three samples suggesting that TIB94 the class II molecule on the surface of these cells acts as receptor for exfoliative toxin A.

Murine Macrophage Activation

We investigated the ability of staphylococcal enterotoxins A and B, the exfoliative toxins A and B and toxic shock syndrome syndrome toxin-1 to activate macrophages. All the toxins tested had the potential to stimulate tumoricidal activity in peritoneal macrophages from lipopolysaccharide responsive C3HeB/FeJ mice. In contrast, none of the toxins activated cytotoxicity in lipopolysaccharide unresponsive macrophages from C3H/HeJ mice. We also studied toxin stimulation of monokine secretion. Staphylococcal enterotoxin A, toxic shock syndrome toxin-1 and both exfoliative toxins triggered C3HeB/FeJ macrophages to secrete tumor necrosis factor-alpha, but enterotoxin B induced only marginal amounts of tumor necrosis factor. All the toxins stimulated interleukin-6 production by macrophages from both strains of mice. Nitric oxide is produced in response to the exfoliative toxins only by the liposaccharide responsive macrophages. These results suggest that each toxin activates macrophages differently. [Details of this work including data are presented in Appendix I]

Staphylococcal exotoxins induce dramatic pathophysiological changes *in vivo*. Previous studies found that exotoxins secreted by *S. aureus* stimulated T cell proliferation (6, 26, 35; all reference numbers refer to citations listed in Appendix I)). In our hands, spleen cells from both C3H/HeJ and C3HeB/FeJ mice proliferated in response to 100 ng/ml of each of our toxin preparations confirming the biological activity of our reagents.

Sea, Eta, Etb and TSST-1 induced C3HeB/FeJ macrophages to produce substantial amounts of TNF. Seb differed from those toxins, by induction of only marginal amounts of TNF. The inability of Seb to induce high levels of TNF secretion contrasts with other recent studies. Seb and TSST-1 have recently been shown to induce human monocytes to transcibe mRNA for IL-1β and TNF (32). Fast *et al.*(11) also showed that TSST-1 and Seb stimulated human monocytes to produce TNF over a period of 1 to 6 days. It is possible that murine macrophages are not stimulated by Seb to the same extent as human monocytes. It is also possible that transcription of TNF genes in human monocytes is not followed by translation and secretion. Transcription and secretion have been separated in human monocytes (17).

Previous investigations have found that nitric oxide production and TNF secretion are concomitantly induced by the calcium ionophore, A23187 (5). Macrophage activation with various toxins resulted in differential secretion of nitric oxide, TNF and IL-6. For example, Eta and Etb were the only toxins we tested that stimulated nitrite secretion by C3HeB/FeJ macrophages. Although the amounts of nitrite produced were less than that induced by an LPS pulse, these data suggest that arginine metabolism is regulated differently by Eta and Etb than by Sea, Seb and TSST-1.

We found that all toxins but Seb induced LPS-responsive macrophages to secrete greater than 15 u/ml of TNF and that only IL-6 is secreted by C3H/HeJ macrophages in response to toxin stimulation. The kinetics of nitrite, IL-6 and TNF secretion after stimulation with various toxins also differs from the response induced by the other toxins. For example, nitrite was only measurable after accumlating for 18 h. in the continuous

presence of exfoliative toxins while IL-6 was measurable within 3 h and was continuously made during the 18 h incubation. In contrast, Sea induced, TNF secretion within the first 3 hr. of stimulation while IL-6 was not secreted in large quantities until after 6 h of exposure. Therefore, these data indicate independent regulation of nitrite, TNF and IL-6 by these exotoxins.

The finding that some toxins can activate macrophages independently of nitrite secretion is consistent with previous studies by Green *et al.* (15). They found that TNF but not the macrophage activators C5 and C5a induced nitric oxide production. The production of one monokine in the absence of another or the independent regulation of monokines is consistent with a number of previous studies. For example, Riessenfeld-Orn *et al.*(27) stimulated human monocytes with *Streptococcus pneumoniae* to induce IL-1 but not TNF. Lonnemann *et al.* (24) found TNF and IL-1 secretion peaked at separate times when human monocytes were stimulated with LPS. Zuckerman *et al.* (37) also found serum TNF peaked prior to IL-1 concentrations after in vivo stimulation. The observations that these staphylococcal exotoxins have such distinct effects on macrophages makes them interesting tools for further investigations on macrophage activation.

Tumoricidal activity induced by the toxins was variable. However, at times when no cytotoxicity was induced by the toxins, tumoricidal activity could be obtained by the same toxin preparations in conjuction with suboptimal concentrations of IFN-Γ. The irregular induction of cytolytic activity may be a result of differences in the induction of IFN-α and subsequent autocrine responses. This would be consistant with multiple signals being required for macrophage cytolysis (16, 33). The finding that Sea, Seb, and TSST-1 induce

cytotoxicity without NO_2^- contrast with the results of Takema (31). This difference may be attributed to the cells used in analyzing cytotoxicity. Our assays measured the cytotoxicity of F5b cells and they measured the killing of P815 cells. Data from our laboratory indicate that macrophage interactions with P815 cells are different from their interactions with F5b (28) and that F5b is not killed by a nitric oxide dependent process

Macrophages from C3H/HeJ mice were not cytotoxic when stimulated with toxins. The lack of activity was not due to toxin breakdown because the same toxin preparations stimulated spleen cells from unresponsive mice to proliferate. Furthermore, when stimulated with LAP-LPS and IFN-Γ, the C3H/HeJ macrophages responded as expected by becoming cytotoxic or secreting monokines.

The finding that LPS^d macrophages were not activated with the exotoxins is consistent with reports of others. C3H/HeJ macrophages do not respond to the calcium ionophore A23187 (29). They also can not be made cytotoxic by *Propionibacterium acnes* (22) or by the lipid-A moiety of Gram-negative bacterial LPS (10). Therefore, the genetic defect of HeJ mice that causes unresponsiveness to other stimuli extends to staphylococcal exotoxins. However, all the toxins induced the LPS^d macrophages to secrete low levels of IL-6 and TSST induced TNF secretion. The finding that unresponsive macrophages can be induced to secrete monokines without becoming cytotoxic is consistent with previous studies (20). In addition, Flebbe *et al.*(13) found C3H/HeJ macrophages could be stimulated by LPS, isolated from rough mutants, to secrete TNF and IL-1, but required an additional signal of IFN-Γ to become cytotoxic. The hypothesis that distinct signals may be provided to macrophages by different toxins is not unprecedented. Smooth LPS activates cells from

C3H/HeJ mice differently than rough LPS (12, 13). The presence of protein in LPS also stimulates macrophages differently than protein-free preparations (9, 19).

It is possible that the exotoxins stimulate different macrophage functions because they trigger autocrine responses. Vogel *et al.* (33) found macrophages produce interferon in response to priming signals. Macrophages require multiple signals to become fully activated (15) and each staphylococcal exotoxin may stimulate interferon differently to provide those signals. Each toxin stimulates uniquely. The kinetics of TNF and nitric oxide secretion in the presence or absence of toxin also indicate that the signals transduced by each exotoxin may be distinct. Additional studies will be required to elucidate the signal transduction pathways for these toxins.

Antiorthostatic suspension

As part of another study involving the effects of prolonged exposure to reduced gravity as experienced in space flight on immunocompetence, we utilized the assays and methodology developed abouve for study of whether antiorthostatic suspension of C3HeB/FeJ mice for a period of 11 days affected macrophage and spleen cell function. We felt one of the potential uses of the assay system we are devising might be to detect immune cell function in the 0-g environment as a potential aid to Astronaut health determinations.

We found that antiorthostatic suspension did not alter macrophage secretion of prostaglandin E_2, thromboxane B_2, superoxide, tumor necrosis factor-α, and interleukin-1. Antiorthostatic suspension also did not affect macrophage-mediated contact-dependent and TNF-mediated cytotoxicity or the expression of Class II histocompatibility molecules or

Concanavalin A and *Bandeiraea simplicifolia* lectin binding sites. The proliferative response of splenic T cells in response to mitogens and staphylococcal exotoxins was significantly enhanced in antiorthostatically suspended mice. We detected significantly higher concentrations of corticosterone in the plasma of antiorthostatically suspended mice. Therefore, there did not appear to be any direct immunosuppressive effects of corticosterone on the parameters tested. [Details of these data are in Appendix II.]

Two major findings have resulted from these investigations: 1) Although antiorthostatic suspension closely mimics some physiological changes associated with space flight, we have shown that it falls short of duplicating some immunological responses affected by spaceflight. The most significant of these immune responses is the reduced post-flight mitogen-induced lymphocyte proliferative response. 2) Glucocorticoids, when elevated to three times normal levels by antiorthostatic suspension, are not inhibitory to macrophage inflammation, secretion, cytotoxicity or membrane molecule expression. Therefore, elevation of these adrenal hormones does not always result in immunosuppression.

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APPENDICES

APPENDIX I

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MURINE MACROPHAGE ACTIVATION BY STAPHYLOCOCCAL EXOTOXINS†

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ABSTRACT

We investigated the ability of staphylococcal enterotoxins A and B, exfoliative toxins A and B and toxic shock syndrome toxin-1 to activate macrophages. All the toxins tested had the potential to stimulate tumoricidal activity in peritoneal macrophages from lipopolysaccharide responsive C3HeB/FeJ mice. In contrast, none of the toxins activated cytotoxicity in lipopolysaccharide unresponsive macrophages from C3H/HeJ mice. We also studied toxin stimulation of monokine secretion. Staphylococcal enterotoxin A, toxic shock syndrome toxin-1 and both exfoliative toxins triggered C3HeB/FeJ macrophages to secrete tumor necrosis factor-alpha but enterotoxin B induced only marginal amounts of tumor necrosis factor. All the toxins used stimulated interleukin-6 production by macrophages from both strains of mice. Nitric oxide is produced in response to the exfoliative toxins only by the lipopolysaccharide-responsive macrophages. These results suggest each toxin activates macrophages differently.

INTRODUCTION

Exotoxins from Staphylococcus aureus cause diseases such as food poisoning, scalded skin syndrome in infants and the multisystem disease, toxic shock syndrome. The toxins are serologically distinct, single polypeptide chains, with sizes ranging from 22 kDa to approximately 35 kDa (21).

The toxins can also induce an immune response and have been termed "superantigens" (36). The superantigens initiate T cell proliferation by the T cell receptor interacting with exotoxins which are bound to Class II molecules of accessory cells (25, 26, 35). Kappler et al. (23) found that T cells which express specific V_β sequences as part of their $\alpha\beta$ T cell receptor are stimulated specifically by staphylococcal enterotoxin A (Sea), staphylococcal enterotoxin B (Seb), and toxic shock syndrome toxin-1 (TSST-1).

Macrophages are also activated by these toxins. Human monocytes stimulated with Seb and TSST-1 for 1-6 days secreted TNF (11). Beezhold et al. (2) used 20 ng/ml TSST-1 to stimulate rat macrophages to produce IL-1. NK cells also become cytotoxic after stimulation with Seb (1).

We studied the ability of Sea, Seb, TSST-1, exfoliative toxin A (Eta), and exfoliative toxin B (Etb) to activate LPS^n (responsive) and LPS^d (unresponsive) murine macrophages. We showed activation of contact-dependent cytotoxicity, TNF and IL-6 secretion and nitric oxide production (as measurable by the stable end product of nitrite) (NO_2^-). We also compared the kinetics of cytokine and NO_2^- production after stimulation by different toxins.

MATERIAL AND METHODS

Mice: C3HeB/FeJ (LPS^n) and C3H/HeJ (LPS^d) mice were bred in the animal facilities in the Division of Biology at Kansas State University.

Tissue Culture Cells: The TNF-sensitive cell line LM929 was obtained from American Type Culture Collection (Rockville, MD 20852). The SV40-transformed cell line F5b is killed by macrophages by a contact-dependent process and has been described previously (8, 28). LM929 and F5b were cultured three times weekly in antibiotic-free Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO, Gaithersburg, MD) supplemented with 2% Fetal Bovine Serum (FBS), 0.3% L-glutamine (Sigma, St. Louis, MO), and 10% Opti-MEM 1 Reduced Serum media (GIBCO). Interleukin-6 was quantitated with the IL-6 dependent, murine B cell hybridoma subclone B9 which was obtained with L. Aarden's permission from R. Nordan (NCI, Bethesda MD). It was cultured in DMEM supplemented with 50 μM 2-Mercaptoethanol, 5% FBS, and 10 pg/ml recombinant IL-6. The hybridoma did not proliferate in response to rTNF, rIL-1, combinations of TNF and IL-1 or in response to any toxin used.

Reagents: Enterotoxins A and B, purified by the procedures of Bergdoll et al. (3) were obtained from Dr. Anna Johnson-Winegar (US Army Medical Research and Development Command, Ft. Dettrick, MD 21701) or Toxin Technology (Madison, Wis). Toxic shock syndrome toxin-1 was obtained from Dr. Peter Bonventre (Dept. Microbiology and Molecular Genetics, University of Cincinnati, College of Medicine, Cincinnati,OH 45267). Exfoliative toxins A and B were purified in our laboratories from culture supernatants of S. aureus strains UT0003 (Eta) and UT0007 (Etb). Extracellular proteins were precipitated with saturated ammonium sulfate and dialysed against water. The toxins were then purified by two cycles of preparative isoelectric focusing. All toxin preparations were biologically active at 100 ng/ml as determined by spleen cell proliferation assay. The exotoxins were endotoxin-free as determined by the Limulus Amebocyte Lysate Assay (Sigma). Our assay was sensitive to an endotoxin concentration of 0.02 ng/ml. The presence of lipoteichoic acid was

determined by Dr. Isaac Ginsburg (Hebrew University Hadassah School of Dental Medicine, Jerusalem, Isreal) using hemagglutination of LTA-sensitized human erythrocytes (14). Our toxin preparations were LTA negative by this method which is sensitive to a concentration of 0.2 μg/ml. Lipid A-associated protein containing LPS (LAP/LPS) was obtained from Dr. David Morrison (Kansas University Medical Center, Dept. of Microbiology, Kansas City, KS 66103). Recombinant, murine TNF and IFN-γ were obtained from Genzyme (Cambridge, MA). Recombinant, murine IL-6 was obtained from R & D Systems (Minneapolis, MN). Experiments were conducted in DMEM supplemented with 2% FBS and 50 μg/ml gentamycin sulfate (DME).

Cytotoxicity Assay: The macrophage cytotoxicity assay was performed as described previously (7). C3HeB/FeJ and C3H/HeJ peritoneal exudate cells (PEC) were obtained 4 or 5 days after injection of 1.5 ml sterile thioglycollate broth (Difco, Detroit, MI). After peritoneal lavage, the PEC were washed and pipetted into flat-bottom, 96 well plates at 1-3 x 10^5 cells per well. After one to two hours the medium was removed and replaced with the appropriate toxin-containing medium (with or without 0.1 u/ml IFN-γ). Approximately, 1 x 10^4 ^{51}chromium-labelled F5b cells were added to each well and the assay was incubated for 16 - 18 h. The microtiter plates were then centrifuged and 90 μl aliquots from each well were counted in a gamma counter. The percent specific release was calculated as follows:

$$\text{Specific release} = \frac{\text{Experimental release - spontaneous release}}{\text{Maximal release - spontaneous release}} \times 100$$

The maximal release and spontaneous release were determined by incubating 1 x 10^4 F5b in 1N HCl (maximal) or medium (spontaneous). The spontaneous release was generally less than 40%. As positive controls, macrophages from C3HeB/FeJ mice were stimulated with E. coli LPS 055:B5 (12.5 μg/ml) (Difco)

and IFN-γ (0.1 u/ml); C3H/HeJ macrophages were stimulated with LAP-LPS (10 μg/ml) and IFN-γ (10 u/ml).

Macrophage Supernatants: C3HeB/FeJ and C3H/HeJ PEC were obtained by peritoneal lavage as described above. The cells were plated at a density of 1 x 10^7 cells per 60 mm tissue culture plate, allowed to adhere for 1-2 hr. and washed with DME. The appropriate toxin in 4 ml DME was added for 30 min. After 30 min., the plates were washed 3 times with 1-2 ml of medium and 4 ml of fresh medium with or without toxin was added. At 3 hr. and 6 hr. after the initial 30 min. incubation period, the supernatants were collected and 4 mls fresh DME with or without toxin was added to the culture plates.

The supernatants were clarified by centrifugation, aliquoted and used immediately or stored at -100°C until assayed. Supernatants collected from cultures pulsed with toxin-containing medium are designated (+) toxin. The supernatants designated (-) toxin came from cultures that received only DME after the initial 30 min. pulse. As a positive control, C3HeB/FeJ macrophages were pulsed with LPS (12.5 μg/ml). Unresponsive, C3H/HeJ macrophages were activated with LAP-LPS (10 μg/ml) and IFN-γ (10 u/ml) as described previously (9). Cytokine and nitrite quantification was based on linear regression of standard curves of rTNF, rIL-6 or $NaNO_2$.

TNF Quantification: Triplicate samples of culture supernatants were serially diluted in DME in 96 well, flat-bottom plates. To each well, 1 x 10^4, ^{51}Cr-labelled LM929 cells were added and incubated for 16 to 18 hr. After centrifugation, 90 μl aliquots were quantitated for chromium release as described.

Nitrite determination: Nitrite concentration was determined by use of the Griess reagent as described previously (30). Briefly, triplicate 100 μl supernatant samples were placed in microtiter plates. To each well 100 μl

Griess reagent (1% sulfanilamide, 0.1% naphylethylene diamine dihydrochloride and 2% H_3PO_4) was added and incubated for 10 min. at 25°C. A microtiter plate reader (Cambridge Technology, Cambridge, MA) was used to read the A_{550}.

IL-6 Quantification: The macrophage supernatants were assayed for IL-6 using the B9 bioassay (18). The B9 cells were washed 3 times in IL-6-free DME to remove residual IL-6. Two thousand B9 cells were added to serially diluted, triplicate samples of culture supernatant and allowed to incubate for 3 days at 37°C. [^{3}H]-thymidine (0.5 μCi/well) was added for 8 hr. prior to harvesting with a PHD Cell Harvester (Cambridge Technology) and liquid scintillation counting.

RESULTS

Toxin induction of macrophage cytotoxicity: The ability of the toxins to induce cytotoxic macrophages was measured by killing of the cell line F5b, previously characterized by our laboratory (8, 28). The cytotoxicity of C3HeB/FeJ macrophages incubated with the exotoxins was variable ranging from 0 to 30 percent. Table 1 is a representative experiment which shows the levels of tumoricidal activity that can be induced by each toxin. None of the toxins stimulated cytotoxicity to the same extent as LPS and IFN-γ. Table 2 shows that the toxins alone did not always induce cytotoxicity (Sea and Seb) or induced very low levels of killing (Eta and Etb). However, when this occurred with either toxin type, cytotoxicity could be induced when used in combination with suboptimal levels of IFN-γ (Table 2). In contrast, no toxin, under any condition, was able to induce C3H/HeJ macrophages to become cytotoxic (Data not shown).

Toxin induction of NO_2^-, IL-6 and TNF secretion: Nitric oxide is released by activated macrophages (4, 30). To determine if arginine metabolism was

involved in toxin-induced activation, we measured the extracellular NO_2^- released in response to the toxins. Sea, Seb, or TSST-1 did not stimulate significant quantities of NO_2^- (Table 3). However, responsive macrophages incubated continuously with Eta or Etb released significant quantities of NO_2^- during an 18 hr. exposure (Table 3). Nitrite was not found in measurable quantities in any other supernatants (Data not shown). The LPS^d macrophages were not stimulated to release measurable nitrite by any of the staphylococcal exotoxins (Data not shown).

To determine if TNF might be secreted independently of macrophage cytolytic activity, we incubated macrophages from C3HeB/FeJ mice with Sea. Within the first 3 hr. TNF appeared in the culture supernatants (Fig. 1). After that time, TNF secretion decreased to background levels. In contrast, responsive macrophages incubated continuously with Seb secreted only marginal amounts of TNF. There appears to be a threshold exposure time because, we found C3HeB/FeJ macrophages pulsed with Sea or Seb for only 30 min. did not secrete TNF.

Tumor necrosis factor was secreted from C3HeB/FeJ macrophages in response to both Eta and Etb stimulation. The continuous exposure to Eta induced approximately 30 u/ml of TNF over the first 3 hr. (Fig. 2). As observed with Sea, the TNF levels dropped to background for the remainder of the 18 hr. experiment. In contrast, Etb induced 30 to 40 units/ml during each collection period. A 30 min pulse of either exfoliative toxin was not enough to induce significant TNF secretion (Fig. 2).

At a concentration of 2.5 μg/ml, TSST-1 stimulated TNF secretion when C3HeB/FeJ macrophages were pulsed for 30 min or incubated continuously with toxin (Fig. 3). When responsive macrophages were continuously incubated with suboptimal concentrations (20 ng/ml) of TSST-1, the macrophages also produced

10 u/ml of TNF. C3H/HeJ macrophages were stimulated to produce a small amount of TNF (18 u/ml) only when incubated continuously with 2 μg/ml TSST-1 (Data not shown). Sea, Seb, Eta, and Etb did not stimulate LPS^d macrophage TNF production (Data not shown).

Interleukin-6 was induced by all the exotoxins tested. Either a 30 min. pulse or the continuous exposure of Sea stimulated the LPS-responsive macrophages to secrete increasing amounts of IL-6 over time, with the greatest accumulation occurring during the last 12 hr (Fig. 4). Interluekin-6 was secreted into the culture supernatant in response to Seb, though the quantities were significantly lower and secretion appeared to peak earlier (Fig. 4). As shown in Figure 5, the exfoliative toxins induced much higher levels of IL-6 than the enterotoxins (Fig. 4). The continued presence of Eta and Etb induced 2 ng/ml or more IL-6 throughout the 18 hr. The kinetics of cytokine secretion induced by the exfoliative toxins also were different from the enterotoxins. Figure 5 shows that a 30 min. pulse of toxin stimulated the most IL-6 to be secreted within the first 3 hr. with decreasing quantities being secreted after this time point. This is opposite to the kinetics of a 30 min. Sea pulse (Fig. 4).

We found macrophages from C3H/HeJ mice could be induced to secrete only IL-6 in response to toxin stimulation. Figure 6 indicates the constant presence of Sea stimulated much higher concentrations of IL-6 than Seb. However, Seb induced significantly more than background levels of IL-6 (8 pg/ml). As found with the LPS^n macrophages, the exfoliative toxins also induced IL-6 secretion by LPS^d macrophages. Eta and Etb induced the secretion of 24 and 55 pg/ml respectively (Fig. 7) of IL-6 which compared favorably with the levels induced by the enterotoxins. However, the macrophages were

stimulated with only 1 μg/ml exfoliative toxin as compared with 10 μg/ml enterotoxin.

The accumulation of IL-6 in the culture supernatant over an 18 hr. incubation was also measured (Table 4). Macrophages from C3HeB/FeJ mice exposed to a 30 min. pulse or incubated continuously with Eta, Etb or Sea secreted large quantities of IL-6. In contrast, the continued presence of Seb was necessary to stimulate greater than background levels of IL-6. The quantities of IL-6 secreted by the LPS^d macrophages were significantly lower (Table 4). Continued presence of each toxin induced IL-6 secretion, but macrophages from C3H/HeJ mice also secreted IL-6 following a 30 min. pulse of Etb or Sea. However, a pulse of Seb or Eta did not yield IL-6 in the culture supernatant (Table 4).

DISCUSSION

Staphylococcal exotoxins induce dramatic pathophysiological changes *in vivo*. We investigated the potential of these toxins to activate murine, peritoneal macrophages as determined by cytotoxicity, production of nitrite, TNF and IL-6. Previous studies found that exotoxins secreted by *S. aureus* stimulated T cell proliferation (6, 26, 35). Spleen cells from both C3H/HeJ and C3HeB/FeJ mice proliferated in response to 100 ng/ml of each of our toxin preparations (Data not shown) confirming the biological activity of our reagents. Sea, Eta, Etb and TSST-1 induced C3HeB/FeJ macrophages to produce substantial amounts of TNF. Seb differed from those toxins, by induction of only marginal amounts of TNF. The inability of Seb to induce high levels of TNF secretion contrasts with other recent studies. Seb and TSST-1 have recently been shown to induce human monocytes to transcibe mRNA for IL-1ß and TNF (32). Fast *et al.* (11) also showed that TSST-1 and Seb stimulated human

monocytes to produce TNF over a period of 1 to 6 days. It is possible that murine macrophages are not stimulated by Seb to the same extent as human monocytes. It is also possible that transcription of TNF genes in human monocytes is not followed by translation and secretion. Transcription and secretion have been separated in human monocytes (17).

Previous investigations have found that nitric oxide production and TNF secretion are concomitantly induced by the calcium ionophore, A23187 (5). Macrophage activation with various toxins resulted in differential secretion of nitric oxide, TNF and IL-6. For example, Eta and Etb were the only toxins we tested that stimulated nitrite secretion by C3HeB/FeJ macrophages. Although the amounts of nitrite produced were less than that induced by an LPS pulse, these data suggest that arginine metabolism is regulated differently by Eta and Etb than by Sea, Seb and TSST-1.

We found that all toxins but Seb induced LPS-responsive macrophages to secrete greater than 15 u/ml of TNF and that only IL-6 is secreted by C3H/HeJ macrophages in response to toxin stimulation. The kinetics of nitrite, IL-6 and TNF secretion after stimulation with various toxins also differs from the response induced by the other toxins. For example, nitrite was only measurable after accumulating for 18 hr. in the continuous presence of exfoliative toxins while IL-6 was measurable within 3 hr. and was continuously made during the 18 hr incubation. In contrast, Sea induced TNF secretion within the first 3 hr. of stimulation while IL-6 was not secreted in large quantities until after 6 hr. of exposure. Therefore, these data indicate independent regulation of nitrite, TNF and IL-6 by these exotoxins.

The finding that some toxins can activate macrophages independently of nitrite secretion is consistent with previous studies by Green *et al.* (15). They found that TNF but not the macrophage activators C5 and C5a induced

nitric oxide production. The production of one monokine in the absence of another or the independent regulation of monokines is consistent with a number of previous studies. For example, Riessenfeld-Orn et al. (27) stimulated human monocytes with Streptococcus pneumoniae to induce IL-1 but not TNF. Lonnemann et al. (24) found TNF and IL-1 secretion peaked at separate times when human monocytes were stimulated with LPS. Zuckerman et al. (37) also found serum TNF peaked prior to IL-1 concentrations after in vivo stimulation. The observations that these staphylococcal exotoxins have such distinct effects on macrophages (See Table 5 for a general summary) makes them interesting tools for further investigations on macrophage activation.

Tumoricidal activity induced by the toxins was variable. However, at times when no cytotoxicity was induced by the toxins, tumoricidal activity could be obtained by the same toxin preparations in conjuction with suboptimal concentrations of IFN-γ. The irregular induction of cytolytic activity may be a result of differences in the induction of IFN-α and subsequent autocrine responses. This would be consistant with multiple signals being required for macrophage cytolysis (16, 33). The finding that Sea, Seb, and TSST-1 induce cytotoxicity without NO_2^- contrasts with the results of Takema (31). This difference may be attributed to the cells used in analyzing cytotoxicity. Our assays measured the cytotoxicity of F5b cells and they measured the killing of P815 cells. Data from our laboratory indicate that macrophage interactions with P815 cells are different from their interactions with F5b (28) and that F5b is not killed by a nitric oxide-dependent process (K. Woods and S.K. Chapes, unpublished observation).

Macrophages from C3H/HeJ mice were not cytotoxic when stimulated with toxins. The lack of activity was not due to toxin breakdown because the same toxin preparations stimulated spleen cells from unresponsive mice to

proliferate. Furthermore, when stimulated with LAP-LPS and IFN-γ, the C3H/HeJ macrophages responded as expected by becoming cytotoxic or secreting monokines.

The finding that LPS^d macrophages were not activated with the exotoxins is consistent with reports of others. C3H/HeJ macrophages do not respond to the calcium ionophore A23187 (29). They also cannot be made cytotoxic by Propionibacterium acnes (22) or by the lipid-A moiety of Gram-negative bacterial LPS (10). Therefore, the genetic defect of HeJ mice that causes unresponsiveness to other stimuli extends to staphylococcal exotoxins. However, all the toxins induced the LPS^d macrophages to secrete low levels of IL-6 and TSST-1 induced TNF secretion. The finding that unresponsive macrophages can be induced to secrete monokines without becoming cytotoxic is consistent with previous studies (20). In addition, Flebbe et al. (13) found C3H/HeJ macrophages could be stimulated by LPS, isolated from rough mutants, to secrete TNF and IL-1, but required an additional signal of IFN-γ to become cytotoxic. The hypothesis that distinct signals may be provided to macrophages by different toxins is not unprecedented. Smooth LPS activates cells from C3H/HeJ mice differently than rough LPS (12, 13). The presence of protein in LPS also stimulates macrophages differently than protein-free preparations (9, 19).

It is possible that the exotoxins stimulate different macrophage functions because they trigger autocrine responses. Vogel et al. (33) found macrophages produce interferon in response to priming signals. Macrophages require multiple signals to become fully activated (16) and each staphylococcal exotoxin may stimulate interferon differently to provide those signals. Each toxin stimulates uniquely (Table 5). The kinetics of TNF and nitric oxide secretion in the presence or absence of toxin also indicate that

the signals transduced by each exotoxin may be distinct. Additional studies will be required to elucidate the signal transduction pathways for these toxins.

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FIGURE LEGENDS

Figure 1. Kinetics of TNF production by C3HeB/FeJ macrophages stimulated by Sea or Seb. C3HeB/FeJ macrophages were incubated in the presence (+) or absence (-) of 10 μg/ml Sea or Seb after an initial 30 min. pulse of the appropriate toxin. Supernatants were collected and assayed for TNF at 3, 6, and 18 h as described in materials and methods.

Figure 2. Kinetics of TNF production by C3HeB/FeJ macrophages stimulated by Eta or Etb. C3HeB/FeJ macrophages were incubated in the presence (+) or absence (-) of 1 μg/ml Eta or Etb after an initial 30 min. pulse of the appropriate toxin. Supernatants were collected and assayed for TNF at 3, 6, and 18 h as described in materials and methods.

Figure 3. Kinetics of TNF production by C3HeB/FeJ macrophages stimulated with TSST-1. C3HeB/FeJ macrophages were incubated in the presence (+) or absence (-) of TSST-1 after an initial 30 min. pulse of TSST-1. Supernatants were collected and assayed for TNF at 3, 6, and 18 h as described in materials and methods.

Figure 4. Kinetics of IL-6 production by C3HeB/FeJ macrophages stimulated by Sea or Seb. C3HeB/FeJ macrophages were incubated in the presence (+) or absence (-) of 10 μg/ml Sea or Seb after an initial 30 min. pulse of the appropriate toxin. Supernatants were collected and assayed for IL-6 at 3, 6, and 18 h as described in materials and methods.

Figure 5. Kinetics of IL-6 production by C3HeB/FeJ macrophages stimulated by Eta or Etb. C3HeB/FeJ macrophages were incubated in the presence (+) or

absence (-) of 1 μg/ml Eta or Etb after an initial 30 min. pulse of the appropriate toxin. Supernatants were collected and assayed for IL-6 at 3, 6, and 18 h as described in materials and methods.

Figure 6. Kinetics of IL-6 production by C3H/HeJ macrophages stimulated with Sea or Seb. C3H/HeJ macrophages were incubated in the presence (+) or absence (-) of 10 μg/ml Sea or Seb after an initial 30 min. pulse of the appropriate toxin. Supernatants were collected and assayed for IL-6 at 3, 6, and 18 h as described in materials and methods.

Figure 7. Kinetics of IL-6 production by C3H/HeJ macrophages stimulated with Eta or Etb. C3H/HeJ macrophages were incubated in the presence (+) or absence (-) of 1 μg/ml Eta or Etb after an initial 30 min. pulse of appropriate toxin. Supernatants were collected and assayed for TNF at 3, 6, and 18 h as described in materials and methods.

Table 1. Cytotoxicity of F5b tumor cells by exotoxin-activated macrophages

Toxin[a]	% Specific Release			
	10:1[b]	20:1	30:1	40:1
Sea (10 μg/ml)	13 ± 0[c]	12 ± 0	15 ± 2	29 ± 1
Seb (10 μg/ml)	9 ± 0	8 ± 1	9 ± 1	18 ± 2
Eta (10 μg/ml)	11 ± 1	18 ± 1	21 ± 0	21 ± 2
Etb (10 μg/ml)	22 ± 0	25 ± 1	27 ± 0	19 ± 1
LPS + IFN-γ	34 ± 2	53 ± 1	58 ± 0	44 ± 1
Medium	8 ± 3	3 ± 1	2 ± 2	6 ± 0

a. Thioglycollate-elicited macrophages were activated with toxin or LPS and IFN-γ as described in the Materials and Methods.

b. Macrophage to target cell ratio.

c. Numbers represent the $\bar{X}$ ± SEM of triplicate samples.

Table 2. IFN-α enhancement of tumoricidal activity

		% Specific Release		
Experiment 1	Toxin[a]	10:1[b]	20:1	30:1
	Eta	6 ± 2[c]	15 ± 3	10 ± 1
	Eta + IFN-γ	14 ± 0	48 ± 3	40 ± 6
	Etb	20 ± 2	19 ± 1	29 ± 0
	Etb + IFN-γ	28 ± 2	33 ± 1	47 ± 2
	Medium	9 ± 2	5 ± 0	6 ± 3
	IFN-γ	5 ± 2	2 ± 2	12 ± 2
	LPS + IFN-γ	26 ± 4	37 ± 3	42 ± 5
Experiment 2	Sea	9 ± 4	6 ± 1	6 ± 1
	Sea + IFN-γ	11 ± 2	18 ± 7	18 ± 2
	Seb	0 ± 1	0 ± 1	0 ± 1
	Seb + IFN-γ	29 ± 2	24 ± 2	21 ± 17
	Medium	0 ± 0	1 ± 3	6 ± 1
	IFN-γ	3 ± 5	0 ± 4	0 ± 2
	LPS + IFN-γ	19 ± 4	22 ± 3	27 ± 0

a. Thioglycollate-elicited macrophages were activated with toxin or LPS in the presence or absence of IFN-γ as described in the materials and methods. Reagents were used in the following concentrations: Eta, 10 μg/ml; Etb, 10 μg/ml; Sea, 10 μg/ml; Seb, 10 μg/ml; IFN-γ, 0.1 μ/ml.

b. Macrophage to target cell ratio.

c. Numbers represent the $\bar{X}$ ± SEM of triplicate samples.

Table 3. Nitric oxide produced by toxin-stimulated C3HeB/FeJ macrophages

Treament[a]	$\mu M\ NO_2^-$ [b]
Sea	1.2 ± 1.3[c]
Seb	1.7 ± 1.5
Eta	26.0 ± 2.6
Etb	7.9 ± 0.6
TSST	1.9 ± 2.8
LPS	152.0 ± 43.3
Media	0.9 ± 0.7

a. The stimulant-containing medium was added to adhered macrophages for 18 hrs. The supernatant was removed and frozen at -100°C until assayed with Griess reagent.

b. Values were calculated by linear regression from a standard of known quantities of nitrite.

c. The numbers represent the $\bar{X}$ ± SEM of the results of 2-6 experiments.

Table 4. IL-6 secreted in response to toxin stimulation

		IL-6 (pg/ml)	
Mouse Strain	Toxin	(+) toxin[a]	(-) toxin[b]
C3HeB/FeJ			
	Eta (1 μg/ml)[c]	> 1600	> 1600
	Etb (1 μg/ml)	> 1600	> 1600
	Sea (10 μg/ml)	1318	1280
	Seb (10 μg/ml)	....	864
C3H/HeJ			
	Eta (1 μg/ml)	24	10
	Etb (1 μg/ml)	56	21
	Sea (10 μg/ml)	33	24
	Seb (10 μg/ml)	19	11
	Medium	..	14

a. Adhered thioglycollate-elicited macrophages were stimulated with toxin for 30 mins, washed 3 times and 4 mls of toxin-containing medium added for 18 hr. The supernatant was collected and IL-6 determined as described in the materials and methods.

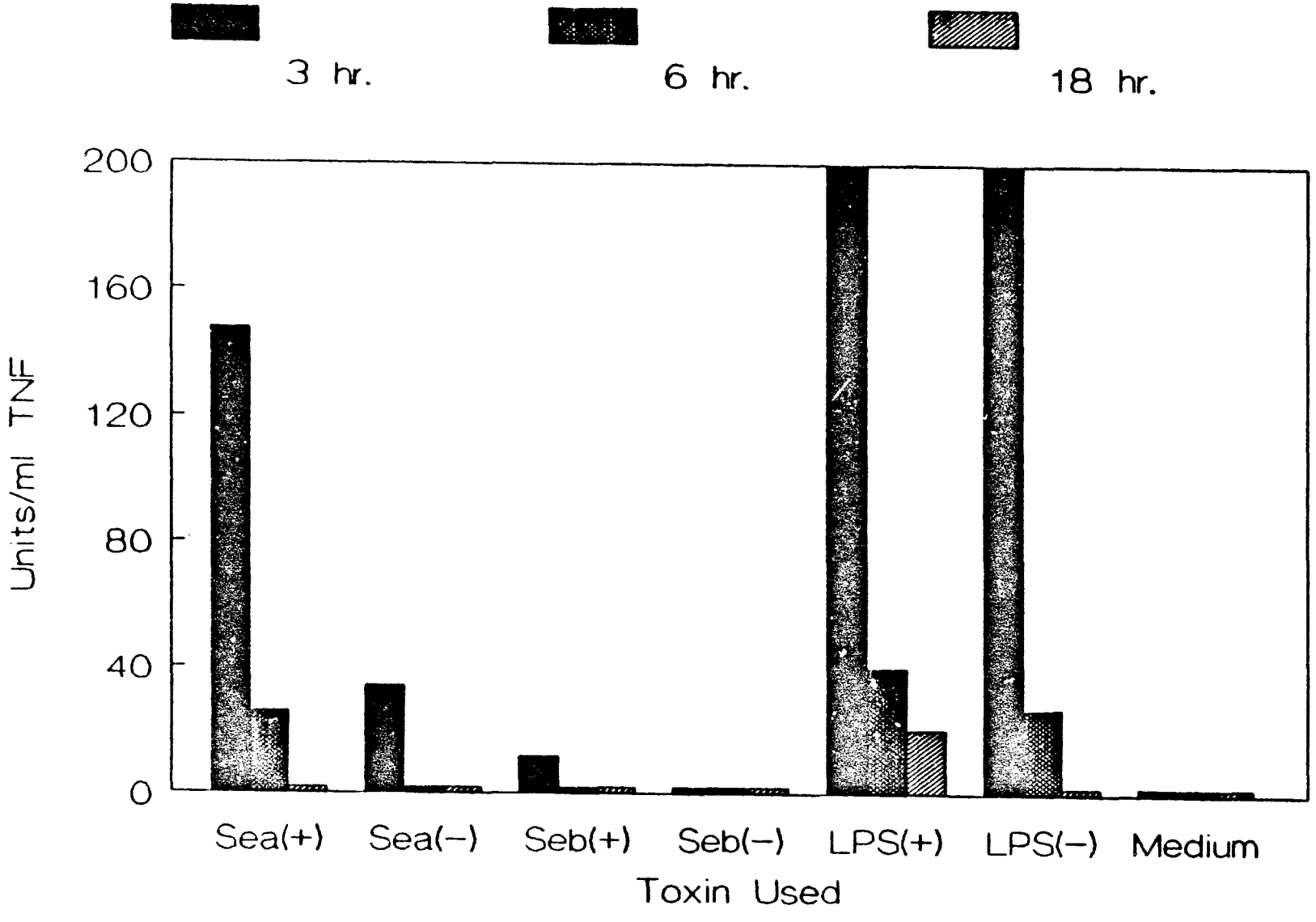
b. Treatment is the same as a) except after the wash, DMEM was added for 18 hr. The supernatant was collected and IL-6 determined.

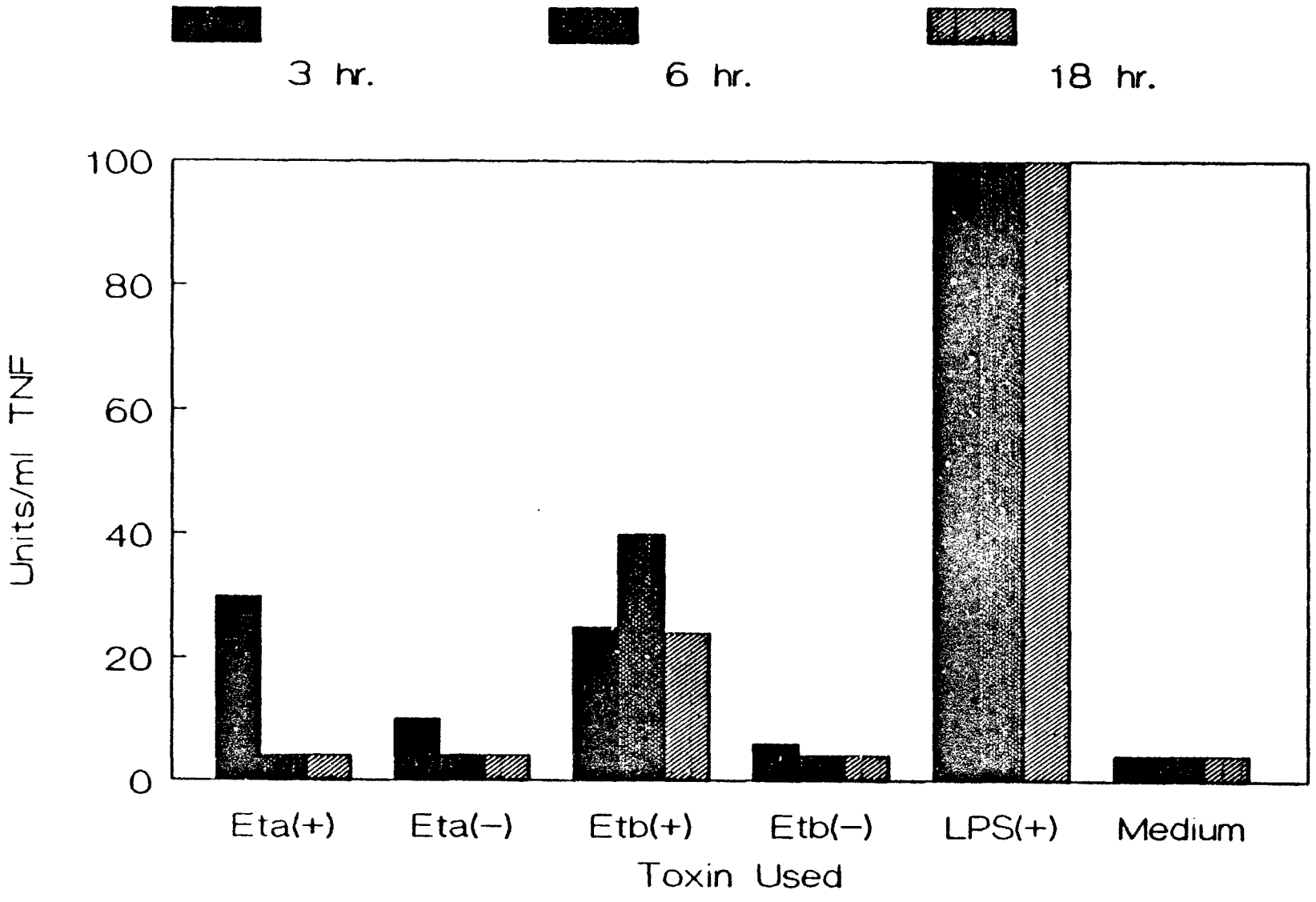
c. The data are representative of 2-5 experiments depending on the toxin.

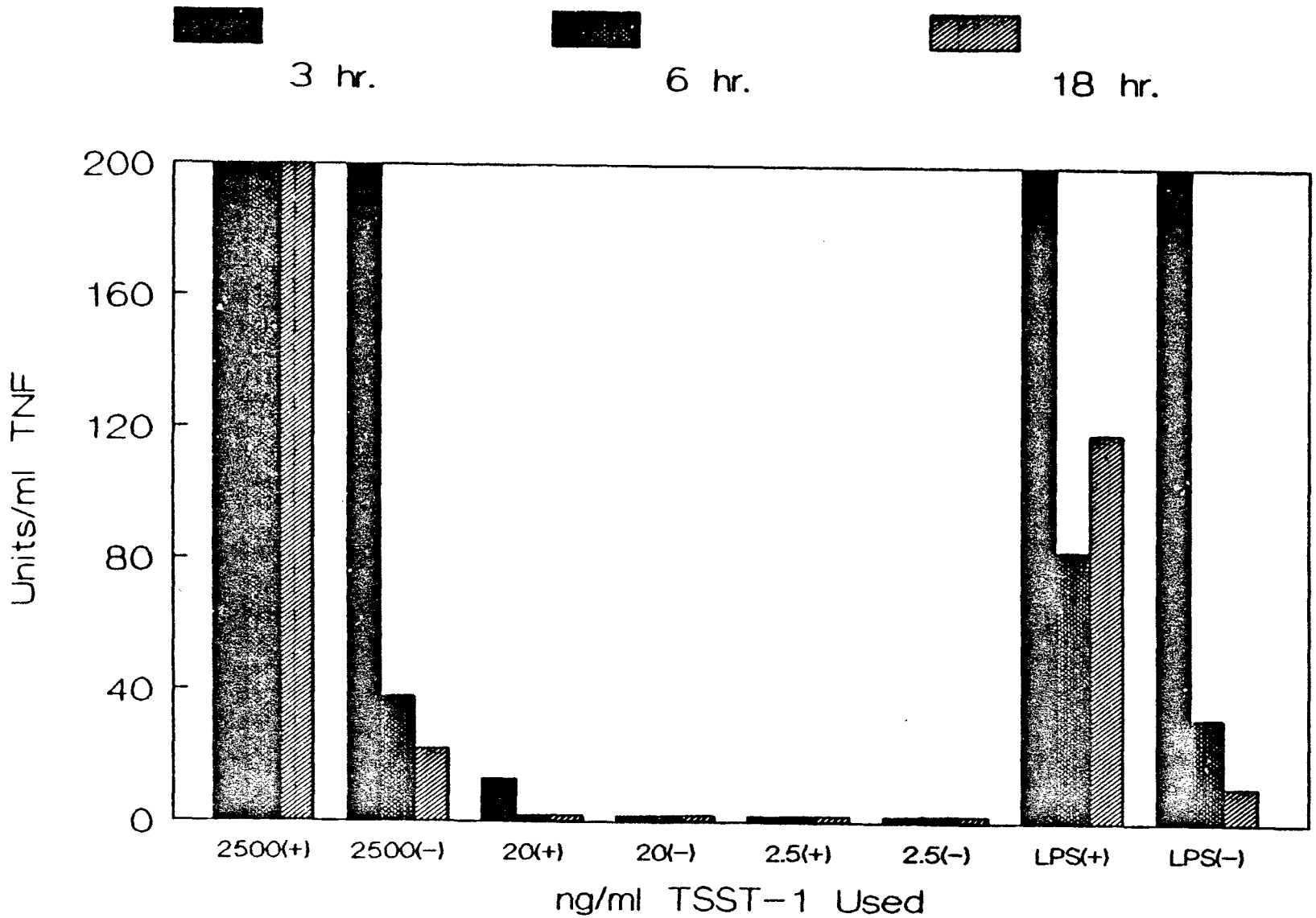
Table 5. Summary of exotoxin activation of macrophages

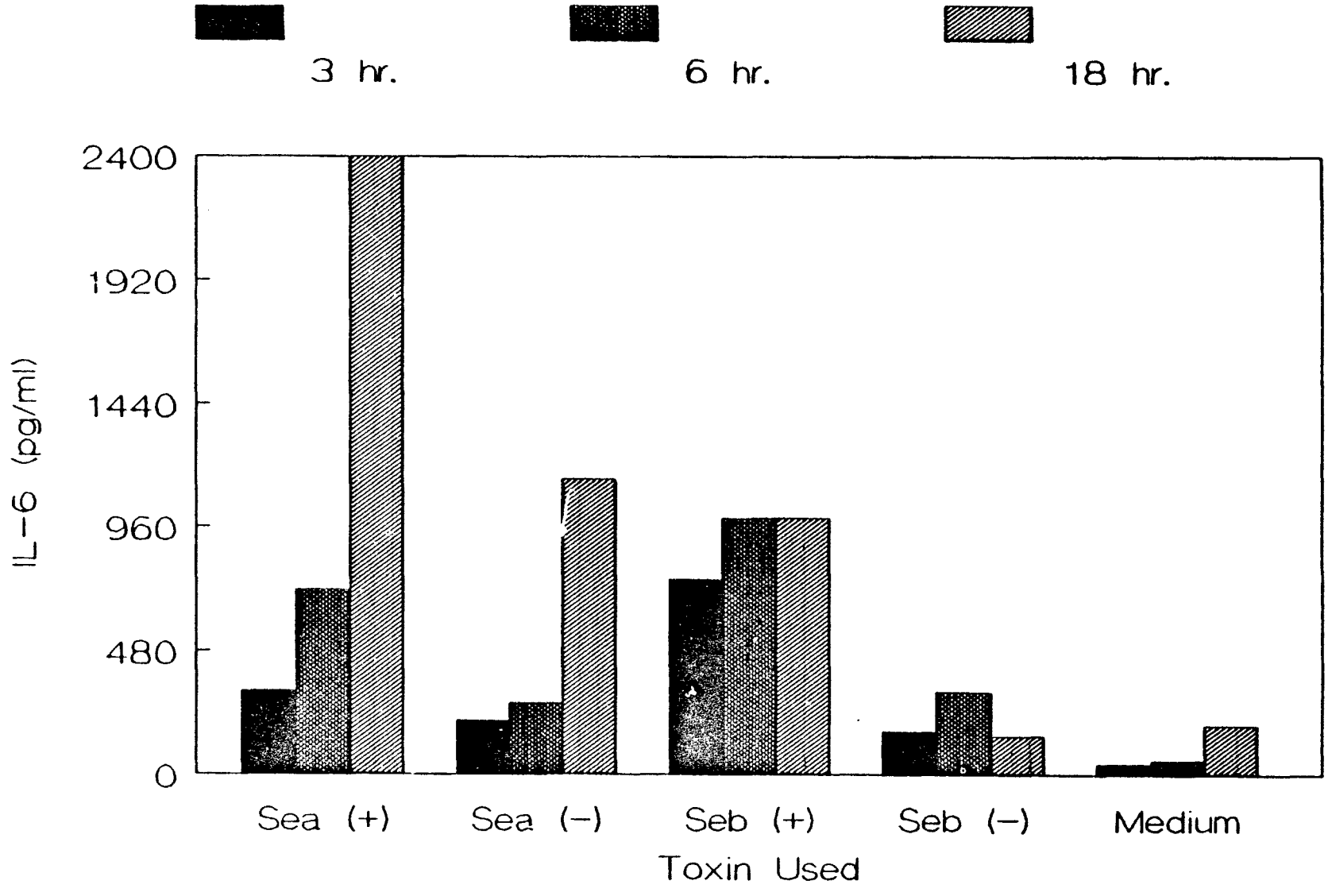
	C3HeB/FeJ (LPS responders)				
Activity	Sea	Seb	Eta	Etb	TSST-1
Tumoricidal	+	+	+	+	+
TNF (u/ml)	150[a]	12	30	40	200
IL-6 (pg/ml)	1318	1600	1600	1600	224
NO_2^-	-	-	+	+	-
	C3H/HeJ (LPS nonresponders)				
Activity	Sea	Seb	Eta	Etb	TSST-1
Tumoricidal	-	-	-	-	-
TNF (u/ml)	-	-	-	-	18
IL-6 (pg/ml)	36	19	24	55	28
NO_2^-	-	-	-	-	-

a. Numbers represent quantity of cytokine/ml in culture supernatants of macrophages incubated with toxin-containing medium for 18 hr.


3 hr.
6 hr.
18 hr.
200
160
120
80
40
0
Units/ml TNF
Sea(+)
Sea(−)
Seb(+)
Seb(−)
LPS(+)
LPS(−)
Medium
Toxin Used


3 hr.
6 hr.
18 hr.
100
80
60
40
20
0
Units/ml TNF
Eta(+)
Eta(−)
Etb(+)
Etb(−)
LPS(+)
Medium
Toxin Used


3 hr.
6 hr.
18 hr.
200
160
120
80
40
0
Units/ml TNF
2500(+)
2500(−)
20(+)
20(−)
2.5(+)
2.5(−)
LPS(+)
LPS(−)
ng/ml TSST−1 Used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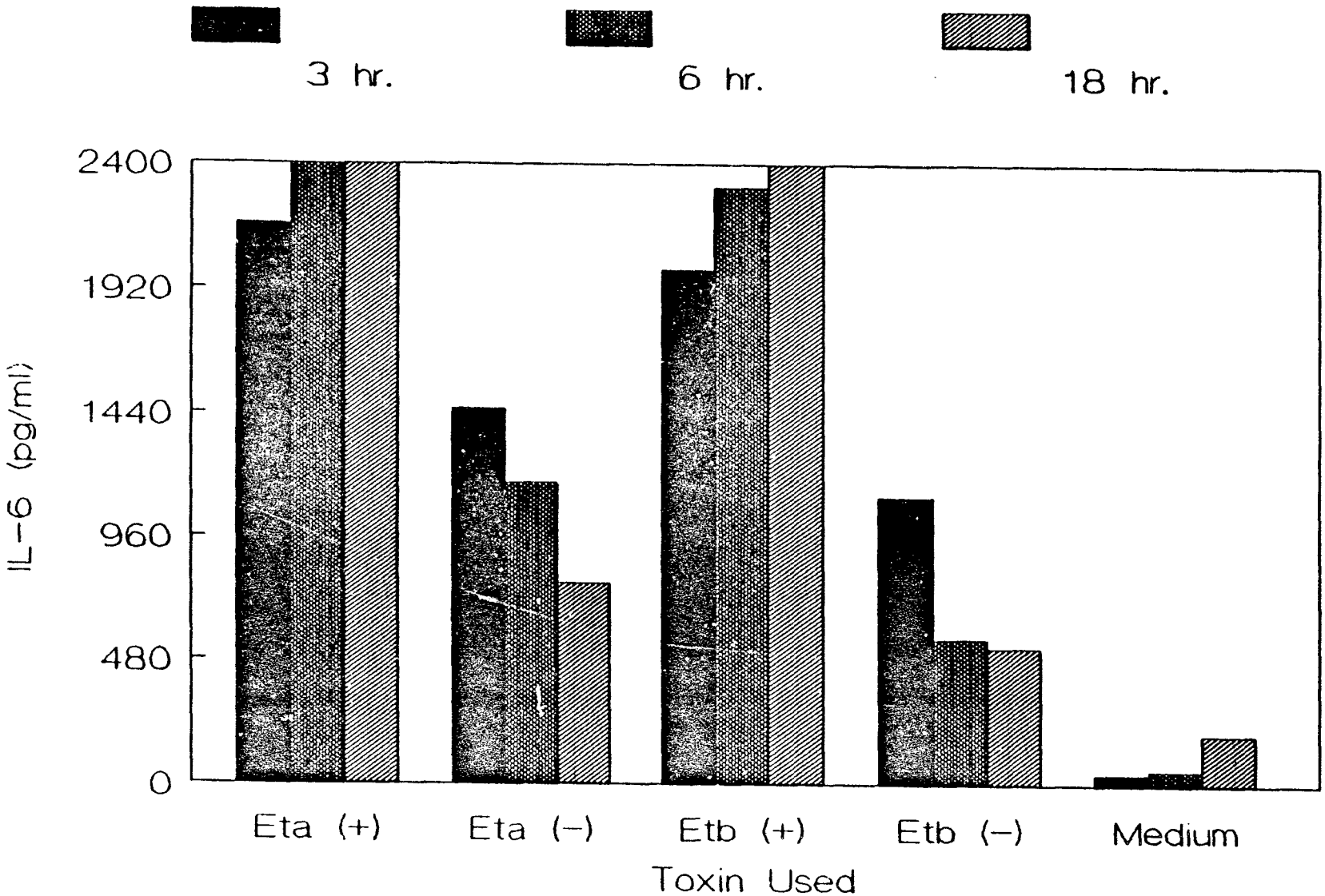
6 hr.
18 hr.
2400
1920
1440
960
480
0
IL-6 (pg/ml)
Sea (+)
Sea (−)
Seb (+)
Seb (−)
Medium
Toxin Used


3 hr.
6 hr.
18 hr.
2400
1920
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480
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IL-6 (pg/ml)
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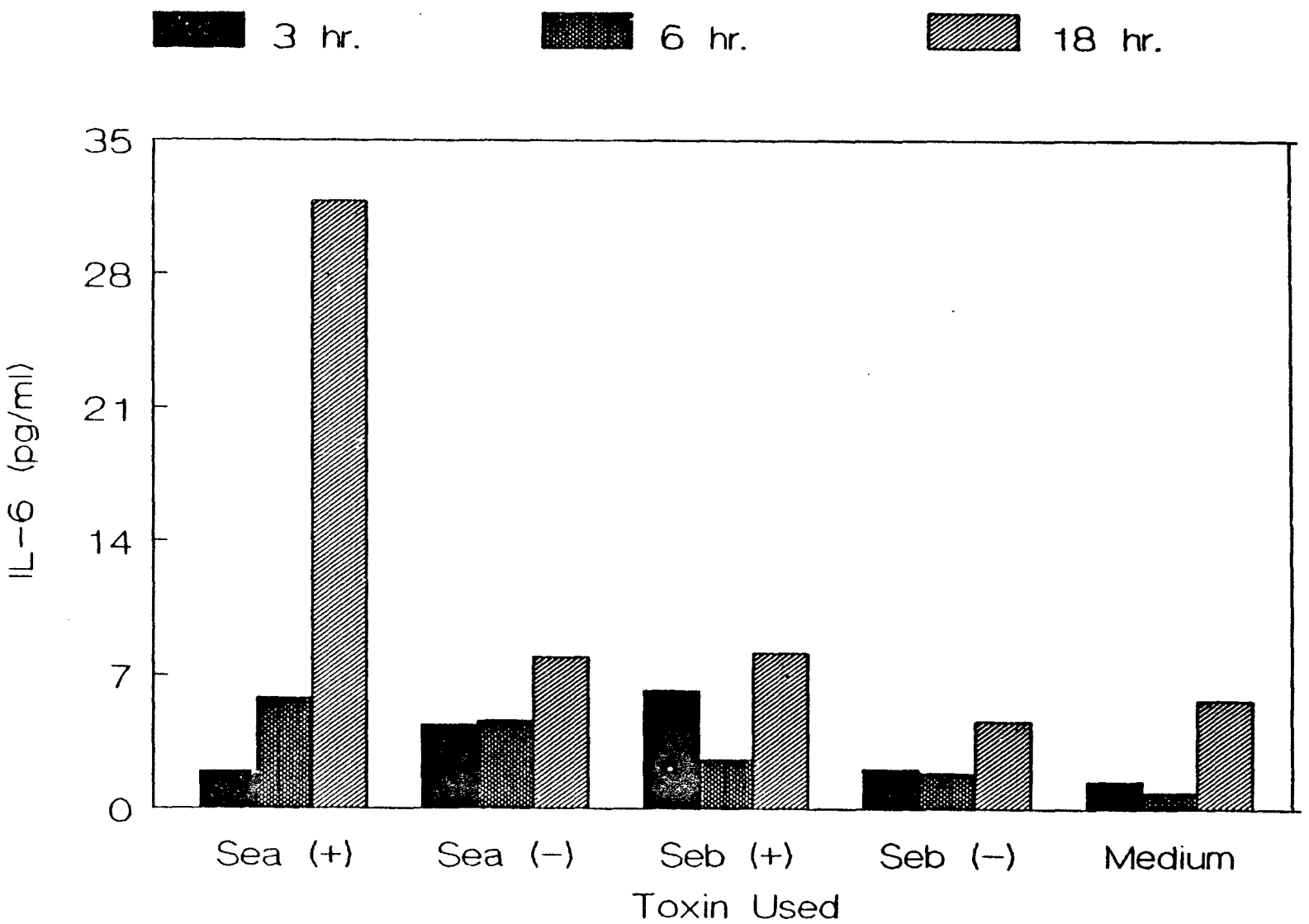
Eta (−)
Etb (+)
Etb (−)
Medium
Toxin Used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6 hr.
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28
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14
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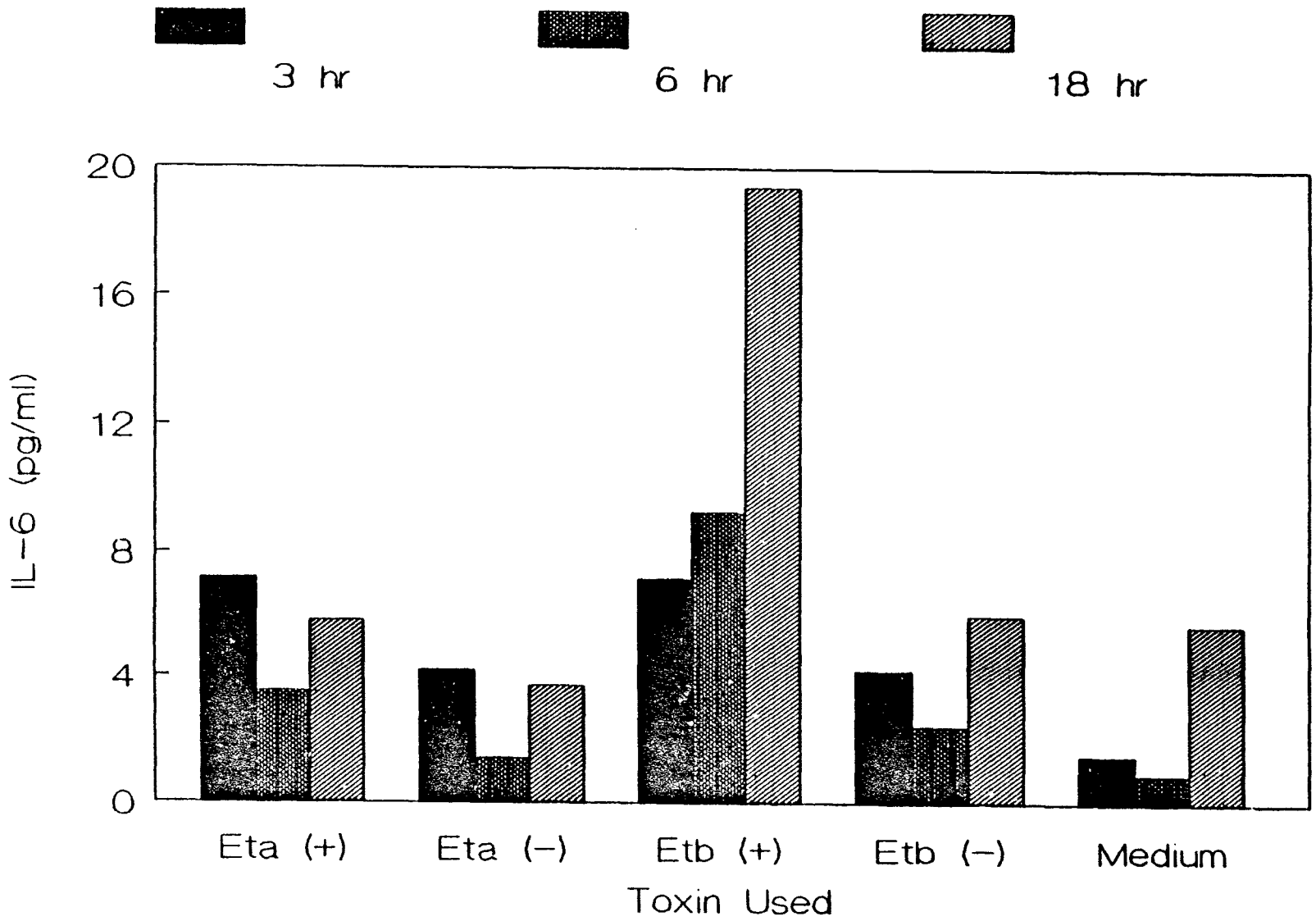
Sea (−)
Seb (+)
Seb (−)
Medium
Toxin Used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6 hr
18 hr
20
16
12
8
4
0
IL-6 (pg/ml)
Eta (+)
Eta (−)
Etb (+)
Etb (−)
Medium
Toxin Used

APPENDIX II

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Effects of Antiorthostatic Suspension and Corticosterone on Macrophage and Spleen Cell Function[1]

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Key Words: Antiorthostatic Suspension, Corticosterone, Stress

Running-Title: Antiorthostatic Suspension and Immunity

ABSTRACT:

The purpose of this study was to determine whether antiorthostatic suspension of C3HeB/FeJ mice for a period of 11 days affected macrophage and spleen cell function. We found that antiorthostatic suspension did not alter macrophage secretion of prostaglandin E_2, thromboxane B_2, superoxide, tumor necrosis factor-α, and interleukin-1. Antiorthostatic suspension also did not affect macrophage-mediated contact-dependent, TNF-mediated cytotoxicity, the expression of Class II histocompatibiblity molecules or Concanavalin A and Bandeiraea simplicifolia lectin binding sites. The proliferative response of splenic T cells in response to mitogens and staphylococcal exotoxins was significantly enhanced in antiorthostatically suspended mice. We detected significantly higher concentrations of corticosterone in the plasma of antiorthostatically suspended mice. Therefore, there did not appear to be any direct immunosuppressive effects of corticosterone on the parameters tested. We discuss the applicability of suspension modeling to study immunological changes that might occur during space flight.

FOOTNOTES:

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INTRODUCTION:

Space exploration has progressed at a rapid pace over the past two decades, yet knowledge of the effects of space flight conditions on the immune system of man is inadequate and limited in its overall scope (5). The opportunity to investigate immunological changes induced by microgravity on actual space flights has often been too infrequent to make firm conclusions. Consequently, ground-based models to simulate biological changes induced by space flight have been designed. Antiorthostatic suspension is a ground-based model developed by Morey (59) which has been shown to closely simulate several physiological parameters (17,59,60,76,83). To date, few studies have investigated the effect of antiorthostatic suspension on the immune system (7,11,33,69,73,76). Rats which were immunized with sheep red blood cells (SRBC) and then suspended for 10-15 days, were examined for alterations in anti-SRBC antibody titers, serum Ig levels, hematocrits, lymphoid organ weights and leukocyte differentials (11). No changes were observed besides reduced thymus and spleen weights. A reduction in the ability of alveolar macrophages of antiorthostatically suspended mice to phagocytose bacteria was observed in a study conducted by Berry et al. (7). Others found decreased production of interferon-alpha/beta when challenged with an intravenous injection of polyriboinosinic-polyribocylidylic acid (69,73). Fleming et al. (33) found an impaired superoxide response of PMNs from antiorthostatically suspended mice accompanied by a reduced ability to kill phagocytosed bacteria.

Little work has been done to examine the effects of antiorthostatic suspension on inflammatory macrophage and spleen cell functions. Therefore, we examined the effects of antiorthostatic suspension on macrophage production of secretory products, cytotoxic function and expression of cell surface receptors as well as proliferative responses of lymphocytes. We found that

the production of the eicosanoids, prostaglandin E_2 (PGE_2) and thromboxane B_2 (TB_2); superoxide, tumor necrosis factor-α (TNF) and interleukin (IL)-1; contact-dependent and TNF-mediated cytotoxicity; and the expression of Class II, concanavalin A (Con A) and Bandeiraea simplicifolia ($BSI\text{-}B_4$) binding sites are not altered by antiorthostatic suspension. In contrast, the mitogen- and toxin-induced proliferation was enhanced for spleen cells taken from anti-orthostatically suspended mice. Although elevated plasma corticosterone levels were detected in antiorthostatically suspended mice, none of the parameters we studied appeared to be negatively affected by the corticosterone.

MATERIALS AND METHODS:

Mice: C3HeB/FeJ mice were bred in the animal facilities in the Division of Biology at Kansas State University. Only mice that had attained a minimum weight of 22 grams were used in these experiments. Their ages ranged from 7 to 14 weeks.

Reagents: The mitogens phytohemagglutinin (PHA) and Con A were purchased from Wellcome Biotechnology (Research Triangle Park, NC) and Sigma (St. Louis, MO), respectively. Exfoliative toxin A (Eta) was purified by isoelectric focusing from ammonium sulfate precipitated culture supernatants of Staphylococcus aureus strain UT0003 (32). Enterotoxin B (Seb), purified by the procedures of Bergdoll (6), was obtained from Dr. Anna Johnson-Winegar (United States Army Medical Research and Development Command, Ft. Detrick, MD 21701). Escherichia coli LPS 055:B5 was purchased from Difco (Detroit, MI). Recombinant murine TNF and IFN-gamma were obtained from Genzyme (Cambridge, MA). PGE_2 and TB_2 standards for reverse phase, high pressure liquid chromatography (HPLC) were purchased from Calbiochem (San Diego, CA).

Reagents for the superoxide assay: phorbol myristate acetate (PMA), superoxide dismutase (SOD), and ferricytochrome C, were obtained from Sigma. Lyophilized corticosterone antiserum B3-163 was purchased from Endocrine Science (Tarzana, CA) and [^{3}H]-corticosterone was purchased from NEN Research Products (Boston, MA). Fluorescein isothiocyanate (FITC) conjugated Con A (#C-7642) and BSI-B_4 (#L-2895) were purchased from Sigma. Goat-anti-mouse IgG conjugated to FITC was obtained from Hyclone (Logan, UT). Protein-A-purified monoclonal antibodies specific for I-A^k (IgG_{2b}) were produced by the hybridoma TIB-94 which was purchased from the American Type Culture Collection (ATCC, Rockville, MD). Propionibacterium acnes (P. acnes) was obtained from Wellcome Biotechnology.

Tissue Culture Cells: Cell line LM929, a TNF-sensitive fibroblast, Balb/c 3T3 fibroblasts and VERO cells were obtained from the ATCC. The SV40-transformed cell line F5b is killed by macrophages through a contact-dependent process and has been described previously (13,70). LM929, VERO, 3T3, and F5b were cultured three times weekly in antibiotic-free Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Gaithersburg, MD) supplemented with 2% fetal bovine serum (FBS), 0.3% L-glutamine (Sigma), and 10% Opti-MEM 1 Reduced Serum Medium (Gibco).

Suspension Technique: We used the antiorthostatic suspension technique that has been described previously (33). Each suspension experiment involved three suspension categories: antiorthostatic suspension (experimental), orthostatic suspension (control), and non-suspended (normal control). Mice were age, weight and gender matched among these three categories. The mice which were antiorthostatically suspended were taped to allow the hindlimbs to be unloaded and unrestrained, while the forelimbs supported approximately 30% of their body weight. Orthostatic suspension involved taping the tail of the

mice, yet allowing 100% of their body weight to be supported by all four limbs. Normal or non-suspended mice were caged alone and unrestrained during the suspension period of 11 days. Four days prior to sacrifice, an inflammatory response was induced in all mice by an intraperitoneal injection of 700 μg of P. acnes. In all experiments, mice were sacrificed between 8:00 and 10:00 a.m. to eliminate differences caused by circadian rhythms.

Blood Collection and Preparation: Immediately prior to animal sacrifice, blood was collected from the retro-orbital sinus using a pasteur pipet. To collect blood samples for plasma arachidonic acid metabolite analysis, the pipet was treated with a disodium ethylenediamine tetraacetate (EDTA)-indomethacin solution (28 nM and 14 μM, respectively). This treatment prevented coagulation and arrested arachidonic acid metabolism. The blood was dispensed into microfuge tubes containing 100 μl of the EDTA-indomethacin solution then mixed well. Immediately thereafter, the blood was centrifuged at 2500 x g for 15 minutes at 4° C. The supernatant (platelet poor plasma) was collected and acid stabilized by combining it with 1/10 volume of 0.1 N HCL in 70% ethanol. Samples were stored at -100° C until they were analyzed for corticosterone, PGE_2 and/or TB_2.

Spleen Cell Proliferation Assay: Spleens were removed from euthanized mice, weighed, and homogenized. Red blood cells were lysed with ice cold 0.17 M NH_4Cl for 5 minutes on ice. The spleen cells were then washed twice with DMEM containing 2% FBS and 5.0 μg/ml gentamycin sulfate (DME). Cells were plated at 1 x 10^6 cells/well (100 μl) in Costar 96-well, flat-bottomed microtiter plates. Wells then received 100 μl of medium (DMEM supplemented with 2% FBS and 5.6 x 10^{-5} M 2-mercaptoethanol (2-ME) with or without one of the following stimulants at the concentration indicated: PHA (9 μg/ml), Con A (2 μg/ml), Eta (1 μg/ml), and Seb (1 μg/ml). Cells were incubated for 48

hours at 37° C in 8% CO_2. Six hours prior to harvest, 0.5 - 1.0 μCi of [^{3}H]-thymidine was added to each well. Cells were harvested on glass fiber discs and added to vials with 1 ml of Scintiverse BD cocktail (Fisher). Incorporation of [^{3}H]-thymidine by the cells was determined by a Tri-Carb 1500 Liquid Scintillation Analyzer (Packard). The stimulation index (SI) was calculated from the following formula:

$$SI = \frac{\text{cpm of experimental}}{\text{cpm of background}}$$

Experimental represents spleen cells incubated in medium plus stimulant. Background represents spleen cells incubated in medium alone.

Peritoneal Cells: Peritoneal exudate cells (PEC) were harvested from euthanized mice by washing the peritoneal cavity twice with 10-12 ml of ice cold PBS. Cytosmears were made with 5 x 10^5 cells and stained with a modified Wright's Stain. Three fields of at least 100 cells were scored for macrophages, neutrophils and lymphocytes.

Peritoneal Macrophage Supernatants: PEC were recovered as described above. Cells were plated at a concentration of 5 x 10^6 cells per 60 mm tissue culture plate. The cells were allowed to adhere for 1.5 - 2.0 hours before medium containing nonadherent cells was removed. After washing the cells, 3 ml of DME alone or with LPS or LPS/IFN-gamma (12.5 μg/ml and 10 units/ml, respectively). The plates were incubated at 37° C at 8% CO_2 and supernatants were collected after 30 minutes, 18 or 24 hours of incubation. The supernatants were clarified by centrifugation and 1/2 volume was immediately frozen and stored at -100° C for TNF analysis. The other 1/2 volume was acid stabilized by combining it with 1/10 volume of 0.1 N HCl in 70% ethanol and then frozen at -100° C until it was analyzed for PGE_2 and TB_2.

Ethanol Extraction of Samples for HPLC Analysis: Acid stabilized plasma samples and macrophage supernatants were ethanol extracted by combining them with 4 times their volume of ice cold 95% ethanol. After incubation on ice for 10 minutes, the samples were centrifuged and supernatants were collected and stored at -100° C.

Separation and Quantitation of PGE_2 and TB_2 Using Reverse-phase, High Performance Liquid Chromatography: Separation and quantitation of arachidonic acid metabolites was achieved by HPLC utilizing a single reverse phase column and a variable wavelength ultraviolet-visible spectrophotometer. An Isco Model 2350 HPLC pump (Isco, Lincoln, NE) was equipped with a C-18 octadecylsilane (ODS) column (Supelcosil LC-18 Column, 15 cm x 4.6 mm, Supelco, Bellefonte, PA) which was guarded with an ODS precolumn (Supelco). Ethanol extracted plasma or supernatant samples were loaded into the injection port with a 1 cc tuberculin syringe fitted with an Acrodisc 0.45 μm prefilter (Fisher, St. Louis, MO) and port injection needle. A single microprocessor-controlled pump isocratically eluted the column at a flow-rate of 1 ml/minute. The mobile phase was a premixed solution comprised of 60/25/15 (v/v/v) distilled water/methanol/acetonitrile and 0.01% trifluoroacetic acid. The acetonitrile was HPLC grade and the water and methanol were filtered and degassed. Only fractions containing PGE_2 or TB_2 were collected from each sample (determined by running PGE_2 and TB_2 standards). The absorbance of the fractions was determined using a spectrophotometer at a wavelength of 193 nm. Quantitation of PGE_2 and TB_2 concentration was calculated by the following formula: $A = Ebc$; where A = absorbance, E = extinction coefficient (PGE_2 = 15,400 and TB_2 = 16,500), b = distance in cm (constant = 1), and c = concentration (mol/l) (82). At the end of each run, the column was flushed

with methanol for approximately 10 minutes and then flushed with mobile phase until it was reequilibrated to intitial conditions.

Cytotoxicity Assay: PEC were washed and resuspended in DME. Cells were added to Costar 96-well, flat-bottomed microtiter plates at 3.0×10^5 cells/well. After 1.5 - 2.0 hours of incubation at 37° C, medium containing nonadherent cells was removed and replaced with 100 µl of fresh DME. F5b target cells, labeled overnight with 150 µCi of ^{51}chromium per 2×10^6 cells in a 100 mm tissue culture plate, were added at a concentration of 1×10^4 cells/well (protocol for vaccinia infected Balb/c 3T3 target cells described below). After an incubation period of 16-18 hours at 37° C, the microtiter plates were centrifuged and 90 µl aliquots were harvested from each well. Samples were counted on a Packard Multi-Prias 1 gamma counter. The percent specific release was calculated from the following formula:

$$\% \text{ Specific} = \frac{\text{experimental release - spontaneous release}}{\text{maximal release - spontaneous release}} \times 100$$

The maximal release and spontaneous release were determined by incubating target cells in 1 N HCl (maximal) or medium (spontaneous). The spontaneous release was generally less than 40%.

Infection of Balb/c 3T3 Target Cells with Vaccinia Virus: The WR strain of vaccinia virus, obtained from ATCC, was propagated, and titered using VERO cells. Balb/c 3T3 cells were plated at a concentration of 1×10^6 cells per 60 mm tissue culture plate then labeled overnight with 100 uCi of ^{51}Cr in 3 ml of DME. One hour prior to addition to the cytotoxicity assay, the Balb/c 3T3 cells were infected at a multiplicity of infection of 2-3 in 1 ml of DME. The plates were rocked at 37° C, then cells were washed, dispersed and added to the assay at a concentration of 1×10^4 cells/well.

TNF Assay: Triplicate samples of peritoneal macrophage culture supernatants were serially diluted in DME in 96-well, flat-bottomed microtiter plates. LM929 cells, labeled with ^{51}Cr as described above, were added at a concentration of 1 x 10^4 cells per well. After an incubation period of 16-18 hours at 37° C, the microtiter plates were centrifuged and 90 μl aliquots harvested from each well were counted on the gamma counter. Percent specific release was calculated as described in the cytotoxicity assay. Quantitation of TNF in the supernatant samples was based on linear regression of standard curves of rTNF.

IL-1 Assay: IL-1 secretion into culture supernatants was assayed by the previously described comitogenic murine thymocyte assay (57). It has been recently suggested that this assay can be influenced by the presence of IL-6; however, the stimulatory concentrations of IL-6 are 1,000-fold higher than for IL-1 (22). Nevertheless, it is possible that some of the proliferation measured is due to the additional presence of IL-6. We have reported our measurements of proliferation as IL-1 with the explicit understanding that IL-6 may be a factor. Thymocytes were aseptically removed from 4- to 8-week old C3H/HeJ mice, teased to release thymocytes, washed and resuspended in DME containing 0.3% glutamine, 3 x 10^{-5} M 2-ME and 5.0 μg/ml gentamycin sulfate. Thymocytes were distributed into 96-well flat-bottomed microtiter plates at a concentration of 1 x 10^6 cells/well in 100 μl of medium. Doubling dilutions of test supernatants (100 μl) were added to the wells, in addition to 0.45 μg PHA/well. Plates were incubated for 48 hours at 37° C and 8% CO_2. Eight hours prior to harvest, 1 μC ^3H-thymidine (20 μl) was added to each well. Incorporation of ^3H-thymidine was measured on a scintillation counter. Background control wells contained thymocytes and PHA only.

Superoxide Assay: Superoxide generation induced in response to PMA was determined by the SOD inhibitable reduction of ferricytochrome C as described previously (33) using a Series 750 Microplate Reader (Cambridge).

Cell Surface Labeling for Flow Cytometric Analysis: Peritoneal cells suspended in DME were plated at a concentration of 1 x 10^6 cells/well in a 96-well, U-bottomed microtiter plate. The plate was centrifuged at 400 x g for 2 minutes and supernatants were removed. Cells were incubated 30 minutes on ice with 50 µl/well of fetal bovine serum to inhibit Fc receptor binding which increased specific binding 80 to 160% (data not shown). Cells were washed with 100 µl/well of sorter buffer (HBSS containing 0.1% bovine serum albumin and 0.1% sodium azide). Plates were centrifuged and supernatants were removed. Labeling with fluoresceinated lectins: A volume of 20 µl of 0.5 mg/ml FITC-Con A or 0.5 mg/ml FITC-BSI-B_4 was added per well. Cells and labeled lectins were pipetted up and down several times to mix, then incubated for 30 minutes at 4° C. After centrifugation at 400 x g for 2 minutes, supernatants were removed and cells were washed twice with 100 µl/well of PBS. Cells were resuspended in 2 ml of PBS containing 1% paraformaldehyde and stored at 4° C. Labeling with primary and secondary antibody: To the appropriate wells, 20 µl of 0.4 mg/ml anti-I-A^k TIB-94 was added. The cells and primary antibody were mixed well and incubated for 30 minutes at 4° C. Plates were centrifuged at 400 x g for 2 minutes and supernatants were removed. Cells were washed twice with 100 µl/well of PBS. A secondary fluoresceinated antibody, goat- antimouse IgG (1/4 dilution) was added in a volume of 20 µl/well and mixed. A 30 minute incubation at 4° C followed before washing twice with PBS. Cells were resuspended in PBS containing 1% paraformaldehyde and stored at 4° C until flow cytometric analysis. As a background control, PEC were labeled with only the FITC-goat-antimouse IgG.

Flow Cytometric Analysis: The proportion of fluorescently labeled PEC was determined using a FACScan Flow Cytometer (Becton-Dickinson, Sunnyvale, CA) equipped with an air-cooled, 15 mW argon ion laser which emitted at 488 nm. Parameters analyzed include forward and right angle light scatter as well as fluorescence properties of the cell. CONSORT 30 Software was utilized for data acquisition and analysis of 5 x 10^3 cells per sample.

Corticosterone Assay: Plasma samples were assayed for corticosterone as described previously (33) except anti-corticosterone antibody was diluted 1:30 to achieve approximately 30% binding.

Statistical Analysis: The Number Cruncher Statistical Package (J.L. Hintze, Kaysvill, UT) was used to perform Student's T test to determine statistical significance on various tests. Chi-square analysis was used to determine statistical significance for PEC differential counts.

RESULTS:

Analysis of Peritoneal Exudate Cell Populations of Suspension Mice: Peritoneal exudate cells elicited by an intraperitoneal injection of P. acnes were examined for three cell types: macrophages, lymphocytes, and neutrophils. The inflammatory cell cascade in response to P. acnes includes a series of distinctive steps characterized by the early immigration of neutrophils followed by macrophages (12). The inflammatory response was studied 4 days after injection with P. acnes to determine if it was altered by antiorthostatic suspension. No significant differences in inflammatory cells were observed between the treatment groups (Table 1). The macrophages were present in the highest proportion: 68%, 69% and 66% for antiorthostatic, orthostatic, and nonsuspended treatment groups, respectively. Percentages of lymphocytes and neutrophils ranged from 14% to 19% for all treatment groups.

We also compared the total peritoneal cell numbers washed from individual mice in the three treatment groups and found no significant differences (Table 1).

Effects of Antiorthostatic Suspension on Body Weight, Spleen Weight, Spleen Cell Number and Plasma Corticosterone Levels: Exposure to stress is known to increase serum glucocorticoid levels (68). In turn, glucocorticoids are responsible for causing the depletion of lymphoid organs and the redistribution of cell populations within those organs (24,27-30). To assess the amount of stress incurred from antiorthostatic suspension, we compared body weights, spleen weights, total spleen cell numbers, and plasmas corticosterone levels of mice from each of the three treatment groups (Table 2). In order to determine how antiorthostatic suspension altered body weight, we calculated body weight as a percentage of initial body weight. Nonsuspended mice demonstrated the lowest reduction in body weight (99% of their initial weight), while antiorthostatically suspended mice demonstrated the highest reduction in body weight (89% of initial weight). Spleen weights are presented as a percentage of final body weight. Once again, spleen weights were most significantly reduced in antiorthostatically suspended mice compared to the mice from orthostatic and nonsuspended treatment groups (Table 2). Total spleen cell numbers of 1.6, 1.8 and 2.0 x 10^3 cells per spleen were determined for antiorthostatic, orthostatic and nonsuspended treatment groups, respectively (Table 2). Since corticosterone is used as an indicator of stress (11), we measured plasma corticosterone concentrations in the treatment mice. The highest concentration of corticosterone (273 ng/ml) was detected in the plasma of antiorthostatically suspended mice, while orthostatically and nonsuspended mice had 135 ng/ml and 94 ng/ml, respectively (Table 2). Significant differences were determined for body weights ($p < 0.01$), spleen

weights ($p < 0.01$), spleen cell numbers ($p < 0.05$), and corticosterone concentrations ($p < 0.01$) between all treatment groups.

Prostaglandin E_2 and Thromboxane B_2 Levels: Inflammatory and immune responses are mediated in part by regulatory substances like eicosanoids, the oxygenated derivatives of arachidonic acid (63,84). We wanted to determine whether antiorthostatic suspension and the associated elevated corticosterone concentrations would affect the production of the eicosanoids, PGE_2 or TB_2, which could also have inhibitory effects on the immune response. We measured the amounts of PGE_2 and TB_2 in the plasma and PEC culture supernatants. The concentrations of PGE_2 and TB_2 in the plasma of mice from the three treatment groups did not differ significantly, except for the TB_2 levels between antiorthostatic and orthostatic treatment groups ($p < 0.05$; Table 3). The individual variation of PGE_2 and TB_2 concentrations is depicted in Figure 1. Peritoneal exudate cells elicited by an intraperitoneal injection with P. acnes were cultured for 24 hours with or without LPS/IFN-gamma. Culture supernatants were collected and analyzed for PGE_2 and TB_2 production. Stimulation of macrophages with LPS/IFN-gamma did not significantly enhance the production of either eicosanoid (Table 4). Prostaglandin E_2 and TB_2 concentrations are only slightly different between treatment groups, none of which were determined significant by statistical analysis (Table 4).

Cytokine Production: Tumor necrosis factor and IL-1 both have pleotropic effects on the immune system and in the pathogenesis of disease (21,22,66). Glucocorticoids were reported to be potent inhibitors of the LPS-induced synthesis of TNF and IL-1 (8,72). In order to determine if the increased amounts of glucocorticoids of antiorthostatically suspended mice affected cytokine production, we measured TNF and IL-1 production by LPS-activated, P. acnes-induced macrophages. Culture supernatants of

LPS/IFN-gamma activated peritoneal macrophages were collected at 30 minutes and 24 hours after the LPS pulse then analyzed for TNF activity. The production of TNF almost doubled in concentration from 30 minutes to 24 hours; however, no significant differences of TNF production were observed between treatment groups (Table 5). The ability of LPS-activated peritoneal macrophages to produce IL-1 was also evaluated. Supernatants were harvested after 18 hours and analyzed for IL-1 production by the comitogenic murine thymocyte assay. Stimulation with LPS doubled IL-1 production (Figure 2). In stimulated samples, IL-1 production did not differ significantly between treatment groups.

Superoxide Production: Macrophages and other phagocytes mediate oxidative killing via superoxide. The impaired production of superoxide by inflammatory cells of antiorthostatically suspended mice was reported previously (33). In addition, some studies indicate that glucocorticoids inhibit superoxide release by mononuclear phagocytes (51,61,65). Therefore, we measured the superoxide response stimulated with PMA to determine if antiorthostatic suspension would impair superoxide production by inflammatory macrophages. Production of superoxide ranged from 2.7 nM for antiorthostatic and orthostatic treatment groups to 2.2 nM for nonsuspended mice (Table 6). No significant differences ($p > 0.1$) in superoxide production were determined between treatment groups.

Cell Surface Molecule Expression: Cell surface molecules are essential for the proper regulation and effector functions of the cell. The presentation of antigen to T-lymphocytes by macrophages requires the expression of Class II molecules on the macrophage (85). The binding and subsequent phagocytosis of certain microbes necessitates the expression of receptors for mannose, galactose, fucose and various other carbohydrate

moieties on the phagocytic cell (78). Glucocorticoids have been found to reduce the expression of Class II molecules (87). We wanted to determine whether antiorthostatic suspension would affect expression of Class II molecules, Con A binding sites (mannose and to a lesser extent, galactose specific) and BSI-B_4 binding sites (galactose specific) on peritoneal exudate cells elicited by P. acnes. Each probe detected different proportions of the total population screened during this analysis. On average, monoclonal antibody specific for I-A^k bound 51% of the total population, while Con A bound 97% and BSI-B_4 bound 59% of the total cell population (Table 7). Maddox et al. (53) have shown that while Con A will bind both resident and stimulated peritoneal macrophages, BSI-B_4 will only bind stimulated peritoneal macrophages. The percentage of cells staining positive for the various probes was not significantly different between any treatment groups (Table 7). Representative histograms of labeled PEC from antiorthostatically and orthostatically suspended mice were superimposed on each other for comparisons (Figure 3). Only slight variations between the two groups were ever detected.

Macrophage Cytotoxicity of Tumor Cells and Virus-Infected Cells: Macrophage cytotoxicity can be mediated by a direct contact mechanism or by soluble macrophage products. Previously, we have shown that the SV40-transformed cell line, F5b, is killed by macrophages via a contact-dependent process (13,70). Alternatively, virus-infected 3T3 cells, unlike normal 3T3 cells, are susceptible to TNF-mediated cytotoxicity (Table 8). Since glucocorticoids have been known to inhibit tumoricidal activity (40), we wanted to determine whether antiorthostatic suspension, which is characterized by elevated concentrations of serum corticosterone, affects either contact-dependent or TNF-mediated cytotoxicity of P. acnes elicited peritoneal

macrophages. The results indicate that contact-dependant cytotoxicity of F5b cells by macrophages from antiorthostatic, orthostatic or nonsuspended treatment groups was not significantly different (Table 9). As illustrated in Table 8, vaccinia-infected cells become susceptible to soluble TNF. Therefore, macrophage cytotoxicity of vaccinia-infected cells does not require contact. Furthermore, P. acnes-elicited macrophages do not produce TNF-α (Table 5). Previous work in our laboratory has discovered that vaccinia-infected target cells can induce the production of TNF by macrophages in a process that probably mimics in vivo macrophage-virus infected cell interactions. The data in Table 9 indicate that neither antiorthostatic suspension nor the elevated glucocorticoid concentrations associated with that process affect the P. acnes macrophage response and cytotoxicity of vaccinia-infected cells. TNF-mediated killing of virus-infected 3T3 cells was not significantly different between treatment groups (Table 8). This also supports that TNF production by the macrophages was not altered by suspension as seen in Table 5.

Lymphocyte Proliferative Response to Mitogens and Toxins: Measurement of the T lymphocyte response to mitogenic stimulation has been a widely accepted in vitro indicator of in vivo competence of T lymphocytes. Space flight studies regularly reported a reduced blastogenic responsiveness of T lymphocytes to mitogenic stimulation (16,45,46,81). Stevenson et al. (77) reported that corticosterone significantly reduced the responsiveness of murine splenic T lymphocytes to mitogenic stimulation. To date, no other antiorthostatic suspension studies tested lymphocyte responsiveness. In our experiments, we studied the effects of antiorthostatic suspension on splenic lymphocyte proliferation stimulated by the mitogens PHA and Con A and staphylococcal exotoxins Seb and Eta. Concanavalin A and PHA are both

polyclonal stimulators of T lymphocytes (4). Both mature and immature T cells are stimulated by Con A, whereas only the mature, corticosteroid-resistant, antigen-committed T cells are stimulated by PHA (23,58). The staphylococcal toxins are powerful stimulators of T lymphocytes (67). These toxins must first bind Class II molecules on accessory cells, then only a small population of T cells which express the appropriate Vß segment as part of the T cell receptor respond (15). Whereas toxins bind to Class II MHC molecules and require extremely low concentrations to activate T cells, mitogenic lectins bind to carbohydrate moieties and require 1,000-fold greater concentrations (49). Therefore, the use of these four probes provides a comprehensive evaluation of the proliferative capacity of spleen cells from suspended mice. Table 10 illustrates these experiments. The toxins, Eta and Seb, did not stimulate the T lymphocytes to the same extent as the mitogens, PHA or Con A (Table 10). This is indicative of the small population that is normally stimulated by these toxins (i.e. only T cells with specific Vß regions: 1, 3, 10, 11, 12 and 17 respond to Sea and 10, 11 and 15 to Eta) (39). Interestingly, both the toxin- and mitogen-induced lymphocyte responsiveness was increased in antiorthostatic compared to orthostatic and nonsuspended treatment groups, although the difference was more apparent with the mitogen-stimulated lymphocytes (Table 10). For instance, Con A stimulated lymphocytes had a stimulation index (SI) of 13.7 for nonsuspended and 21.6 for antiorthostatic, while Seb-stimulated lymphocytes had a SI of 2.7 for nonsuspended and 2.9 for antiorthostatic (Table 10). Statistical analysis determined that there was a significant difference in SI between the treatment groups: $P \leq 0.10$ for toxin stimulated lymphocytes; $P < 0.01$ for PHA; and $P < 0.05$ for Con A (Table 10). Scattergrams of these results are shown in

Figures 4 and 5 to demonstrate the considerable variation in proliferation of spleen cells assayed from individual mice.

DISCUSSION:

This study examined the effects of 11-day antiorthostatic suspension, orthostatic suspension or normal housing (nonsuspended) and the subsequent rise in corticosterone on various aspects of the immune response in C3HeB/FeJ mice. This study differed from an earlier one done in our laboratory (33) where we studied the inflammatory cells induced 1 day after P. acnes injection which were predominantly neutrophils. The current investigation focused on the inflammatory cells induced 4 days after P. acnes injection, when macrophages were present in the highest proportion. The proportion of macrophages, lymphocytes and neutrophils was not different between treatment groups suggesting that neither antiorthostatic suspension nor elevated corticosterone levels affected the inflammatory response by these cell populations.

Experiments were done to determine the impact of antiorthostatic suspension on the macrophage production of TNF, IL-1, superoxide and the eicosanoids PGE_2 and TB_2. None of these mediators were significantly affected by antiorthostatic suspension. To date, few studies have investigated the effect of antiorthostatic suspension on the production of cytokines. Those studies found decreased production of IFN-α/β by mice when challenged with an intravenous injection of polyriboinosinic-polyribocylidylic acid (69,73). The reduction of interferon production correlated with a loss of resistance to the diabetogenic strain of encephalomyocarditis virus (73,80). Our laboratory has previously reported an impaired superoxide production by peritoneal PMNs of antiorthostatically suspended mice (33).

Because those studies focused on the PMN while macrophages were the subject of the current study, the results may not be directly comparable. To our knowledge, no other studies have investigated the effects of antiorthostatic suspension on the production of any eicosanoid, TNF or IL-1. However, cold water stress has been found to inhibit macrophage production of both TNF and IL-1 (1,2).

We also examined the effect of antiorthostatic suspension on two cytotoxic pathways of macrophages, neither of which have been evaluated in other suspension studies. One lytic pathway involves the induction and secretion of TNF. In the course of these studies, vaccinia-infected 3T3 cells, as opposed to normal 3T3 cells, were found to be sensitive to TNF-mediated killing. This is the first report, to our knowledge, that demonstrates that infection of cells with vaccinia virus makes them susceptible to TNF-mediated cytotoxicity. However, this is consistent with studies that show that other viruses (25,50) and intracellular pathogens (47) can alter cellular sensitivity to TNF. This study, similarly to another (44), has found that P. acnes-elicited macrophages do not produce IL-1 and TNF. However, they are still capable of TNF-mediated cytotoxicity of vaccinia-infected 3T3. The virus-infected cell produces interferon which stimulates macrophage secretion of TNF. Since neither this complex process nor the contact-dependent lytic pathway (as measured by cytotoxicity of F5b) were affected by antiorthostatic suspension, it appears that the physiological changes that occur in response to antiorthostatic suspension do not alter macrophage cytotoxicity as we have measured them.

Consistent with our studies on macrophage secretion and cytotoxicity, the expression of Class II histocompatibility molecules and Con A and BSI-B_4 binding sites was not significantly altered by suspension treatments. No

direct comparisons to our results can be found in the literature. However, in various stress studies, Ia expression may or may not be affected by elevated corticosterone (43,90). Zwilling et al. (90) suggest that factors, other than corticosterone, associated with stress contribute to Ia antigen expression. Interestingly, Steffen and Mussachia (76) reported an increased expression of glucocorticoid receptors on muscle tissue in antiorthostatically suspended rats. Macrophages from cold water stressed animals also have been reported to have increased amounts of prothrombinase complex enzymes on the cell surface compared to controls (1). However, the different experimental system makes comparisons difficult.

Exposure to stress is known to increase serum corticosterone concnetrations (68). We found that mice in both antiorthostatic and orthostatic treatment groups had significantly elevated plasma corticosterone levels indicating the stressful environment the rodents were subjected to during the 11 day study period (31,33,68,76). However, based on corticosterone concentration and the extent to which body weight, spleen weight and spleen numbers decreased, we conclude that antiorthostatic suspension poses the most physiologically stressful situation to the mice. Because antiorthostatic suspension induced elevated levels of corticosterone, we were concerned that it might affect our studies. Glucocorticoids generally exert suppressive effects on the immune system (64) especially production and/or secretion of a variety of monokines. Corticosteroids inhibit the production of IL-1 by monocytes (88). Inhibition may occur either at the level of transcription of IL-1 mRNA or through cAMP inhibition of post-transcriptional IL-1 synthesis (48). Tumor necrosis factor secretion is also decreased by glucocorticoids (8,9). Glucocorticoids also inhibit the release of arachidonic acid as well as superoxide (14,34,38,41,52,79). Hogan

and Vogel (40) reported that glucocorticoids inhibited the tumoricidal activity of IFN-gamma/LPS activated macrophages in a dose-dependent manner by interfering with either the "priming" or "triggering" signals responsible for macrophage activation. The expression of Class II histocompatibility antigens on macrophages has also been reported to be reduced after glucocorticoid treatment (35,42,87,89).

Despite numerous studies demonstrating the suppressive effects of glucocorticoids, our results did not show any correlation between elevated corticosterone concentration and reduced secretion, cytotoxicity or membrane receptor expression. It is possible that we did not see depression in secretory responses because the P. acnes-activated macrophages were less susceptible to corticosteroid-mediated inhibition. Others have found that glucocorticoid-induced inhibition of TNF production by porcine alveolar macrophages and human monocytes could be reversed if the cells were incubated with IFN-gamma (26,79). Arachidonic acid release by A23187- or zymosan-treated alveolar macrophages also was not inhibited by glucocorticoids if the cells were simultaneously incubated with other stimulants (75). It is possible that the conditions under which corticosteroids are exposed to cells can influence whether they are inhibitory, stimulatory or ineffective. In many studies where glucocorticoids were inhibitory, the effects were seen in vitro using concentrations (75) or incubation times (79) that are not physiological. Another explanation for the lack of macrophage suppression by elevated corticosterone in our experiments could be because during the 11 day suspension period the cells became desensitized or habituated (54). Chiara and Sobrino (14) found that murine macrophage respiratory bursts were inhibited by dexamethasone if the drug was given only hours before macrophage

recovery, but not if the cells were tested one or two days after the dexamethasone was administered.

In addition to examining the effects of antiorthostatic suspension on macrophages, we also studied the effects on lymphocytes, namely the proliferation of splenic lymphocytes in response to the mitogens, PHA and Con A; and the staphylococcal exotoxins, Seb and Eta. We found that anti-orthostatic suspension caused splenic lymphocytes to exhibit an increased proliferative response when stimulated with either mitogens or toxins. However, the mitogens induced a more dramatic proliferative response when compared to the toxins. No other studies have investigated the effects of antiorthostatic suspension on toxin- or mitogen-induced lymphocyte proliferation, so we cannot make any direct comparisons. However, space flight studies with human and animal subjects and ground-based clinostat studies examined peripheral blood lymphocyte proliferative responses. Almost all these investigations including post-flight evaluations (16,45,46,81), in vitro space studies (16-18), and clinostat experiments, reported reduced mitogen-induced proliferative responses (19). Only one study, in which rats were flown on board Cosmos 7820 for 20 days, reported a post-flight increase in mitogen-induced blastogenic responsiveness (56). Interestingly that study was done with splenic lymphocytes. Others have suggested that glucocorticoids and stress affect splenic T cells differently than blood T cells (20,54). Therefore, it is possible that splenic lymphocytes do not mimic the responses of peripheral blood lymphocytes. This concept is also indirectly supported by the findings that rat spleen lymphocyte membrane molecule changes do not completely parallel changes in human blood lymphocytes in response to space flight (74,81). Alternatively, antiorthostatic suspension may not accurately duplicate the effects of space flight on lymphocytes. Therefore, more work

with splenic lymphocytes in space must be done for comparative reasons. Until then, caution should be used in attempting to extrapolate splenic lymphocyte data to the effects of flight and to studies with blood lymphocytes.

The increased mitogen- and toxin-induced proliferation of splenic lymphocytes taken from antiorthostatically suspended mice with significantly elevated corticosterone is also not consistent with studies in which decreased proliferative responses were observed after exposure to glucocorticoids. Vischer (86) found that after administering hydrocortisone to mice, splenic lymphocytes exhibited a decreased proliferative responses to PHA, pokeweed mitogen and allogeneic cells. Stevenson et al. (77) also reported that the T lymphocytes demonstrated significantly reduced proliferative response to mitogens when corticosterone was administered in vivo. Blomgren and Andersson (9) reported that exposure to prednisone in vitro also had the same effect. Interestingly, the increased glucocorticoid receptor expression on mitogen-stimulated lymphocytes did not increase glucocorticoid-induced inhibition of lymphocyte proliferative responsiveness (71).

Although our lymphocyte response data differ from the results of several groups, our findings are supported by a number of investigations. Gillis et al. (36) found that glucocorticoid-induced inhibition could be ameliorated by IL-2. Almawi et al. (3) found that IL-1, IL-6 and interferon-gamma, together could counteract the suppressive effects of dexamethasone. Therefore, it may be possible that the in vivo exposure to cytokines counteracted the suppressive effects of corticosterone in our experiments. Nakano et al. (62) reported that glucocorticoid secretion may be mediated by histamine and under certain conditions enhance mitogenesis. They found that one type of histamine agonist prevented Con A-induced lymphocyte proliferation, while one other histamine agonist and one antagonist enhanced the proliferative response (62).

Another study by Goodwin and Atluru (37) reported enhanced immunoglobulin production of peripheral blood lymphocytes when stimulated with glucocorticoids. In addition, investigators using various stress models, specifically electric shock and cold water immersion, have shown enhanced proliferative responses of lymphocytes after mice were subjected to the stressor (2,53). Enhanced mitogenesis was dependent on the intensity and frequency of the stress (2,53). Interestingly, Cunnick et al. (20) using a shock model, found that glucocorticoids may be involved in suppressing peripheral blood T cell responses but that adrenal gland hormones, like corticosterone, are not involved in suppressing spleen cell mitogenesis. These studies suggest that multiple variables contribute to an altered immune response such as the type of stressor, the duration of exposure to the stressor, the frequency of treatment with the stressor, as well as the strain of the mouse subject and the lymphocyte subpopulation.

In conclusion, two major findings have resulted from these investigations: 1. Although antiorthostatic suspension closely mimics some physiological changes associated with space flight, we have shown that it may fall short of duplicating some immunological responses affected by spaceflight. The most significant of these immune responses is the reduced post-flight mitogen-induced lymphocyte proliferative response.
2. Glucocorticoids, when elevated to three times normal levels by antiorthostatic suspension, are not inhibitory to macrophage inflammation, secretion, cytotoxicity or membrane molecule expression. Therefore, elevation of these adrenal hormones does not always result in immunosuppression.

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FIGURE LEGENDS:

Figure 1. Concentrations of TB_2 and PGE_2 in plasma of antiorthostatically suspended (A), orthostatically suspended (O), and nonsuspended (N) mice. Each dot represents an individual mouse and the dash represents the mean.

Figure 2. Interleukin-1 (IL-1) production of P. acnes-elicited peritoneal macrophages of antiorthostatically suspended (experiment), orthostatically suspended (control), and nonsuspended (normal) C3HeB/FeJ mice. Macrophages were incubated for 18 hours in DME with or without LPS (12.5 μg/ml).

Figure 3. Comparison of expression of Class II molecules and lectin receptors on peritoneal exudate cells between antiorthostatically and orthostatically suspended mice. Histograms of antiorthostatically suspended mice (denoted by solid line) were superimposed on histograms of orthostatically suspended mice (denoted by dotted line). Histograms A, B, and C represent cells labeled with TIB-94 (anti-H-2^k), Con A and BSI-B_4, respectively, as described in Materials and Methods. Note: x-axis is on a logarithimic scale.

Figure 4. Spleen cell proliferation in response to mitogens. Proliferative responses were induced in spleen cells of antiorthostatically suspended (A), orthostatically suspended (O) or nonsuspended (N) mice with 2 μg/ml Con A or 9 μg/ml PHA. Stimulation index was determined by measuring incorporation of ^{3}H-thymidine in a 48 hour assay. Each dot represents an individual mouse and the dash represents the mean.

Figure 5. Spleen cell proliferation in response to Staphylococcal exotoxins Eta and Seb. Proliferative responses were induced in spleen cells of antiorthostatically suspended (A), orthostatically suspended (O), or nonsuspended (N) mice with 1 μg/ml Eta or 1 μg/ml Seb. Stimulation Index (SI) was determined by measuring incorporation of ^{3}H-thymidine in a 48 hour assay. Each dot represents an individual mouse and the dash represents the mean.

Table 1. Peritoneal Exudate Cell Differential Counts of Antiorthostatically Suspended Mice

Suspension[2]	Total Cell Number (10^7)	% Cell Population[1]		
		Macrophages	Lymphocytes	PMNs
Antiorthostatic	1.1 ± 0.1[3,4]	68 ± 1[5]	17 ± 1	15 ± 1
Orthostatic	1.0 ± 0.1	69 ± 1	17 ± 1	14 ± 1
None	0.9 ± 0.1	66 ± 1	19 ± 1	15 ± 1

1. Peritoneal exudate cells were elicited by i.p. injection of C3HeB/FeJ mice with 700 μg P. acnes 4 days prior to cell recovery.
2. Mice were suspended or caged normally for 11 days prior to sacrifice.
3. Numbers represent mean ± S.E.M. N = 29-36 mice per treatment group.
4. No significant difference in total cell number between antiorthostatic vs orthostatic and orthostatic vs none (normal control) as determined by two-tailed matched T-test analysis.
5. Chi-square analysis of differentials indicates no significant differences between treatment groups.

Table 2. Effect of Antiorthostatic Suspension on Body Weight, Spleen Weight, Spleen Cell Numbers and Plasma Corticosterone Levels

Suspension[1]	Body Weight as % Initial Body Weight[2]	Spleen Weight as % Final Body Weight[3]	Total Spleen Cell Count (10^3)	Corticosterone[4] ng/ml
Antiorthostatic	89 ± 0.6[5,6]	.67 ± 0.02	1.6 ± 0.1	273 ± 26
Orthostatic	93 ± 0.8	.85 ± 0.02	1.8 ± 0.1	135 ± 16
None	99 ± 0.6	.91 ± 0.03	2.0 ± 0.1	94 ± 18

1. Mice were suspended or caged normally for 11 days prior to sacrifice.
2. Percentage is caculated by: $\frac{\text{wt. mouse post-suspension}}{\text{wt. mouse pre-suspension}} \times 100$
3. Percentage is calculated by: $\frac{\text{wt. spleen post-suspension}}{\text{wt. mouse post-suspension}} \times 100$
4. Corticosterone levels in plasma were determined by RIA.
5. Numbers represent mean ± S.E.M. N = 43-51 mice per treatment group.
6. Significant differences between antiorthostatic vs orthostatic vs none (normal control) as determined by two-tailed matched T-test analysis. $P < 0.01$ for Body Weight, Spleen Weight and Corticosterone. $P < 0.05$ for spleen cell count.

Table 3. Prostaglandin E_2 and Thromboxane B_2 Concentration in Suspension Mouse Plasma

Suspension[1,2]	nmol/ml PGE_2	nmol/ml TB_2
Antiorthostatic	8.6 ± 0.6[3,4]	8.7 ± 1.3
Orthostatic	7.6 ± 0.9	5.9 ± 1.0
None	6.4 ± 0.8	7.7 ± 1.0

1. Plasma was separated from whole blood collected from mice after suspension. Plasma was acid stabilized and ethanol precipitated before separation by HPLC and quantitation (see Materials and Methods).
2. Mice were suspended or caged normally for 11 days prior to sacrifice.
3. Numbers represent mean ± S.E.M. N = 10 mice per treatment group.
4. The concentration of TB_2 of anticrthostatic mice is significantly different from the concentration of TB_2 of orthostatic mice ($P < .05$). No other significant differences were determined by two-tailed matched T-test analysis.

Table 4. Prostaglandin E_2 and Thromboxane B_2 Production by Peritoneal Macrophages of Antiorthostatically Suspended Mice.

	nmol/ml PGE_2[3]		nmol/ml TB_2	
Suspension[1,2]	-24 hr[4]	+24 hr	-24 hr	+24 hr
Antiorthostatic	4.5 ± 0.9[5,6]	3.5 ± 0.8	5.6 ± 0.9	5.6 ± 1.0
Orthostatic	4.5 ± 0.9	4.0 ± 1.1	8.1 ± 1.0	5.9 ± 0.9
None	5.8 ± 0.7	4.0 ± 0.9	7.5 ± 1.9	7.3 ± 1.1

1. Peritoneal macrophages were elicited by i.p. injection of C3HeB/FeJ mice with 700 μg *P. acnes* 4 days prior to sacrifice.
2. Mice were suspended or caged normally for 11 days prior to sacrifice.
3. Separation and quantitation of PGE_2 and TB_2 by reverse-phase HPLC as discussed in Materials and Methods.
4. Peritoneal macrophages were cultured in medium or medium containing LPS and IFNγ (10 μg/ml, 10 u/ml, respectively) for 24 hours.
5. Numbers represent mean ± S.E.M. N = 8 mice per treatment group.
6. No significant differences between any treatment group as determined by two-tail matched T-test analysis.

Table 5. Tumor Necrosis Factor-Alpha Production by Peritoneal Macrophages of Antiorthostatically Suspended Mice

Suspension[1,2]	TNF (units/ml)[3] (± LPS/IFNγ) Stimulation Period[4] (-) 30 min	(+) 30 min	(-) 24 hour	(+) 24 hour
Antiorthostatic	0.0	2.5 ± 0.4[5,6]	0.0	4.7 ± 0.6
Orthostatic	0.0	2.7 ± 0.3	0.0	5.4 ± 0.4
None	0.0	2.8 ± 0.2	0.0	5.5 ± 0.4

1. Peritoneal macrophages from C3HeB/FeJ mice were elicited by i.p. injection of 700 μg P. acnes 4 days prior to sacrifice.
2. Mice were suspended or caged normally for 11 days prior to sacrifice.
3. TNF determined by multiple regression analysis of unknowns compared to recombinant TNF standard cytotoxicity of ^{51}Cr-labelled LM-929 target cells.
4. Peritoneal macrophages were cultured in medium or LPS and IFNγ (10 μg/ml, 10 u/ml, respectively) for 30 minutes or 24 hours.
5. Numbers represent mean ± S.E.M. N = 12 per treatment group.
6. No significant differences between any treatment group as determined by two-tailed matched T-test analysis.

Table 6. Superoxide Production by Peritoneal Macrophages of Antiorthostatically Suspended Mice

Suspension[3]	Superoxide Production[2] nM O_2^-
Antiorthostatic	2.7 ± 0.2[4,5]
Orthostatic	2.7 ± 0.1
None	2.2 ± 0.2

1. Peritoneal macrophages were elicited by i.p. injection of C3HeB/FeJ mice with 700 μg P. acnes 4 days prior to sacrifice.
2. Superoxide radical generation induced in response to PMA was determined by SOD inhibitable reduction of ferricytochrome C.
3. Mice were suspended or caged normally for 11 days prior to sacrifice.
4. Numbers represent mean ± S.E.M. of triplicate samples. N = 6 mice per treatment group.
5. No significant differences between any treatment group as determined by two-tailed matched T-test analysis.

Table 7. Expression of Class II Molecules and Lectin Receptors on Peritoneal Exudate Cells of Antiorthostatically Suspended Mice

	Mean % positive cells[2]		
Suspension[1,3]	anti-I-A^k	ConA	BSI-B_4
Antiorthostatic	52 ± 3[4]	96 ± 1	62 ± 3
Orthostatic	49 ± 3	97 ± 1	63 ± 4
None	53 ± 4	97 ± 1	53 ± 4

1. Peritoneal exudate cells were elicited by i.p. injection of C3H3B/FeJ with 700 μg P. acnes 4 days prior to sacrifice.
2. Percent fluorescence is expressed as the mean ± S.E.M. N = 9 mice per treatment group.
3. Mice were suspended or caged normally for 11 days prior to sacrifice.
4. No significant difference between any treatment as determined by two-tailed matched T-test analysis.

Table 8. Sensitivity of Vaccinia Virus-Infected Balb/c 3T3 Cells to TNF-Mediated Cytotoxicity

Target[1]	Treatment	Specific ^{51}Cr Release U/ml rTNF			
		16	8	4	2
LM 929	none	78 ± 3[2]	87 ± 3	80 ± 2	82 ± 5
Balb/c 3T3	none	9 ± 1	5 ± 0	7 ± 1	7 ± 0
Balb/c 3T3	vaccinia[3]	41 ± 1	39 ± 4	45 ± 1	42 ± 0

1. Targets were plated at 1 x 10^4 cells/well in a 96-well, flat-bottomed microtiter plate.
2. Numbers represent mean ± S.E.M.
3. Cells were infected with vaccinia virus at an MOI of 2-3.

Table 9. Suspension Mouse Macrophage Cytotoxicity of Virus-Infected 3T3 Cells and F5b Tumor Cells

	% Specific Release[2]		
Suspension[1]	3T3	3T3v[3]	F5b
Antiorthostatic	8 ± 2[4,5]	32 ± 1	37 ± 2
Orthostatic	5 ± 1	30 ± 1	38 ± 3
None	5 ± 1	29 ± 1	32 ± 3

1. Mice were suspended or caged normally for 11 days prior to sacrifice.
2. Cytotoxicity was determined in a 16 hour assay using a macrophage:target ratio of 30:1.
3. 3T3 cells were infected at an MOI of 2.
4. Numbers represent mean ± S.E.M. N = 12 mice per treatment group where 3T3 or $3T3_v$ were target cells and N = 9-11 mice per treatment group where F5b are target cells.
5. No significant differences between any treatment group with any target as determined by two-tailed matched T-test analysis.

Table 10. Suspension Mouse Spleen Cell Proliferation in Response to Mitogens and Staphylococcal Exotoxins

	Stimulation Index[1]							
Suspension[2]	Eta	Stats[3]	Seb	Stats	PHA	Stats	Con A	Stats
Antiorthostatic	3.3±0.3[4]	(P=.10)	2.9±0.3	(P<.10)	9.3±1.6	(P<.01)	21.6±2.4	(P<.05)
Orthostatic	2.5±0.2		2.5±0.3		3.2±0.7		12.5±2.0	
None	2.9±0.3		2.7±0.3		4.5±0.7		13.7±1.8	

1. Spleen cell proliferation in response to Eta (1 μg/ml), Seb (1 μg/ml), PHA (9 μg/ml), and Con A (2 μg/ml), was determined by measuring incorporation of ^{3}H-thymidine in a 48 hour assay.
2. Mice were suspended or caged normally for 11 days prior to sacrifice.
3. Significant differences between antiorthostatic and orthostatic as determined by two-tailed matched T-test analysis.
4. Numbers represent mean ± S.E.M. Spleen cells used from: N = 9 mice (per treatment group) stimulated with Eta, N = 12 mice (per treatment group) stimulated with Seb, N = 16 mice (per treatment group) stimulated with PHA and Con A.

Figure 1

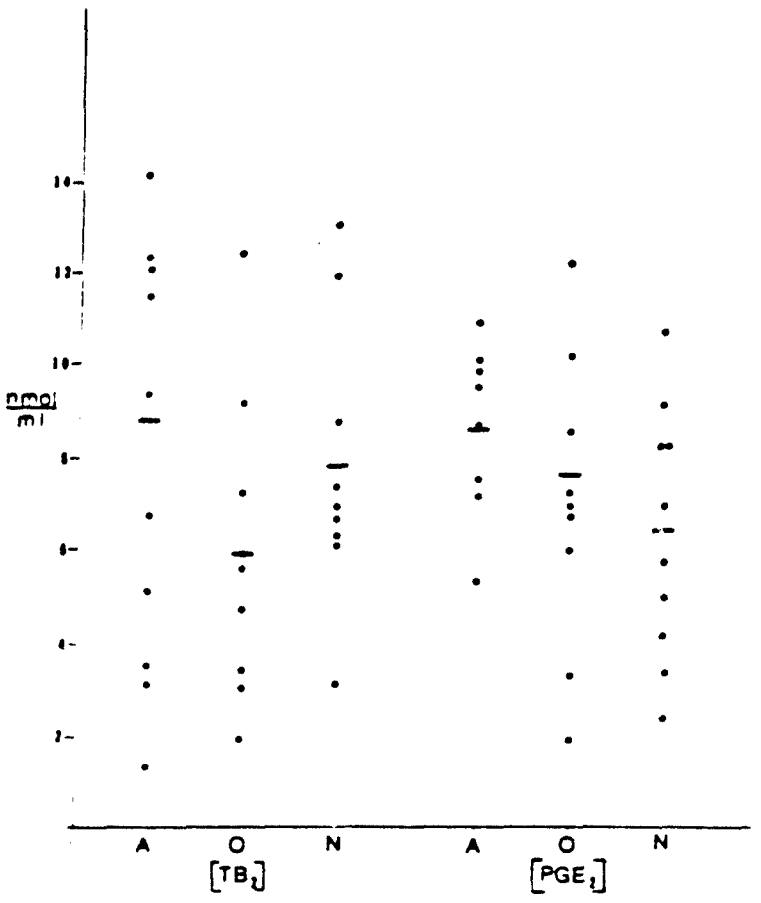
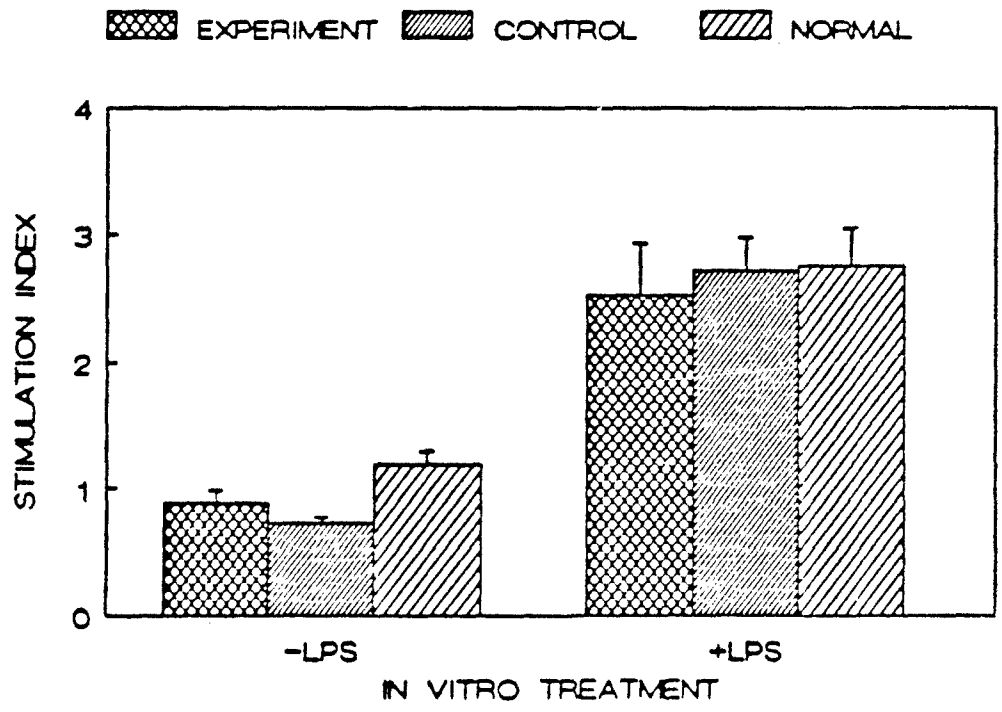


Figure 2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CONTROL
NORMAL
STIMULATION INDEX
4
3
2
1
0
-LPS
+LPS
IN VITRO TREATMENT

Figure 3

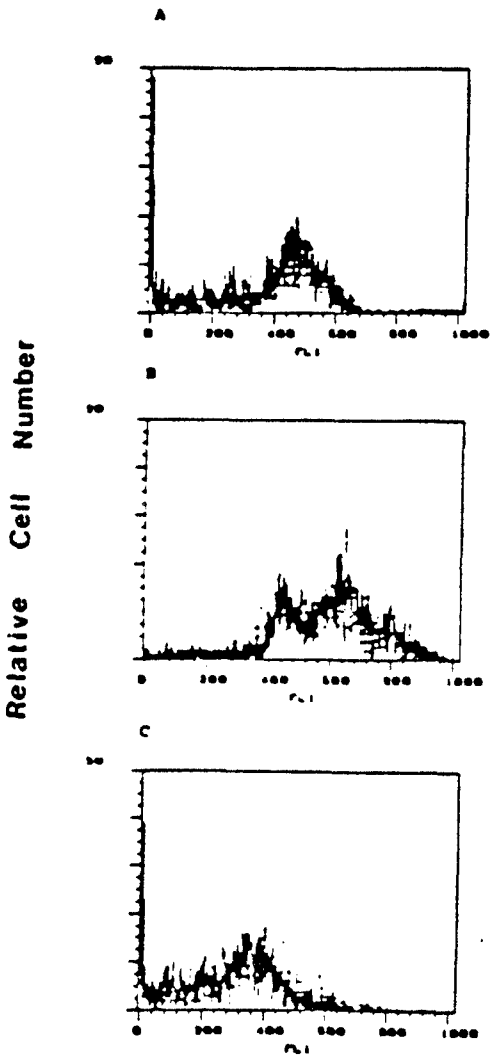

Figure 4

Figure 5