Naval Medical Research and Development Command

Independent Research Annual Report FY94

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WORK UNIT NUMBERING

Work units are coded using the numbering series listed below to indicate the laboratory or detachment at which the research was performed.

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	Naval Dental Research Institute Detachment, San Antonio San Antonio, TX
1000	Naval Medical Research Institute Bethesda, MD
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2000	U.S. Naval Medical Research Unit No. 2 Jakarta, Indonesia
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INTRODUCTION

The Naval Medical Research and Development Command (NMRDC) is the primary Navy R&D organization responsible for managing research and development programs concerning the health, safety, readiness and effective operational performance of Navy and Marine Corps personnel. In FY94, the NMRDC organization consisted of eight echelon-4 laboratories and five echelon-5 detachments, staffed by military and civilian scientists and located in the continental U.S. and overseas. The main research topics studied by NMRDC scientists include combat casualty care and combat dentistry, infectious diseases and AIDS, diving and submarine medicine, aviation medicine, human performance and environmental/occupational medicine.

NMRDC views the Independent Research (IR) program as unique among research programs, with its central focus on fostering the in-house laboratory investigators' scientific creativity, enthusiasm, and pride in conducting top-notch biomedical reserach in support of the Fleet. Through the IR program, NMRDC strives to provide these investigators with a mechanism for pursuing their novel research ideas and approaches, for broadening their expertise in state-of-the-are and emerging technologies and for initiating new efforts in research areas that are compatible with projected Navy biomedical needs. NMRDC believes the opportunities provided by the IR program are critical for stimulating and maintaining the creative and innovative thinking of Navy scientists and will result in a heightened return on the investment of the Navy's precious basic research dollars.

"BEST" INDEPENDENT RESEARCH PAPER OF FY94

LIPOPOLYSACCHARIDE-INDUCED DIFFERENTIAL CELL SURFACE EXPRESSION OF INTERCELLULAR ADHESION MOLECULE-1 IN CULTURED HUMAN UMBILICAL CORD VEIN ENDOTHELIAL CELLS

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ABSTRACT

The effects of endotoxin on cell surface ICAM-1 expression in human umbilical cord vein endothelial cells (HUVEC) was examined using solid phase radioimmunoassay, immunocytochemistry and electron microscopy. At various incubation times (e.g. 3, 6, 12, 24 hours), the ICAM-1 expression was enhanced by lipopolysaccharide (LPS, or endotoxin) from one ng/ml to 100 ug/ml with maximal enhancement at 0.1-1 ug/ml. The kinetics at 1 ug/ml LPS showed that the maximum ICAM-1 expression occurred at 24 hours. The LPS-induced ICAM-1 expression was not inhibited by the neutralizing rabbit polyclonal antibodies against human IL-1α, IL-1β and TNF-α, either alone or in combination. This indicated that the mechanism of this induced expression was not an autocrine effect mediated by the LPS-induced IL-1 or TNF-α. The LPS-induced cell surface ICAM-1 exhibited a polarized distribution shown in immunoelectron micrographs with higher density on the luminal surface. DNA synthesis activity of HUVEC was, contrary to the ICAM-1 expression, suppressed by LPS. Immunocyto-chemical studies indicated that ICAM-1 was not uniformly expressed in the culture, i.e. some cells expressed more surface ICAM-1 than others, and some of the ICAM-1-expressing cells had an uneven patchy distribution of this antigen. Combined immunocyto-chemical and ^{3}H-thymidine incorporation studies showed that cells with strong ICAM-1 expression had little DNA synthesis activity, while those with little ICAM-1 expression synthesized DNA actively. ICAM-1 on endothelial cells serves as an anchor for the leukocytes in cell-cell adhesion. Its differential expression among the cells in the endothelial population may explain the localized and scattered sites of leukocytes sticking to endothelium observed at wound sites and during extravasation.

Key Words: Endotoxin, ICAM-1, HUVEC, gene expression, radioimmunoassay, immunocytochemistry, electron microscopy

INTRODUCTION

Intercellular adhesion molecule (ICAM-1) was first identified as a protein species distinct from lymphocyte function-associated antigen (LFA-1) in the homotypic adhesion of phorbol ester-stimulated lymphocytes (1). Since its discovery, ICAM-1 has been found in lymphocytes, monocytes, granulocytes, endothelial cells and epithelial cells, etc. (1-3) The ICAM-1 expression in these cells can be induced by interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), interferon gamma (IFN gamma), lymphotoxin (LT), phorbol myristate acetate and lipopolysaccharide (LPS, or endotoxin) (1-7). Increased expression of ICAM-1 on the surface of these cells leads to the increase of leukocyte-endothelial adhesion (7-11). The sticking of leukocytes to endothelium via ICAM-1 binding to CD11a/CD18 and CD11b/CD18 on leukocytes has been implicated as the third step of leukocyte extravasation process following the steps of rolling and chemotactic stimulation (12, 13). It has been found that LPS-induced ICAM-1 expression in endothelial cells is mediated by serum factor, soluble CD14 (sCD14) (14-16). In order to further understand the detailed mechanisms of this LPS-induced ICAM-1 expression and the role of ICAM-1 in the enhancement of endothelial-leukocyte interactions, we used the methods of radioimmunoassay, immunocytochemistry and immuno-electron microscopy to examine the LPS-induced ICAM-1 expression in cultured human umbilical cord vein endothelial cells (HUVEC). Dose dependency, kinetics, autocrine effects of LPS-induced IL-1 and/or TNF-α, DNA synthesis activity of the cells during ICAM-1 induction, ICAM-1 expression in individual cells, and the detailed distribution of ICAM-1 on the surface of HUVEC were studied.

MATERIALS AND METHODS

Culture of HUVEC cells

HUVEC cells (CRL 1730, American Type Culture Collection) were cultured to confluence in 90% F12K medium, 10% fetal bovine serum, 100 ug/ml heparin and 30 ug/ml endothelial cell growth supplement in 48-well tissue culture plates, 8-chamber slides or 13 mm diameter glass and plastic cover slips in a humidified 5% CO_2 incubator at 37°C. Endotoxin (E. coli 0111:B4, prepared as 1 mg/ml stock in sterile phosphate buffered saline with calcium and magnesium (PBS) was diluted into various concentrations with the endothelial culture medium.

Radioimmunoassay of surface ICAM-1

Two hundred and fifty ul of tissue culture medium containing various concentration of LPS were added to triplicate wells of 48-well tissue culture plate containing confluent HUVEC. After the tissue culture plate was returned to the incubator for the desired incubation time, 100 ul mouse anti-human ICAM-1 antibody (Gen Trak, Inc.; 1:10 dilution with PBS) was added to the designated wells and the plate was incubated on ice for one hour. Background control wells were filled with either 100 ul PBS or 50 ug/ml mouse IgG in PBS (less than 5% difference between both cases shown in the experiments). After the plate was blocked with 150 ul 3% sheep serum in PBS on ice for 30 minutes, 100 ul of ^{125}I-sheep anti-mouse IgG was added to each well. After incubation for one hour on ice, the plate was washed three times with PBS and the cells in each well were dissolved in 0.5 ml 0.1 N NaOH at 37°C for 30 minutes. Aliquots were withdrawn to assay the radioactivity of the samples by a gamma counter and the protein concentration of each sample by BCA assay (Pierce) using bovine serum albumin as a standard. The specific radioactivity per unit protein of each sample was calculated from total cpm and the total protein content of the sample. After subtraction of the background, the resulting specific radioactivity was normalized to the basal expression.

Effects of the neutralizing rabbit polyclonal antibodies against human IL-1α, IL-1β and TNF-α on the LPS-induced ICAM-1 expression

One ml of neutralizing rabbit anti-human IL-1α (IgG fraction; Genzyme), IL-1β (IgG fraction; Genzyme) and TNF-α (IgG fraction, purified from antiserum; Genzyme) were lyophilized and re-dissolved with 1 ml tissue culture medium. After dialysis against tissue culture medium for 24 hours with three changes of the medium, the antibody solutions were sterilized by passing through 0.2 um filters and used for experiments. ICAM-1 expression in each well was induced by the addition of 100 ul of culture medium containing either 0.1 ug/ml LPS, 40 units/ml human TNF-α, 10 units/ml human IL-1α or 10 units/ml human IL-1β. Antibody (10 ul) against each cytokine or the combination of the anti-cytokine antibodies (10 ul of each) was incorporated in the culture medium with stimulus in order to determine the effects of these anti-cytokine antibodies on the induced ICAM-1 expression. Background controls and the basal expression of ICAM-1 were always carried out in the same plate. After 4 or 24 hours incubation at 37°C, the plate was assayed for surface ICAM-1 expression as previously described. The data in each data set were normalized to the corresponding sample treated with such stimulus, but without antibodies.

^{3}H-Thymidine incorporation measured by scintillation counting

To the HUVEC cells treated with various doses of LPS for 23 hours, aliquot of ^{3}H-thymidine was added and the culture was further incubated for one hour. After washing three times with PBS, the cells were treated with 0.5 ml ice-cold 10% trichloroacetic acid (TCA) and incubated for 30 minutes on ice. After washing three times with 0.5 ml 5% ice-cold TCA, the cells were dissolved with 0.5 ml 0.1 N NaOH at 37°C for 30 minutes and neutralized with 0.5 ml 0.1 N HCl. Sample aliquots (0.5 ml) were taken for determining the radioactivity with 10 ml scintillation counting cocktail (Formula-989, Du Pont) and for protein assay by BCA method. The specific radioactivity per unit protein was calculated from the total cpm and the total protein of the samples. The ^{3}H-thymidine incorporation was normalized using the specific radioactivity of the non-treated (control) sample as the basis.

Immunocytochemical study

^{3}H-thymidine incorporation and peroxidase staining

HUVEC cells cultured in 8-chamber slides and treated with varying doses of LPS in culture medium for the desired times followed by treatment with 2 uCi/ml ^{3}H-thymidine for one hour were incubated with anti-ICAM-1 (Gen Trak) on ice for one hour. After blocking with 3% goat serum on ice for 30 minutes and washing three times, goat anti-mouse IgG conjugated with horseradish peroxidase (HRP) was added. After one hour incubation on ice and washing three times with PBS, the sample was fixed on ice with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 for 30 minutes. The fixed samples were washed twice with cacodylate buffer, once with 0.1 M Tris buffer, pH 7.4, and 0.3 ml substrate solution containing diaminobenzidine and H_2O_2 was added followed by a 30 minutes incubation at room temperature. After washing three times with PBS, the chamber divider on the slides was removed. The slides were washed with distilled water, air-dried, coated with a layer of silver bromide emulsion and air dried in the dark. After an appropriate exposure time, the slides were developed and color pictures were taken to depict the ^{3}H-thymidine incorporation (silver grains around the nucleus of the cells) and the surface expression of ICAM-1 (brown staining on the cell surface).

Immunofluorescence

HUVEC cultured on 13 mm diameter glass cover slips were incubated with mouse anti-ICAM-1 (Gen Trak) for one hour on ice, fixed with 2% paraformaldehyde on ice, and blocked with 3% goat serum for 30 minutes. After washing three times with PBS, the samples were incubated with FITC-goat anti mouse IgG in the dark for one hour. After washing twice with PBS followed

by washing twice with deionized water, the specimens were mounted to glass slides. The fluorescent staining of the cells was observed and photographed by a Zeiss fluorescent microscope attached with a camera.

Immunoelectron microscopy

HUVEC cultured on 13 mm plastic cover slips were incubated with mouse anti-ICAM-1 for one hour, blocked with 3% goat serum for 30 minutes, and incubated with HRP-goat anti-mouse IgG for one hour on ice. The samples were fixed with 2% glutaraldehyde for 30 minutes on ice and blocked with 3% goat serum for an hour. After three washes with PBS, the samples were stained by a solution containing diaminobenzidine and H_2O_2 for 30 minutes. The samples were further processed with osmium treatment, dehydration, embedding with plastics, and microtome sectioning, etc. and then examined under an electron microscope.

RESULTS

LPS dose dependency on ICAM-1 expression

HUVEC, after subtracting the background count, clearly expressed ICAM-1 on the cell surface without LPS stimulation. Such basal expression was also observed in the immunocytochemical staining with FITC and HRP products shown in the later sections. The observation of low level expression of ICAM-1 in the untreated HUVEC is consistent with previous reports (4, 5). The ICAM-1 expression was increased by exposing the cells to 0.001-100 ug/ml LPS. Figure 1 shows the LPS dose-dependent ICAM-1 expression of the experiments carried out with 3, 6, 12 and 24 hours exposure to LPS. The ICAM-1 expression drastically increased as the LPS concentration was increased from 0.001 to 0.1-1 ug/ml, then decreased as the LPS concentration was increased from 1 to 100 ug/ml. Subsequent experiments employed an LPS dose of 0.1 or 1 ug/ml to stimulate ICAM-1 induction in HUVEC.

Kinetics of LPS-induced ICAM-1 expression

The kinetics of the ICAM-1 expression induced by 1 ug/ml LPS is shown in Figure 2, and can be described in three phases, i.e. a sharp rising phase (0-12 hours), a plateau phase of high expression (12-24 hours) and a declining phase (24-96 hours).

Roles of cytokines IL-1α, IL-1β and TNF-α in LPS-induced ICAM-1 expression

It is known that endotoxin can stimulate the endothelial cells to produce interleukin 1 (IL-1) (17, 18), which can also induce endothelial cells

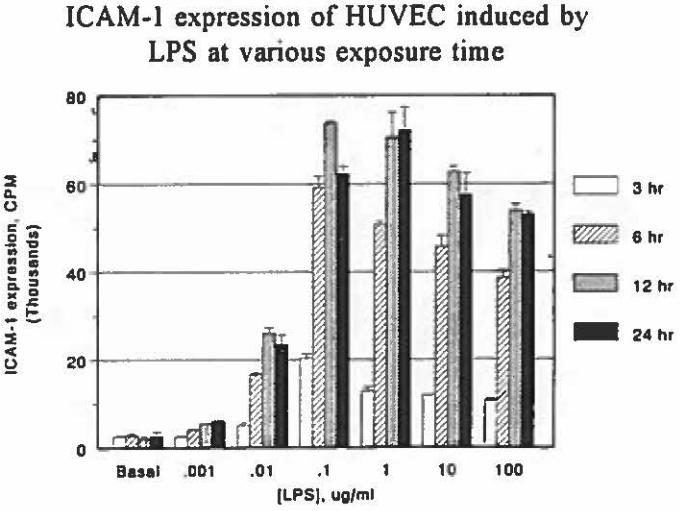

Fig. 1. **Dose dependency of LPS-induced ICAM-1 expression on the surface of HUVEC cells at 3,6,12 and 24 h incubations.** The maximal response was at the dose range of .1-1 μg/mL.

to express ICAM-1 (3, 5, 6). In order to segregate the effects of the LPS-induced IL-1 and/or TNF-α from the effect of LPS on ICAM-1 expression in HUVEC, neutralizing rabbit polyclonal antibodies specific to human IL-1α, IL-1β and TNF-α, respectively, were added to the culture with LPS to inhibit the ICAM-1 induction by these cytokines. The results in Figure 3 indicate that while each neutralizing antibody readily suppressed the cytokine-induced ICAM-1 expression by its respective antigen (IL-1α, IL-1β or TNF-α), LPS-induced ICAM-1 expression was not inhibited by these antibodies either alone (data not shown) or in combinations.

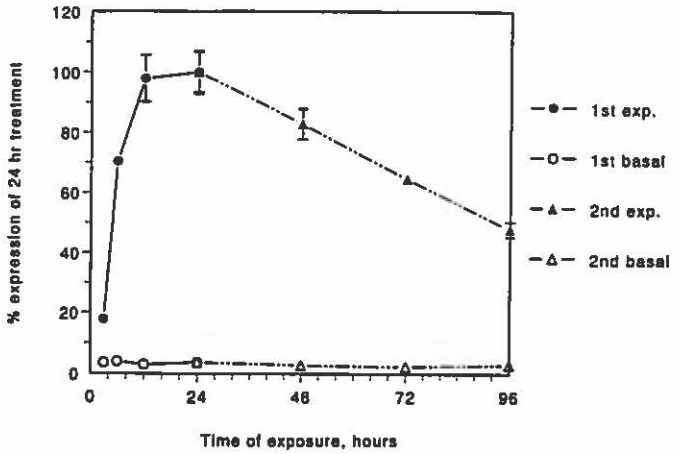

Fig. 2. **Kinetics of ICAM-1 expression on the surface of HUVEC cells induced by 1 μg/mL endotoxin.** The experiments were carried out separately for 0-24 h and 24-96 h. The data were normalized to the samples of highest expression, i.e., the 24 h data points, with cpm values of 72,167 ± 5232 and 150,648 ± 9814 for the 0-24 h and the 24-96 h experiments, respectively.

This suggests that the LPS-induced ICAM-1 expression was not exerted by the autocrine and/or paracrine effects of LPS-induced IL1 and/or TNFα.

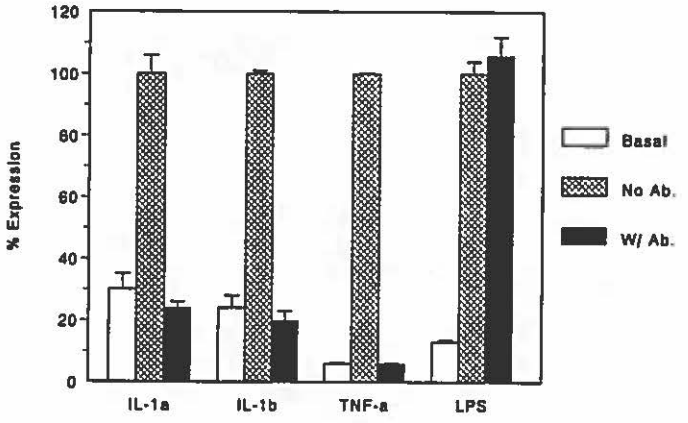

Fig. 3. **Effects of neutralizing rabbit polyclonal antibodies specific to IL-1α, IL-1β, TNFα and their combinations, respectively, on the IL-1α-, IL-1β-, TNFα-, and LPS-induced cell surface ICAM-1 expression in HUVEC during 4 h incubation.** The data were normalized to the control where the cells were treated with stimulant but without antibody with the control cpm values of 16,289 ± 1048,20,050 ± 94,21,339 ± 64 and 9372 ± 362 for IL-1α, IL-1β, TNFα, and LPS respectively.

Correlation between ICAM-1 expression and DNA synthesis

The effects of endotoxin on the DNA synthesis activity of HUVEC was measured by ^{3}H-thymidine incorporation. Figure 4 shows the results of the experiment carried out twice in triplicate for each data point. Compared to the control in the absence of LPS, the DNA synthesis activity of HUVEC was progressively suppressed from 90% to 30% when the LPS concentration was increased from 0.001 to 100 ug/ml, suggesting that DNA replication or cell division was not the cause of the LPS-induced ICAM-1 expression. This line of evidence was further investigated in cytochemical experiments described in the following section.

Differential ICAM-1 expression by individual cells

In the previous sections, the LPS-induced ICAM-1 expression of HUVEC was studied immunobiochemically. The data represent the average of all the cells in the samples. The following sections describe the data from experiments carried out to acquire information on individual cells.

(a) ^{3}H-thymidine incorporation and HRP products staining

The effects of endotoxin on DNA synthesis activity and surface ICAM-1 expression is shown in Figure 5. The cells actively synthesizing DNA are shown with silver grains in the nucleus derived from the incorporated ^{3}H-thymidine. The cells with surface ICAM-1 expression are shown with brown staining from the HRP reaction products. Interestingly, the silver grain and the brown staining are rarely seen in the same cells. The unstimulated samples showed strong ^{3}H-thymidine incorporation but weak ICAM-1 basal expression (Figure 5a). In the LPS-treated samples, the ^{3}H-thymidine incorporation was reduced and the surface ICAM-1 expression was significantly enhanced (Figures 5b and 5c).

(b) FITC staining

Immunofluorescence staining of surface ICAM-1 expression in HUVEC is shown in Figure 6. The unstimulated sample showed little staining (Figure 6a), while the LPS-stimulated sample exhibited considerable increased levels of green fluorescent staining of the bound FITC-labelled

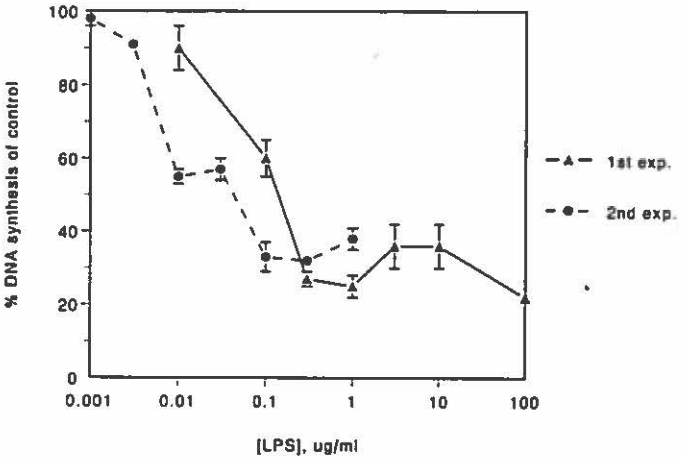

Fig. 4. Effects of LPS on DNA synthesis activity of HUVEC cells at 24 h treatment, determined by [^{3}H]thymidine incorporation and expressed as percentage of the control. Two separate experiments were carried out independently in triplicate for each LPS dose with control cpm values 22,573 ± 1689 and 17,949 ± 338 for the first and the second experiments, respectively.

antibody (Figure 6b). The cells in the sample were not uniformly stained, indicating that some cells expressed more surface ICAM-1 than the others. Distinct spotty and patchy staining patterns were also observed within individual cells, suggesting the presence of clusters of ICAM-1 freshly deposited from the secreting vesicles to the cell surface and overlapping of the cell surface, in line with the electron micrographic observations described in the next section.

(c) Immunoelectron micrograph

The detailed distribution of the surface ICAM-1 in HUVEC is shown in the electron micrographs in Figure 7. The control cell had little staining on the cell surface (Figure 7a), while the LPS-stimulated cell showed intense staining, stronger

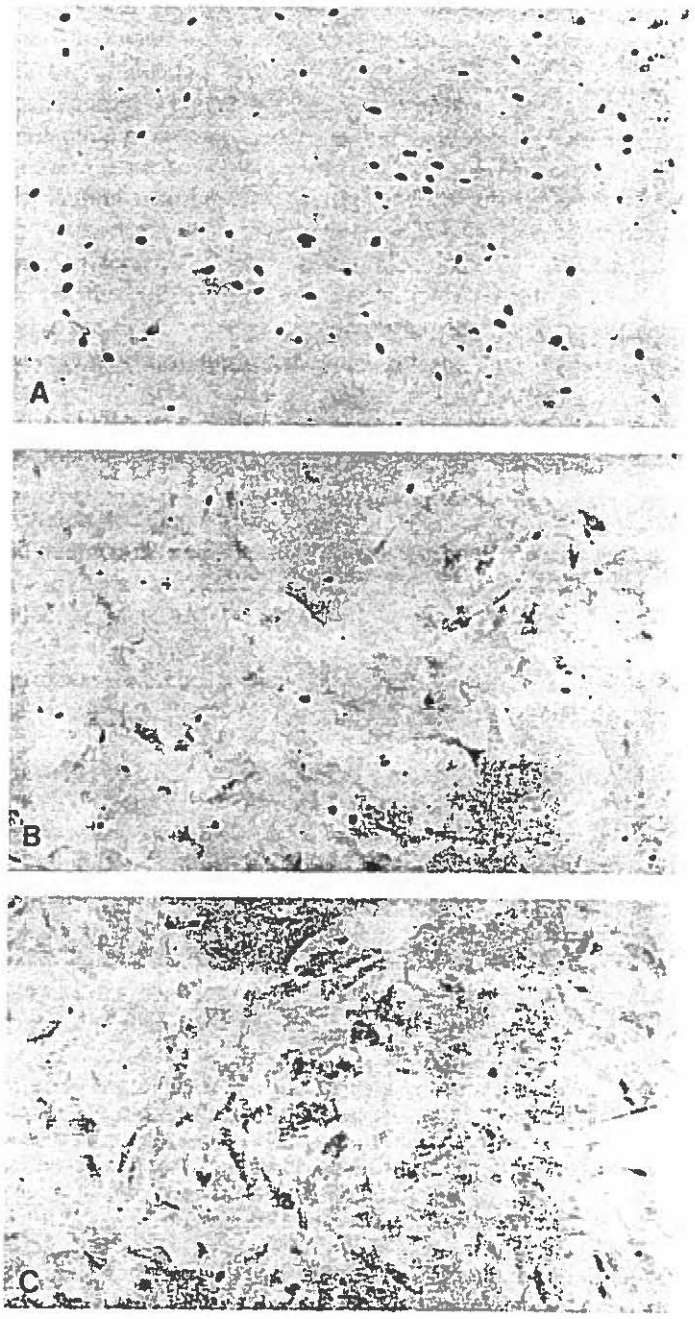

Fig. 5. **Double labeling of HUVEC cells with the HRP products (brown color) staining for cell surface ICAM-1 and the silver grains from [^{3}H]thymidine-incorporated DNA of the nuclei.** Basal expression of ICAM-1 is shown as trace brownish HRP products staining in the control sample with most silver grains showing the [^{3}H]thymidine incorporation in the nuclei (A). Exposure of the samples to LPS (.1 µg/mL, B; 1 µg/mL, C) increased the ICAM-1 expression and reduced the [^{3}H]thymidine incorporation.

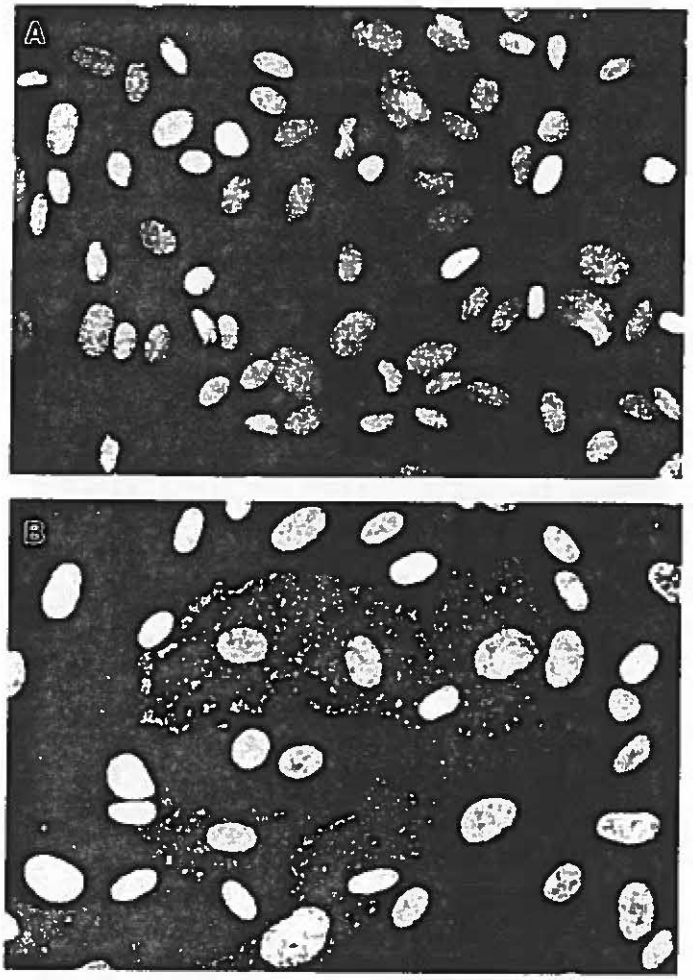

Fig. 6. **Effects of LPS (1 μg/mL, 24 h) on surface ICAM-1 expression in HUVEC shown by FITC staining.** Little staining was observed in the control (A), while intense staining with spotty and patchy patterns was observed for the LPS-treated sample (B).

(Figure 7b). Higher population of ICAM-1 on the luminal surface would favor the anchoring or trapping of leukocytes. The newly erupted secreting vesicles were also clearly shown on the luminal and basal surfaces (Figure 7b), demonstrating the translocation of the ICAM-1 molecules from its synthesis sites to the luminal and basal membranes. Figure 7c shows the ICAM-1 distribution on the luminal surface at a higher magnification. The newly erupted secreting vesicles (arrows) and the overlapping of cell surface resulted in the spotty and patchy patterns observed in the FITC-stained cells in Figure 6b.

DISCUSSION AND CONCLUSIONS

The surface expression of ICAM-1 in HUVEC was inducible by endotoxin in a dose-dependent manner. At 1 ng/ml LPS barely induced the ICAM-1 production above basal expression. The induction was significant at 10 ng/ml, peaked at 0.1-1 ug/ml and dropped off above 1 ug/ml. A similar trend of the LPS-dose dependency of ICAM-1 expression was observed for different batches of HUVEC. The doses of LPS used in this paper are about 100-1000 fold higher than those observed in vivo during inflammation conditions. However, the in vivo data represent the average values of LPS concentration in blood and may not reflect the concentration of certain localized areas, especially at wounds and focal infections. Effective LPS concentrations at these focal sites may attain levels in the range we have tested. LPS-induced ICAM-1 expression in culture continuously exposed to 1 ug/ml of LPS increased sharply within 12 hours, maintained a plateau of high expression between 12-24 hours and decreased after 24 hours. However, the induced ICAM-1 level at 96 hours was still a remarkable 50% of the maximal expression. Co-incubation of the cells with LPS and neutralizing antibodies specific to IL-1α, IL-1β and TNF-α failed to inhibit the LPS-induced ICAM-1 expression, indicating that the induction of ICAM-1 expression by endotoxin was not mediated by extracellular IL-1 and/or TNF-α. A similar phenomenon was also observed for the LPS-induced endothelial-leukocyte adhesion molecule-1 (ELAM-1) and vascular cell adhesion molecule 1 (VCAM-1) expression in HUVEC (unpublished data of the authors'). Thus, the LPS-induced ICAM-1, ELAM-1 and VCAM-1 expression in endothelial cells is either due to the direct interaction of the cells with the LPS/sCD14, or due to the possible autocrine or paracrine effects of LPS-induced IL-2, IL-8, interferon-gamma or other cytokines as yet to be shown. However, it is clear that LPS rather than LPS-induced IL-1 and/or TNF-α plays a major role in the early recruitment of neutrophils in the local LPS response (19).

LPS inhibits DNA synthesis activity in HUVEC as shown by ^{3}H-thymidine incorporation. The

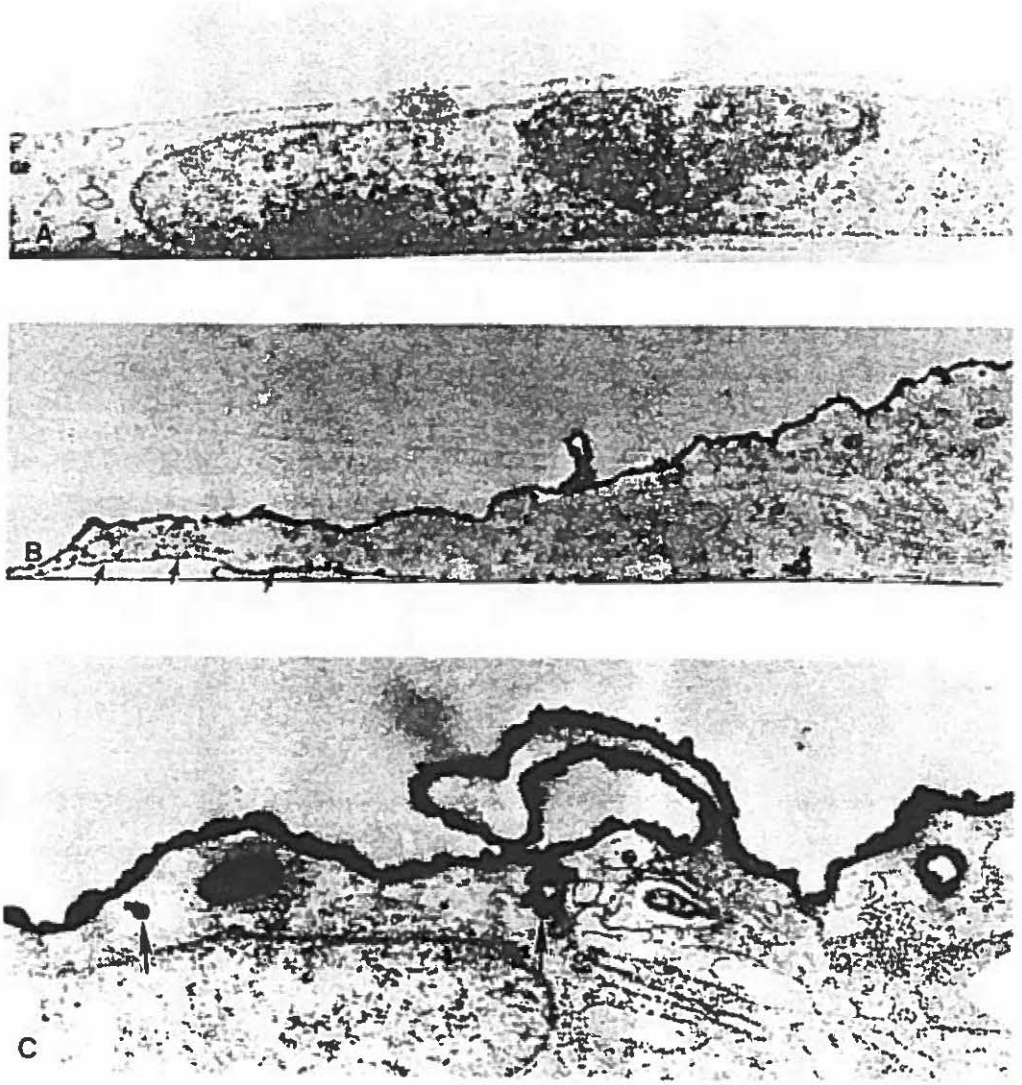

Fig. 7. **Distribution of LPS-induced surface ICAM-1 in HUVEC shown by electron micrograph.** The cell of the control sample (A) showed little ICAM-1 on the cell surface. The cell treated with LPS (1 μg/mL, 24 h) clearly showed the polarized distribution of cell surface ICAM-1 with higher density on the luminal surface than the basal surface (arrows) in cell body and cell extension (B). A section of the luminal surface area was further enlarged to show the details of the cell surface distribution of ICAM-1 (C). The spotty and patchy pattern of the FITC staining observed in Fig. 6 was due to the ICAM-1 of the newly erupted secretion vesicles (arrows) and the overlapped structure of the cell surface.

LPS-induced ICAM-1 expression and DNA synthesis inhibition in HUVEC were unevenly distributed throughout the cells in the culture, and were mutually exclusive within a cell. This may indicate that ICAM-1 induction and DNA synthesis inhibition can only occur during separate and limited times in the cell cycle.

Electron micrographs indicated that ICAM-1 was distributed all over the surface of the cell. There was a somewhat polarized distribution favoring the luminal surface, which would facilitate interactions with other cells types. Newly erupted vesicles arrying secreted ICAM-1 to the membrane and overlapping area of the cell surface, observed in the electron micrograph and in the FITC-stained samples as spotty and patchy patterns, suggest the translocation of ICAM-1

from its synthesis sites to high density areas on the cell surface. The high density, polarized cell surface distribution (favoring the luminal surface) and the non-uniform expression of ICAM-1 by individual cells in the culture are consistent with a role for ICAM-1 in the scattered, localized and selective sites for leukocyte-endothelial interactions and adhesion

(i.e. sticking) followed by transmigration in the processes of leukocytes extravasation. Thus, induction of ICAM-1 expression on endothelial cells by LPS (also by IL-1, TNF and IFN-gamma, etc.) will enhance endothelial-leukocyte adherence and leukocytes extravasation, and contribute to the pathophysiology of sepsis and septic shock.

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Title

PLASMA VOLUME, VASOPRESSIN AND THE GENETICS OF MOTION SICKNESS

Principal Investigator

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Objective

Motion sickness, spatial orientation and vestibular dysfunction in aircrews and shipboard personnel are significant operational concerns for the Navy because they affect personnel performance. Since changes in plasma volume may affect susceptibility to motion sickness, these factors have become increasingly problematic as troops are deployed to hot weather regions (such as the Middle East) and are exposed to extreme operational stressors (such as the high gravitational forces experienced in modern fighter aircraft).

Two key aspects of the motion sickness problem are studied in this project, one physiological and the other genetic. The first objective is to determine whether a decrease in plasma volume contributes to vestibular dysfunction and leads to disorientation and motion sickness in susceptible personnel. The physiological effects of diuretic and natriuretic hormones (atrial natriuretic factor, vasopressin) influencing tolerance to untoward motion in humans are studied. A synthetic hormone (1-desamino-8-D-arginine vasopressin (DDAVP)) is used to expand plasma volume in hopes of decreasing personnel susceptibility to disorientation and motion sickness.

The second objective of this study is to determine the possible genetic basis for susceptibility to motion sickness. If it is determined that this susceptibility is genetically controlled, the gene(s) responsible for the increased predilection of some individuals to motion sickness will be identified. Ultimately, understanding the gene(s) and gene product(s) that modulate susceptibility to motion sickness and disorientation may lead to pharmaceutical approaches for modifying these genetic effects.

Approach

This research initiative was examined by a hypothesis that motion sickness is a heritable trait and using the candidate gene approach, progress was made at identifying one of the molecular mechanisms responsible for motion sickness. Using Coriolis stress susceptibility as a marker for tolerance to untoward motion, the PI reported that a genetic polymorphism of the alpha-2 adrenergic receptor (A2AR) encoded on chromosome 10 (C10) is associated with a propensity for motion sickness. Polymorphisms are variant forms of a gene that exist in a population in high enough frequency that they have not occurred by chance mutation alone. These findings have obvious operational relevance. By implicating a role for the genes encoding the A2AR, a ubiquitous receptor that is activated by endogenous catecholamines such as epinephrine and

norepinephrine, we can now formulate a more coherent understanding of the mechanisms by which motion sickness develops, and understand the reason why some individuals are relatively immune to the untoward effects of shipboard or aircraft motion which may affect task performance.

Through efforts to understand the physiologic and molecular actions of genetic polymorphisms of the A2AR in mediating susceptibility to Coriolis stress, novel observations were made that also impact clinical medicine. For example, the A2AR, in addition to being expressed in neurons receiving vestibular input, is also expressed on platelets, in the pancreas, in the kidnes and on the vasculature. These receptors control platelet aggregation, glucose-mediated insulin secretion, salt and water metabolism and blood pressure and response to orthostatic stress. In the course of studies, findings having tremendous implications have been made for operational commands outside of the aviation and the surface warfare community. For example, a heretofore unknown mechanism of thermogenesis in man, that is relevant to special forces operating in the cold, have been identified.

A genetic basis, for differences in renal salt and water excretion that may impact land warfare troops exposed to hot climates, has been discovered. These findings are also relevant to the civilian community. A polymorphism of the A2AR that is associated with a genetic predisposition for hyperinsulinemia and diabetes mellitus has been identified. These studies have not only increased one's understanding on the pathogenesis of motion sickness, they have resulted in products useful to civilians as well as the Office of Naval Resesarch and the Department of Defense.

Results

No association was found with Coriolis stress susceptibility among polymorphic markers of the C10 beta-1 adrenergic receptor gene which is in tight linkage disequilibrium with the C10 A2AR gene. Considering this result, it is likely that the C10 A2AR indeed governs Coriolis stress susceptibility.

The investigators now report that the C10 A2AR polymorphisms do not encode differences in A2AR number or affinity binding constants. Despite similar expression of A2AR number and affinity binding constants of the C10 A2AR from subjects with variant genotypes, it was found that the polymorphism of the C10 A2AR are associated with three intermediate phenotypes. These intermediate phenotypes of results from genetic polymorphisms which by themselves may not determine susceptibility to motion sickness, but coupled with other environmental or epistatic factors, determine the physiologic responses to untoward motion. These intermediate phenotypes include epinephrine-medicated platelet aggregation, salt excretion and baroreceptor sensitivity in healthy subjects. These findings are highly significant and relevant. It was previously demonstrated that increased susceptibility to Coriolis stress was associated with higher blood pressure, and polymorphisms of the C10 A2AR are also associated with increased blood pressure. Accordingly the investigators have demonstrated a common mechanism by which increased susceptibility to motion sickness, orthostatic intolerance, high blood pressure and tendency towards thrombotic stroke are all inherited. Furthermore it is suggested from these findings that volume status, salt balance and hydration may affect an individual's predisposition for motion sickness as well as orthostatic tolerance.

A polymorphism of the C2 A2AR gene has been identified. Sequencing of this polymorphism reveals an expansion of a trinucleotide repeat encoding a string of glutamic acids. This C2 A2AR polymorphism is found in the structural region of this single exon gene. Specifically, this polymorphism is found in the region of the gene which encodes the third intracytoplasmic loop of this G-protein linked receptor and is coupled to signal transduction. No association between the variant forms of this trinucleotide repeat and Coriolis stress susceptibility was found. However, because of the functional significance attached to the expansion of trinucleotide repeats in other genes, and because the C2 A2AR is specifically expressed in the pancreas and modulates glucose-stimulated insulin release, the investigators had to determine whether their polymorphism was functional. They report that the C2 A2AR polymorphism is associated with hyperinsulinemia and diabetes mellitus in man. Also, no association was found between Coriolis stress and an anonymous polymorphic marker in linkage disequilibrium with the C4 A2AR gene. The investigators have not been able to identify a polymorphic marker of the M2 muscarinic, cholinergic receptor.

Fiscal Data ($K)

FY92	FY93	FY94	FY95	FY96
120.0	112.0	108.0	0.0	0.0

Publications

Lockette W, Peraino D, Farrow S. A Genetic Polymorphism of the C2 Alpha-2 Adrenergic Receptor is Associated with Hyperinsulinemia and Diabetes Mellitus in Blacks. J. Clinical Investigation. (Submitted)

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Lockette, W. et. al. Alpha-2 Adrenergic Agonists Increase Cellular Lactate Efflus. Hypertension. (Submitted)

Freeman, K. et. al. A Genetic Polymorphism of the Alpha-2 Adrenergic Receptor is Associated with Increased Exercise-Induced Sweat Sodium Excretion in Humans.

Title

THE ROLE OF CYTOKINES IN RECOVERY FROM ENTERIC SHOCK COMPROMISE FOLLOWING HEMORRHAGIC SHOCK

Principal Investigator

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Objective

The objective of this project is to examine the possibility of eliminating gut origin sepsis following hemorrhagic shock in mice by the oral administration of cytokines. This treatment regimen may be useful in ameliorating the profound immunosuppression seen following hemorrhagic shock.

Approach

- Mouse model, controlled hemorrhage to 35mm Hg for 1 hour, resuscitate
- Administer oral experimental prophylactic substances
- Measure bacterial colonies/gm liver, spleen and MLN
- Measure magnitude of immune responses.

Results

- The number of bacterial colonies per gram of tissue in liver, spleen and mesenteric lymph nodes is decreased by at least 3-4 logs following administration of cytokine as late as two hours following hemorrhage.
- Hemorrhage has been shown to reduce immunological capacity.
- Oral administration of cytokines restores immunologic capacity to spleen, mesenteric lymph nodes and Peyer's patches.
- Cytokines given orally also improved the intestinal integrity of the small intestine, which is badly damaged after hemorrhage.
- Intestinal areas supplies by the superior mesenteric artery (ileum and jejunum) are the most compromised by ischemic events.
- Administration of oral IL-6 or crude cytokine alleviates the histological picture, restoring the gut epithelial layer and increasing circulation. The iv and oral administration of IL-6 reduces blood pressure, a mechanism believed to be responsible for increasing gut circulation.
- Local vasorelaxing events may explain the increased circulation in the areas supplied by the superior mesenteric artery. Ex vivo experiments on rat intestine show that intraintestinal administration of IL-6 increases fluid flow across the intestinal bed supplied by the mesenteric artery.

Oral administration of IL-6 to shocked mice:

1. reduces bacterial translocation
2. restores immune potential
3. restores intestinal integrity

A treatment modality for reduction of long term sepsis can be given on the battlefield (i.e., FIRST ECHELON) to a hemorrhaged casualty. This treatment will increase survival be decreasing the risk of gut origin sepsis following hemorrhage.

Fiscal Data (SK)

FY92	FY93	FY94	FY95	FY96
0.0	0.0	108.0	109.0	0.0

Publications

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Title

EVALUATION OF THE INFLUENCE OF SUPERANTIGENS AND POLYCLONAL B-CELL ACTIVATORS IN PERIODONTAL DISEASES

Principal Investigator

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Objective

Periodontal diseases are localized inflammatory conditions which may include sufficient destruction of the periodontium to result in loss of teeth. Periodontal destruction, loss of attachment, and loss of bone are all attributes of this disease. It is well accepted that periodontal diseases are prevalent among military personnel and present serious problems for Navy and Marine Corps effectiveness as well as for the general public. Such diseases are initiated by bacteria present in the gingival tissue.In addition to an infective etiology the presence of an immunological component of periodontal disease has long been suspected, and extensive studies have been made to delineate the nature of this component consistent with the hypothesis that severe periodontal disease pathology is B-cell mediated. A basic disagreement, however, concerns the difference between the nonspecific and the specific plaque hypothesis of disease initiation. Essentially, this relates to one of two alternatives. The first is whether the etiology of periodontal disease is primarily a result of disturbances in the balance set up between host and the endogenous flora which under certain conditions causes tissue damage opportunistically. Whereas alternatively, that a single pathogen, or small group of pathogens, infects a possibly susceptible host in a manner consistent with any of a number of conventional bacterial infections.

Our preliminary studies and those of others have demonstrated that as a possible result of the presence of potent polyclonal B-cell activators associated with bacteria localized in the periodontal lesion, B-lymphocytes in the periodontium may be activated non-specifically. It is becoming clear, however, that such responses are under exquisite T-cell and macrophage control and that substances influencing the function of these cells may also play a role in the establishment of a B-cell inflammatory response. As a second but related example of a localized oral inflammatory disease, periapical periodontitis, although not as well characterized, also shows an immunological component responsible for disease. This situation also derives from an initial bacterial infection and can result in bone loss.

The primary objective of the proposed work is to investigate the following general hypothesis: Bacteria associated with periodontal diseases have as a part of their makeup substances that can modulate the host immune response and in so doing establish the conditions necessary for the development of periodontal disease. It is probable that these virulence factors for periodontal disease are carried by many different bacteria. Therefore, it would not be necessary for periodontal disease or other localized inflammatory disease to be caused by a specific indicator organism but only that the virulence factor be present and that the host be responsive. It is possible that these virulence factors may be superantigens or polyclonal B-cell activators or both. Recently, and with the use

of indirect methods, superantigens have been associated with the development of periodontitis.

Finally, it is increasingly clear that the oral cavity can act, especially in immune-compromised patients, as the origin for systemic dissemination of pathogenic organisms. Within the past few decades endogenous anaerobic infections have become far more common due in part to improved diagnostic techniques and increased awareness on the part of health care professionals. Many of these infections involve the anaerobic bacteria associated with periodontal disease and periapical lesion development. Increased knowledge and understanding of the immunomodulatory factors associated with such microbes could permit a better understanding of how bacteria modulate host inflammatory responses. This could permit more effective treatment regimens to be devised based on the counteraction of immunomodulation. Should superantigens and/or polyclonal B-cell activators function as virulence factors in the etiology of periodontitis they could be used as markers to produce probes for the development of various diagnostic tests.

Approach

The primary method is to investigate the following general hypothesis: bacteria associated with periodontal disease have as a part of their makeup substances that can modulate the host immune response and in doing so establish the conditions necessary for the development of periodontal disease. It is probable that these virulence factors for periodontal disease are carried by many different bacteria. Therefore, it would not be necessary for periodontal disease or other localized inflammatory disease to be caused by a specific indicator organism but only that the virulence factor be present and that the host be responsive. It is possible that these virulence factors may be superantigens or polyclonal β-cell activators or both.

Superantigen activity associated with periodontal pathogens is being identified using reverse transcriptase polymerase chain (rt-PCR) methodology to identify T-cell receptor Vβ messenger RNA. Polyclonal B-cell activators are being identified by evaluating production of polyclonal antibody.

Results

Bacterial Antigens:

In addition to the various periodontopathogens previously indicated we have now extended the project to include antigens from a group of oral *Treponema denticola* spirochetes and are evaluating these agents for lymphocyte blast transformation, cytokine production, polyclonal B-cell activation, and superantigen synthesis.

Peripheral blood responses:

Recent results suggest that P.g. and A.a. contain potent immunosuppressants which have differential effects on B-cells and T-cells independent of the periodontal health of an individual and exert their influence through other than toxic effects.

In addition, studies with T. Denticola, recent studies with the *T. denticola* antigen preparations have suggested that patients diagnosed with severe adult periodontitis show elevated lymphocyte blast transformation after culture with bacterial components.

Recent studies with the *T. denticola* antigen preparations have suggested that patients diagnosed with severe adult periodontitis show elevated lymphocyte blast transformation after culture with bacterial components.

Identification of superantigen activity:

A variety of methods are being used to determine if the various periodontopathogens carry superantigens. Work which started during the previous reporting period utilizing flow cytometry are being continued. A variety of monoclonal antibodies with specificity's toward the various Vb determinants on the surface of the T-cell are now available and have been labeled with fluorescent tags. Bacterial antigens and control T-cell stimulators are being used to transform T-cells into blasts which are subjected to two-color flow cytometry. Attempts are currently being made to identify if T-cells carrying specific Vb regions are specifically elevated.

Production of large quantities of IL-2 would be expected if superantigens were present in bacterial extracts. In order to test for this a variety of studies were designed to ascertain if IL-2 levels were elevated in PBMCs obtained either from diseased or non-diseased individuals. Sufficient work has been done with PBMC from non-diseased individuals to demonstrate that significant IL-2 is present in culture media after these cells were cultured with P. gingivalis, A. actinomycetemcomitans, and toxic shock syndrome I toxin (TSST-1).

Freeze storage of lymphocytes.

Work continues to progress in this area with the evaluation of PBMC from five additional subjects. We have now determined that frozen and stored lymphocytes retained functional activity after more than 60 weeks in frozen storage. In addition, studies were conducted to determine whether the autologous immune response demonstrated by unstimulated lymphocytes also retained activity. Our results indicate that frozen and stored lymphocytes retain a variety of functional activities, including autologous responses and production of IL-1, IL-2, IL-6 and TNF-β, even after more than a year in liquid nitrogen storage. Frozen storage continues to represent a viable method to permit extending immunological assays on clinically characterized subjects.

Level of progress:

Significant progress has been realized including the probable identification of superantigen activity in A.a. and P.g. homogenates. The establishment of the quantitative rt-PCR utilizing fluorescent tagged primers should unequivocally prove this.

Fiscal Data (SK)

FY92	FY93	FY94	FY95	FY96
0.0	111.0	108.0	104.0	0.0

Publications

Miller GA, DeMayo T and Hutter J. Production of Interleukin-1 by Polymorphonuclear Leukocytes Present in Inflammatory Periradicular Tissues. Journal of Endodontics. 1995. (Accepted for Publication)

Presentations and Abstracts

Getka, T. Immunomodulation by Components of Bacterial Associate with Adult Periodontitis. National Naval Medical Center. 1994.

Miller, G.A. and D.C. Alexander. 1995. Evidence for superantigen production by bacteria associated with adult periodontitis. Journal of Dental Research, 74:125 (Abstract No. 906)

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Miller GA. Involvement of Superantigens in the Development of Periodontal and Endodontal Inflammation. National Institute of Dental Research, National Institutes of Health. 1995.

Title **MODULATION OF CELL SURFACE ADHESION MOLECULES AND CYTOSKELETAL REORGANIZATION BY MONOCYTE-ENDOTHELIAL CELL INTERACTION**

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Objective Navy personnel engaged in combat and hazardous operations often suffer from traumatic injuries and infections which inflammatory reactions may lead to septic shock, impaired wound healing and death. The aim of this proposed study is to establish basic understanding of the cellular and molecular mechanisms that regulate inflammation and to develop a pharmacologic and/or immunologic means for modulating the intensity of inflammation.

Approach Excessive leukocyte adhesion and accumulation mediated by adhesion molecules during inflammation often causes severe tissue injury and subsequent development of multiple organ failure in septic shock syndrome. We hypothesized that optimization of leukocyte-endothelial interaction by regulation of the expression of adhesion molecules may provide a practical mechanism for protection of endotoxin-related organ failure. This study was designed to explore a pollible application of cytokines to control or modulation of leukocyte-endothelial interactions during inflammation induced by endotoxin or gram-negative bacteria. Thus, the endothelial injury induced by excessive leukocyte adhesion and accumulation can be ameliorated and septic shock syndrome can be controlled. Cytokines, such as IL-8, IL-10, and TGF-β, have been evaluated in vitro for modulation of adhesion molecules expressed by human monocytes and endothelial cells by radioimmunoassay, immunofluorescence, immunoelectron microscopy and autoradiography and for control of monocyte adhesion to LPS-activated endothelial cells by radioactive chromium retention and morphometry.

Results During the three-year funding period three major studies were performed: (1) induction of endothelial adhesion molecules by gram-negative bacterial endotoxin on monocyte/macrophage expression of β1 and β2 integrins, (2) effect of cytokines such as TGF-β1. IL-8, and IL-10 on endotoxin induction of endothelial adhesion molecules and β1 and β2 integrins on monocytes/macrophages, and (3) effect of cytokines on monocyte adherence to endotoxin-activated endothelial cells.

I. Endotoxin (Lipopolysaccharide, LPS) of Adhesion Molecules Expressed on Human Endothelial Cells and Monocytes/Macrophages

Induction of the expression of endothelial adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1), endothelial leukocyte adhesion molecule 1 (ELAM-1, E-selectin), and vascular cell adhesion molecule 1 (VCAM-1) by E. coli endotoxin was studied by immunocluorescence and radioimmunoassay. Endotoxin induced expression of these endothelial adhesion

molecules in a dose-related pattern. ICAM-1 expression was more prominent than others. These results provided important information on the role of endotoxin in the pathogenesis of multiple organ failure in septic shock syndrome.

Human monocytes/macrophages constitutively express β1 and β2 integrins and L-selectin (LECAM-1). Effects of endotoxin on the expression of adhesion molecules such as β1 integrins (VLA-5, VLA-3, VLA-2), β2 integrins (CD11/CD18), and fibronectin on human peripheral blood monocytes and bronchoalveolar lavage macrophages were investigated. In human macrophages, the expression VLA-5. VLA-3 and VLA-2 which are the receptors for extracellular matrix proteins such as fibronectin, laminin and collagen was downregulated after endotoxin stimulation. However, endotoxin increased expression of fibronectin by the macrophages. The diminishment of these receptors may reduce or impair macrophage migration in the lung tissue that may weaken the natural defense system in the lung.

The modulatory effect of LPS on the expression of VLA-5 and surface fibronectin in human peripheral blood monocytes were also investigated. We have found that endotoxin increases expression VLA-5 and surface fibronectin. The enhancement of VLA-5 and surface fibronectin would facilitate monocyte migration and accumulation in the tissues during inflammation. The endotoxin-induced increase of monocyte migration and accumulation may have the following two functions: (1) to increase monocyte phagocytosis and enhance wound healing by production of wound healing related growth factors or cytokines, and (2) to increase secretion of inflammatory cytokines such as IL-1, IL-6 and TNF which may exert detrimental effects on the host.

The effect of LPS of LPS on CD11b and L-selectin in human monocytes was investigated to determine whether LPS may interfere with the normal inflammatory responses of the host. Both CD11b and L-selectin are constitutively expressed on monocytes. CD11b is a receptor for ICAM-1 expressed by endothelial cells. L-selectin is a counter part of E-selectin expressed by endothelial cells, which mediates rolling of neutrophils and monocytes along the luminal surface of blood vessels prior to firm attachment of endothelium. LPS diminished expression of CD11b and L-selectin on human monocytes, which implied that LPS may reduce monocyte adhesion to endothelial cells.

II. Cytokine Modulation of the Expression of Adhesion Molecules by Human Endothelial Cells and Monocytes

Modulation of the expression of endothelial adhesion molecules by TGF-β1, endothelial IL-8 and IL-10 was investigated. TGF-β1 alone induced expression of all three adhesion molecules in a dose-dependent manner. However, TGF-β differently affected endotoxin induction of the adhesion molecules. The effects varied with type of the molecules, time and mood of treatment. Pre-treatment of endothelial cells with TGF-β1 increased endotoxin induction of ICAM-1 and VCAM-1 but decreased E-selectin expression. TGF-β1 enhanced expression of all the adhesion molecules when cells were primed with 1 μg/ml endotoxin. Concomitant treatment of endothelial cells with endotoxin and TGF-β1 resulted in significant increase in ICAM-1 expression but decrease in VCAM-1. The

results indicate the TGF-β1 may play an important role in modulation of leukocyted adherence and emigration by up- or down- regulation of endothelial adhesion molecules in endotoxin-induced inflammation and that TGF-β1 dose.

There are two types of IL-1: monocyte IL-8 produced by activated monocytes/macrophages and endothelial IL-8 produced by activated endothelial. We have conducted experiments on the effect of endothelial IL-8 on the expression of ICAM-1 and E-selectin by human endothelial cells. We found that IL-8 showed a significant dose-related suppressive effect on LPS induction of E-selectin, but had no significant effect on ICAm-1 induction by LPS. The results suggest that endothelial IL-8 may be useful for control of endotoxin induced inflammation.

IL-10 is a pleiotropic cytokine displaying pro-inflammatory and anti-inflammatory effect on many cells. We have studied the effect of IL-10 on the expression of ICAM-1, E-selectin, and VCAM-1 by endotoxin in order to understand whether IL-10 may be potentially important for preventing excessive leukocyte adhesion and accumulation in endotoxin mediated inflammation. IL-10 exhibited differential effects on endotoxin induction of endothelial adhesion molecules. IL-10 alone at higher doses stimulated expression of ICAM-1 but suppressed endotoxin induced ICAM-1 expression at lower doses. No significant effect of IL-10 on E-selectin was found. IL-10 significantly inhibited LPS induction of VCAM-1. The results show that IL-10 may prevent or suppress leukocyte adhesion to endotoxin activated endothelial cells via suppression of ICAM-1 and VCAM-1 expression.

The influences of TGF-β1, IL-8 and IL-10 on β1, β2 integrins and L-selectin (LECAM-1) on human monocytes was studied. TGF-β1 did not show any significant effect of the cytokine on the expression of VLA-5 and CD11b on monocytes. However, we found that IL-8 significantly downregulated CD11b and L-selectin. The results suggest that IL-8 may suppress monocyte adhesion to endothelial cells via downregulation of CD11b and L-selectin. Il-10 was also found to reduce expression of CD11b by monocytes, indicating that IL-10 may also suppress monocyte adhesion by down regulation of CD11b expression.

III. Cytokine Effect on Monocyte Adhesion to LPS-Activated Endothelial Cells

Cytokines TGF-β1, endothelial IL-8, and IL-10 were tested for modulation of monocyte adhesion to LPS-activated human endothelial cells. We found that TGF-β1 did not have significant suppressive effect on monocyted adherence to endothelial cells; however, both IL-8 and IL-10 had significant inhibition on monocyted adhesion to LPS-activated endothelial cells. These findings indicate that IL-8 and IL-10 are important cytokines which are involved in regulation of LPS-induced inflammatory responses and they may be useful for in vivo control of inflammation.

In summary, we have found: (1) LPS induces endothelial expression of ICAM-1, E-selectin and VCAM-1 in a dose-related manner; (2) TGF-β1 regulates differentially LPS-induced expression of the endothelial adhesion molecules; (3) β1 and β2 integrins are constitutively expressed on human monocytes and

macrophages; (4) LPS shows differential effects on the expression of β1 and β2 integrins; (5) endothelial IL-8 and IL-10 suppress monocyte adhesion via downregulation of L-selectin and CD11b and inhibition of ICAM-1 and VCAM-1 expression; and (6) IL-8 and IL-10 are potentially important cytokines which can utilize for control of host inflammatory response.

Fiscal Data (SK)

FY92	FY93	FY94	FY95	FY96
100.0	86.0	97.0	0.0	0.0

Publications

Kang YH, Lee CH, Brummel SE, Newball HH and Forrester J. Effects of Endotoxin on Expression of VLA Integrins by Human Bronchoalveolar Lavage Macrophages. Journal of Leukocyte Biology. 1995: 57.

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Kang YH, Dao L, Brummel SE, Harlan D, Lee CH. IL-10 inhibits monocyte adhesion to LPS-activated endothelial cells by suppression of VCAM-1 and CD11b. Will be presented at the 18th Annual Conference on Shock.

Title

THE USE OF LEET AND BRIGHT LIGHT SEPARATELY AND TOGETHER FOR SHIFTING THE WORK-REST CYCLE

Principal Investigator

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Objective

The general purpose of this proposal is to develop interventions for optimizing performance of military personnel. The major objective is to identify measures which decrease fatigue and performance deterioration and optimize sleep in personnel required to perform night work or to work after shifting time zones. We seek to determine the relative and combined value of two interventions: timed bright light exposure and electromagnetic sleep induction (LEET), for improving quality of sleep and performance after a shift in the sleep-wake cycle. Hypotheses to be tested include:

- Both LEET and bright light will improve the quality of sleep after a 10-hour shift of the work-rest cycle.
- Both LEET and bright light will improve the quality of cognitive performance, both speed and accuracy, after a 10-hour shift of the work-rest cycle.
- Both LEET and bright light will decrease sleepiness during the work period, after a 10-hour shift of the work-rest cycle.
- Bright light alone will be more effective than LEET alone.
- The combination of bright light and LEET will be more effective than either intervention alone.
- Bright light will promote the shift of physiological rhythms (temperature and melatonin) to the new circadian phase.

Approach

Volunteers underwent a 10-hour phase delay of their work rest cycle. Subjects received either bright (>2000 lux) white or dim red light from 2200-0200 each night after the phase shift. They received either inactive or active LEET prior to the daytime sleep periods. Sleep was recorded during a baseline night and during the 3 daytime sleep periods after the phase shift. The circadian rhythms of 6-sulphatoxymelatonin (6-SM) excretion in the urine and core temperature were monitored throughout the study. Repeated cognitive performance testing was performed during the nighttime work periods.

Results

Both bright light and LEET decreased the number of arousals during the first daytime sleep period after the phase shift. After 3 days of treatment, the bright light exposure significantly increased the duration of sleep and sleep efficiency, and decreased wake time after sleep onset but not sleep latency, indicating bright light was improving ability to stay asleep rather than ability to fall asleep initially.

The results of this study are unique in providing polysomnographic proof of improved sleep with three days of bright light treatment. There was a nondignificant trend for a similar additive effect of LEET in that the group that received both bright light and LEET slept longer than those receiving only bright light, and those who received only LEET slept longer than those who received no intervention.

Most performance tasks showed some evidence of improvement with the bright light intervention. The results were most consistent with phase shifting rather than direct alerting effects of light. Measures involving accuracy more often affected than those involving only speed. There was no consistent evidence of any performance effects of LEET on performance, either beneficial (as might have been expected secondary to improved sleep) or detrimental (as might have been expected if LEET had 'hangover' effects as are seen with pharmacological sleep agents).

Bright light promoted adjustment of the 6-SM rhythm to the new work rest schedule. There was no effect on temperature. It is thought that masking related to activity level may have obscured the temperature data. LEET did not affect either 6-SM or temperature.

Fiscal Data (SK)

FY92	FY93	FY94	FY95	FY96
69.1	106.0	108.0	0.0	0.0

Publications

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Presentations Abstracts

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Title **CULTURING PLASMODIUM SP. SPOROZOITES *IN VITRO***

Principal Investigator Eileen D. Franke, LCDR, MSC, USN

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Objective Malaria is a major threat to deployed troops in endemic areas. Currently available methods of chemoprophylaxis are rapidly being undermined by the widespread development of drug resistant malaria. Immunization with irradiated sporozoites provides complete protection against malaria challenge, but because of the inability to culture malaria sporozoites in vitro it has been entirely unfeasible to consider large scale production of sporozoites for use in vaccines. Our goal is to develop a method for culturing infective malaria sporozoites in vitro; sporozoites that can be used to successfully immunize against malaria.

Approach We will produce *Plasmodium yoelli* ookinetes using methods established for other *Plasmodium* species. These ookinetes will be cultured with *Drosophila melanogaster* line 2 (L2) cells in ookinete culture medium on solidified Matrigel for 14-21 days and evaluated for development of oocysts with sporozoites. The sporozoites will be tested for infectivity, immunogenicity and capacity to induce protection. If the sporozoites are non-infectious, mosquito salivary gland preparations will be tested for their capacity to provide the signal required to convert them to the infectious form. If capable of inducing protective immunity, a similar system will be developed for the human parasite, *P. falciparum*, and transitioned to advanced product development.

Results Ookinetes and oocysts of *Plasmodium yoelii* have been produced in vitro from gametocytes obtained from infected mouse blood. These oocysts express the circumsporozoite protein (CS) as shown by immunofluoresence assay (IFA) using anti-CS specific monoclonal antibodies. This accomplishment is significant because ookinetes and oocysts are consecutive stages preceding the sporozoite stage in the *Plasmodium* sporogonic cycle; the cytoplasm and nuclei of the oocysts divide to form the elongated sporozoites. The finding that the oocysts produced in vitro express CS protein is significant in that this is also observed in oocysts developing in mosquitoes.

In order to obtain a higher rate of ookinete development from zygotes, several modifications of the culture conditions were investigated, including centrifugation of cultures, increasing the concentration of wheat germ agglutinin, and removing red blood cells from the mixture after exflagellation by gradient centrifugation on Lymphocyte Separation Medium. None of these methods increased the rate of ookinete development.

Oocysts usually do not survive or develop beyond four days in vitro. In vivo *P. yoelii* sporosoites form in the oocysts 9-14 days after the bloodmeal. Modifications to the culture medium were tested in an effort to increase the survival and development of oocysts; these included: adding conditioned medium

prepared from four-day cultures of *Drosophila* L2 cells, removing red blood cells from the culture, and adding other defined insect cell culture media (Schneider's Drosophila Medium and M3 Medium) in 1:1 (v/v) ratios to the basic oocyst culture medium. None of these modifications resulted in measurable improvement in oocyst survival or development.

These data, are important in that they help us to understand the factors involved (or not involved) in ookinete transformation and oocyst development, both of which are critical for sporozoite development.

Fiscal Data (SK)

FY92	FY93	FY94	FY95	FY96
0.0	118.0	123.0	126.0	0.0

Title

CHARACTERIZATION OF THE ROLE OF COSTIMULATORY MOLECULES IN ORGAN TRANSPLANTATION AND IN SEPTICEMIA

Principal Investigator

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Objective

The primary factor limiting the widespread adoption of cadaveric organ transplantation for the treatment of battle casualties remains graft rejection. Another major cause of morbidity and mortality after combat injury is septic shock. These undesired immune responses are cause by graft expressed allo-antigens or exotoxin stimulating T-cell activation. We have been exploring a novel therapeutic approach to inhibit, in an antigen specific way, the T-cell activation underlying the pathological conditions.

Approach

T-cell activation and functional differentiation requires that the cells be costimulated through the CD28 T-cell receptor. We have used several reagents to block the interaction of CD28 with its ligand B7 to determine if this will prevent skin graft rejection, the graft versus host reaction and septic shock induced mortality of mice.

Results

We have produced and confirmed the functional specificity of all the reagents required for this study (antibodies and fusion proteins). In addition, a mouse colony genetically lacking the CD28 receptor has been established. Using these materials, we have shown that CD28 plays a critical role in T-cell alloantigen and bacterial superantigen recognition. Further, that blocking the CD28 and B7 interaction strongly inhibits T-cell activation following alloantigen or bacterial exotoxin stimulation in vitro. These data have laid the groundwork for in vivo testing, presently being pursued. We have for example established several mouse models for testing including an allogeneic skin graft model, graft versus host reaction models following bone marrow transplantation, and lethal septic shock.

Fiscal Data ($K)

FY92	FY93	FY94	FY95	FY96
0.0	0.0	118.0	114.0	96.0

Publications

Ishida Y, Chused TM, Murakami S, Abe R. Antigen-Specific cell conjugate formation and long-lasting calcium responses in recognition of M1s Cellular Superantigen by Cloned Murine T-Lymphocytes. Cellular Immunology. 1994. 155: 414-427.

Presentations and Abstracts

Abe R. Preclinical Studies Costimulatory Pathway Research Progress Report. Repligen. Corp. Report. 1994.

Abe, M.D., Ph.D. R. Distinct Signal Transduction in Mouse CD4+ and CD8+ Splenic T Cells After CD28 Ligation. Branch Seminar Experimental Immunology

Branch, National Cancer Institute. 1994.

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Title

ANTIMICROBIAL ACTIVITIES OF POLYMORPHONUCLEAR GRANULE COMPONENTS IN HUMAN PERIODONTAL DISEASES

Principal Investigator

Donald W. Turner, D.D.S., Ph.D.

Naval Dental Research Institute

Objective

To study the microbicidal effects of granule factors located in polymorphonuclear neutrophils on certain putative periodontal pathogenic bacteria. To determine if differences exist between activities of these factors in PMNs isolated from health subjects compared to subjects with clinically diagnosed periodontal disease.

Approach

Neutrophils, isolated from peripheral blood of healthy subjects with diagnosed specific periodontal pathology will be challenged with Treponema denticola (Td) and Porphyromonas gingivalis (Pg). The bacteria will be opsanized with autologous or heterologous serum before challenge. It is anticipated that supernatant from PMNs of diseased subjects will be deficient or void in microbicidal activity. Further, differences chromatographic profiles, electrophoretic properties and enzymatic activities of fractioned supernatant will be examined.

Results

In milestone 1, neutrophil granule factors from healthy subjects and commercial sources was tested with putative periopathogen cultures.

Blood samples from healthy control and periodontally diseased volunteers have been obtained. Neutrophils, their granule components and monocytes have been isolated. Supernatant from monocyte cultures have been prepared for use in neutrophil stimulation studies.

A method for identifying live and dead Treponemas has been developed using two fluorescent stains. The stains will be used to determine percentage of killed Treponemas with commercial and isolated neutrophil granule components.

In milestone 2, neutrophil granule factors from healthy and diseased subjects are being examined for enzyme and biochemical characteristics along with commercial PMN enzymes. The pharmacia high performance liquid chromatography "SMART" system is currently being used to fractionate and isolate possible neutrophil stimulatory factors from monocyte cultures and isolates of possible neutrophil granule microbicidal factors.

Fiscal Data ($K)

FY92	FY93	FY94	FY95	FY96
0.0	0.0	99.0	108.0	115.0

Presentations and Abstracts

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Pederson ED, Turner DW, Merritt C, Glynn CT and Simonson LG. Aerobic Cultivation of Porphyromonas gingivalis. J. Dent. Res. 74: Abstract #918. 1995.

Turner DW, Pederson ED, Glynn CT, Merritt C, and Simonson LG. Fluorescence Stain for Estimating Treponema denticola Viability. J. Dent. Res. 74: Abstract #1902, 1995.

Publications

Pederson ED, Turner DW, Merritt C, Glynn CT, Simonson LG. Reducing Medium for Cultivation of Anaerobic Porphyromanas gingivalis Under Aerobic Conditions. Oral Microbiology and Immunology. Submitted.

Title **VALIDATION OF A COMPUTER-BASED PSYCHOMOTOR TEST (CBPT) FOR THE SELECTION OF FEMALE NAVAL AVIATORS**

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Objective

The Navy needs to determine the efficacy of enhanced computer-based performance test batteries (CBPTs) in the selection of female naval aviation candidates. As women are considered for new and more challenging roles in military aviation, evidence of abilities and skills relevant to gender will be needed to ensure accurate and fair selection procedures. A gender-referenced data-base for the proposed Naval CBPT may improve such decisions. Such information will also provide information about the performance of women that can be used in the evaluation and planning of women in Aircrew Cockpit Management (ACM) training. Specifically, understanding more about the strengths or weaknesses of various groups of individuals in certain tasks can be used to focus cockpit training to target crew coordination more effectively.

The current naval aviation selection system is based entirely on the paper-and-pencil Aviation Selection Test Battery (ASTB), a revision of the Aviation Qualification Test/Flight Aptitude Rating (AQT/FAR). The ASTB has demonstrated acceptable predictive validity in the selection of naval aviators. However, recent research at the Naval Aerospace Medical Research Laboratory (NAMRL) has found that the paper-and-pencil selection system based on the AQT/FAR could be significantly improved with the addition of computer-based performance tests (CBPT). A variety of U.S. Navy (USN), U.S. Army (USA), and U.S. Air Force (USAF) research has demonstrated that computer-based tests can increase the accuracy of decisions regarding aviator training success. CBPTs are extremely sensitive to individual differences in performance.

Approach

The proposed research will be accomplished using the existing NAMRL CBPT. The software for this system is compatible with the USAF BAT micro 386 system. A proposed Navy acquisition of twenty-five USAF BATs was not funded. Acquisition of the USAF BAT micro 386 system will be pursued on a smaller scale so that the research could proceed on the BAT simultaneously. Use of the BAT with both USAF BAT and NAMRL CBPT software would allow direct comparison of two selection systems with the same sample of female naval aviators. Data collection with the NAMRL CBPT would begin immediately,

while data collection with the BAT would begin when, and if, the BAT hardware is acquired and implemented at the Naval Aviation Schools Command (NASC) and NAMRL in Pensacola, Florida.

The sample would consist of at least 75 women enrolled in naval aviation training or already designated as naval aviators. This sample size would be sufficient for multivariate trend analysis. Testing with the NAMRL CBPT would take place primarily at the NASC and NAMRL in Pensacola, Florida. Additional testing would be accomplished at NAS Whiting, NAS Meridian and NAS Corpus Christi according to sample needs. The duration of the planning and data-collection phase would be for one year. Data analyses, collection of related flight training performance information and final report generation would require one additional year. Appropriate multivariate statistical transformations and analyses will be conducted to determine performance differences and predictive validity of the CBPTs when performance training criteria are available.

Results

Operability and reliability testing of the test software and hardware with subjects has been completed at VP-30 in Jacksonville, FL. Forty subjects were tested; eight of which were female aviators. This data has not yet been analyzed.

Milestone slippage occurred due to software development problems and personnel turnover. Performance differences between male and female aviators or aviation candidates will be collected, analyzed and reported, but the predictive validity of the NAMRL CBPT with regard to female aviation candidates cannot be assessed by the end of this FY due to the length of time required to obtain related flight training performance information.

Fiscal Data ($K)

FY92	FY93	FY94	FY95	FY96
0.0	0.0	82.0	59.0	0.0

Title

EFFECTS OF AVERMECTINS (MECTIZAN') ON ACQUISITION AND TRANSMISSION OF SPECIFIC ARTHROPOD-BORNE RICKETTSIAL, SPIROCHETAL AND ARBOVIRAL PATHOGENS (PHASE I: TICKS)

Principal Investigator

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Objective

The general purpose of this study is to determine the effectiveness of avermectins as prophylactic treatment against specific tick-borne disease pathogens via the impedance of tick vector feeding, stadial progression, fecundity and transovarial infection of offspring.

Specifically, this study is broken down into four major objectives, the first being to determine if currently approved subcutaneously injected dosages/formulations of avermectins will effectively interrupt pathogen acquisition by ticks from treated, infective mammalian hosts.

The second objective for this study is to determine if currently approved dosages/formulations of avermectins will effectively interrupt transmission of arthropod-borne pathogens from infective ticks to treated mammalian hosts.

The third objective is to determine the effects of avermectins, administered subcutaneously to the mammalian host, on various aspects of tick biology, including tick feeding rate and period, morphological development of specific internal organs while feeding, stadial progression and fecundity (Oviposition rate, egg character and egg hatch).

The fourth and final objective is to determine the presence or absence of specific pathogens transmitted transovarially to the tick larvae and whether avermectins effectively interrupt this transmission.

Ivermectin has been reported to be extremely efficacious at approved mammalian dosages against most hematophagous ectoparasites and has been shown to effectively reduce the human disease threat in specific human communities. The results of this study will be used as preliminary data in the development of dosage regimes and application methodology for thick-borne disease prevention among deployed military personnel.

Approach

Preliminary studies will be conducted to determine the time of maximum bacteremia/viremia for these selected pathogens in the New Zealand White rabbits. Pathogens selected for use in this study and the corresponding tick vector species include:

1. Quaranfil fever virus and *Hyalomma dromedarii*
2. *Borelia burgdorferi* and *Amblyomma americanum*, and
3. *Rickettsia conori* and *Rhipicephalus sanguineous*.

Two rabbits will be inoculated (infected) with each of the proposed pathogens and monitored for 14d. Serology samples will be collected from the rabbits at daily post-infection for determination of the circulating bacteremia/viremia/rickettsemia in the host animals. These data will be used to establish the optimum tick infestation date(s), post-infection.

For each pathogen-acquisition/transmission stage within each phase of this study, four rabbits will be inoculated (or otherwise infected) with a specific pathogen. Two of the four rabbits will be randomly selected to receive ivermectin treatment. Immediately prior to being infected with the select pathogen, the two rabbits selected to receive ivermectin treatment will be subcutaneously injected with ivermectin at a dosage of 0.2 mg/kg BW. Additionally, serum sample aliquots from ivermectin-treated rabbits will be assayed to determine ivermectin blood levels. Arthropod vectors (i.e., flat nymphs) will be exposed to the infective rabbits at specific dates post-infection as determined by specific pathogen incubation periods. Nymphal ticks will be allowed to bloodfeed on the infective rabbits until repletion, after which they will be allowed to complete ecdysis under standard tick rearing procedures/conditions. The resultant flat adults will then be used to reinfest a separate group of clean (specific pathogen-free) rabbits. Two clean, ivermectin treated, and two clean, untreated rabbits will be used for pathogen transmission attempts from adult ticks fed on each of the infective rabbits previously (4 X 2 = 8 rabbits). A random sampling of replete vectors will be screened for pathogen detection following each arthropod feeding stage. Surviving female arthropods will be maintained for fecundity observations, after which their offspring will be screened for pathogen presence.

Results

Baseline experiments to determine "control" information (i.e., untreated/uninfected physiology, biology and histology) for both *Rhipicephalus sanguineous* and *Hyalomma dromedarii* have been performed to evaluate the following specific parameters when these tick species are fed on New Zealand white rabbits:

- Attachment (feeding) period of nymphs
- Premolting period of nymph/adult transition stage
- Attachment (feeding) period of adult females
- Preoviposition (gestation) period of females; postfeeding
- Egg-mass/egg-weight determinations; postoviposition
- Sperm production (morphology and volume) by males , and morphology in female spermathecae

Infection and monitoring of "clean" rabbits with Quaranfil fever virus and Borrelia burgdorferi for 14-day observation period to determine peak viremia was completed. Gross clinical symptomology of infection included "droopy ears" and increased irritability, however increased rectal temperatures were not noted.

Colonies of *Amblyomma americanum Rhipicephalus sanguineous* and *Hyalomma dromedarii* have been enhanced and increased to sufficient maintenance levels to provide adequate numbers for all phases of the study.

All pathogen cultures/isolates necessary for completion of the project (i.e., *Borrelia burgdorferi, Rickettsia conorii*, and Quaranfil virus) are being maintained at this laboratory pending use.

Fiscal Data (SK)

FY92	FY93	FY94	FY95	FY96
0.0	0.0	76.0	66.0	0.0

Publications

Presley SM, Abbassy M, Ashmawy Z, Mohammed F and Osman M. Occurrence of Sperm in Female Spermatheca of *Hyalomma dromedarii* (Koch) and *Rhipicephalus sanguineous* (latreille) (ACARI:Ixodidae). J. Med. Entomol. 1995. (In Revision).

Title

IMMUNITY TO MALARIA INFECTION AND T CELL REACTIVITY[3] TO THE CIRCUMSPOROZOITE PROTEIN

Principal Investigator

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Objective

Malaria is a major threat to Naval/Marine Corps forces in tropical areas. The rapid development of drug resistance has stimulated research in malaria vaccines. However, the components of the immune system which can effectively control the malaria parasite have not been determined, and until this is done vaccine development will be difficult. This project will study naturally immune volunteers living in Kenya, for evidence that their T cell response to the malaria Circumsporozoite (CS) protein is contributing to their immunity. The results of this research will help direct malaria vaccine development based on the CS antigen.

Specific goals of the project are:

- To identify naturally protected and susceptible subjects in Kenya
- To determine the patterns of in vitro T cell reactivity to the CS protein.
- To determine the amino acid sequence of the CS protein in each infected person looking for variants.
- To determine the T cell reactivity to any CS protein variants, as evidence of immune evasion.

If T cell reactivity to the CS protein is found to correlate with natural protection, this will be a benchmark for malaria vaccine efforts. If infections are due to parasites with variant CS molecules, this will define a specific challenge for vaccine makers to overcome.

Approach

Persons living in Western Kenya, a highly endemic area for falciparum malaria, will first be treated with anti-malarial drugs, and then be followed for the occurrence of new malaria infections. As these new malaria infections are discovered, parasites will be harvested for DNA analysis of their gene coding the circumsporozoite (CS) protein. Peripheral blood lymphocytes will be donated by these study volunteers for analysis of in vitro T lymphocyte reactivity to the CS protein.

Patterns of T cell reactivity will be compared between persons not getting malaria and persons quickly infected. Also, for each infected person the CS protein variant will be determined, and that person's T cell reactions analyzed for evidence of escape mutants. This should give a good picture of the importance of T cell immunity to the CS protein in protecting naturally exposed persons.

Results

A red blood cell enzyme defect, pyruvate kinase deficiency, was found to correlated strongly with time to malaria reinfection. Persons with high levels of PK got malaria faster. This is the first demonstration of a role for PK in malaria.

Persons getting malaria later had T cells which produced more IL-4 and less INF-gamma in response to the TH3R epitope of the malaria CS protein. Thus, not all types of T cell responses to the CS protein are associated with protection. This has importance for malaria vaccine development.

Naturally occurring amino acid changes in the CS Th3R epitope profoundly affect recognition by human T cells. This again will have important implications for vaccine development.

Fiscal Data ($K)

FY92	FY93	FY94	FY95	FY96
0.0	96.0	90.0	87.0	0.0

Publications

Njunge L, Oppolo M, Orago AS and Weiss W. Resistance to Infection by P. Falciparum: An IL-4 Response to the Circumsporozoite Protein by CD4+ Cells Correlates with Protection, While an Interferon-Gamma Response by CD8+ Cells Does Not. Journal of Immunology. Submitted.

Werimo K, Mulayi N, Chek S, Martin S and Weiss W. Pyridoxyl Kinase Deficiency: A New Red Cell Enzyme Defect Which is Associated with Resistance to Falciparum Malaria. American Journal of Tropical Medicine and Hygiene. 1994. Submitted.

Ohas E, Ogola J, Udahaykumar V, Lal A and Weiss W. Naturally Occurring Amino Acid Substitutions in the P. Falciparum Circumsporozoite Protein TH3R Epitope Determine the T. Proliferative Response in Kenyan Volunteers. Journal of Immunology. 1994. Submitted.

Presentations

Njunge L, Oppolo M, Orago AS and Weiss W. Protective Responses in Plasmodium Falciparum Malaria: Interferon-gamma and Interleukin-4 Production by CD4+ and CD8+ T Cells from individuals Resistant and Those Susceptible to Reinfection with P. Falciparum. American Society of Tropical Medicine and Hygiene Meeting. 1994

Werimo K, Mulayi N, Chek S, Martin S and Weiss W. Red Cell Pyridoxyl Kinase Deficiency is Important in Protecting Kenyans from Reinfection by Falciparum Malaria. American Society of Tropical Medicine and Hygiene Meeting. 1994.

Ohas E, Ogola J, Udhaykumar V, Lal A and Weiss W. Control of Proliferative T Cell Responses to P. Falciparum Circumsporozoite Protein Epitope TH3R: The Importance of Naturally Occurring Amino Acid Variation. American Society of Tropical Medicine and Hygiene Meeting. 1994.

Maklobongo M, Oloo A, Orago AS and Weiss W. Lymphocyte Subsets During Symptomatic and Asymptomatic Infections with P. Falciparum. American Society of Tropical Medicine and Hygiene Meeting. 1994.

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and CD8+ T Cells from Individuals Resistant and Those Susceptible to Reinfection with P. Falciparum. 15th African Health Sciences Congress. 1994.

Copeland RS, Onyango RK, Oyaro C, Omondi M, Asago C, Kamanza K and Weiss W. Variation in Malaria Challenge Over Short Distances in a Holoendemic Area in Western Kenya: Implications for Vaccine Trials and Immunology Studies. 15th African Health Sciences Congress. 1994.

Title **IDENTIFICATION AND ANALYSIS OF HUMAN RECEPTORS FOR DENGUE VIRUS**

Principal Investigator

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Co-Investigators

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Objective

The virus host cell receptor interaction is the key first step in the infection process and could be used as a target for antiviral therapy. Most viruses initiate infection by attaching to target cell surface proteins prior to entry and replication. The objectives of this study were to:

a. identify the cell surface receptors to which dengue virus attaches to gain entry into the cell;
b. analyze in detail the nature and specificity of virus receptor interactions.

The aim of this work was to identify dengue virus receptors on permissive tissue culture cells that included the human monocytic cell lines U937 and K562. Since dengue virus readily infects mosquito cells, the mosquito cell line C6/36 was also included. It has been postulated that the envelope protein (E) and the membrane glycoprotein (M) of dengue may be involved in the attachment of the virus to the cell surface receptor. Based on this postulate, purified E and M proteins were to be used to define the dengue-specific cell surface receptor. If the cell surface receptor was determined to be an unknown protein, DNA clones containing the cell receptor gene were to be established using cDNA libraries. The interactions between the virus attachment protein and the cell surface receptor were to be studied by utilizing mutant forms of the cloned attachment protein genes to define the specific protein domains that are essential for this process.

Approach

Intact dengue type 2 (New Guinea C strain) virions were used in the study. Crude membrane preparations were made from both U937 and K562 cells. The membrane proteins were resolved on a SDS-polyacrylamide gel and transferred onto nitrocellulose membranes. The nitrocellulose membranes were reacted with dengue virus followed by incubation with anti-dengue antibodies. A second

incubation was then performed with phosphatase-labeled cell lysate subsequently made. The 35S- labeled proteins were separated into two fractions: one that binds the immobilized virus and one that does not. These fractions were then analyzed by SDS-PAGE.

Results

This project was aimed at the identification of human cell receptors for dengue virus. Although human cell lines U937 and K562 were originally intended to be used, other dengue virus permissive cell lines were also used. These included LLCMK/2, and C6/36 cells. Significant progress was not made because of the failure to identify a specific cellular protein as the receptor. A variety of different techniques were employed without much success. The techniques and approaches used are summarized as follows.

Using crude U937 and K562 membrane preparations immobilized on nitrocellulose, a protein of about 40 kD appeared to specifically interact with the virus. Both cell lines produced identical results. There also appeared a series of non-specifically interacting proteins. This was expected because these cells carry Fc receptors on their surface which will interact with the antibody used in the assay irrespective of virus binding. Repetition of the assay revealed that whereas the 40 kD band was difficult to reproduce, some low molecular weight bands, in the range of 12-18 kD, consistently appeared. To reduce background, the membrane proteins were pre-adsorbed to IgG-agarose. This did not lead to any significant reduction in background levels. Also, the pattern of bands was different from what was previously seen. These experiments were repeated using LLCMK/2 cells as well as 125I-labeled protein A for detection instead of phosphatase-labeled antibody. A similar Western blot type analysis was done using 35S-labeled virus for direct detection, obviating the need for antibody. Despite repeated attempts, the results were inconsistent.

Dengue virus immobilized on a solid matrix was reacted with a U937 cell lysate preparation containing 35S-labeled proteins. In the protein fraction that bound to the dengue virus matrix, a 30 kD protein was specifically enriched.

In other experiments, membrane fractions from surface-iodinated U937 cells were reacted with virus followed by reaction with anti-dengue antibody and protein A-sepharose. With this approach no specific protein band was observed, indicating that the 30 kD protein, identified previously, was no the receptor protein.

The negative results obtained with the above experiments could have been due to the denaturation and/or destruction of the putative receptor during the processing of the samples. To correct for this possibility, chemical cross-linking experiments were performed using three different bifunctional reagents; disuccinimidyl suberate, dithiobis (succinimidyl propionate and ethylene glycol-bis-(succinimidyl succinate). Treating with these cross-linking agents should allow protein structure and conformation to remain intact. The experiments were performed with either 35S-labeled virus or 125I-labeled cells. The results were inconclusive.

Fiscal Data ($K)

FY92	FY93	FY94	FY95	FY96
0.0	96.0	85.0	0.0	0.0