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EFFECT OF SPACE FLIGHT ON INTERFERON PRODUCTION - MECHANISTIC STUDIES

Gerald Sonnenfeld, Ph.D. - Principal Investigator*

Department of Microbiology and Immunology

School of Medicine

University of Louisville

Louisville, KY 40292

*The NASA Technical Monitor for this grant is:

Dr. Gerald R. Taylor

Mail Code SD-5

NASA Johnson Space Center

Houston, TX 77058

INTRODUCTION

We are completing a study to determine the effects of suspension of rats in an antiorthostatic, hypodynamic, hypokinetic model [1]. This model simulates some aspects of weightlessness [1]. While not a perfect model, suspension studies can yield useful information for planning of experiments for studies on astronauts actually undergoing space flight. Stress factors in suspension can, in part, be controlled for by suspending rats in an orthostatic position that does not result in effects similar to those of weightlessness [1]. Preliminary studies we have carried out indicated that antiorthostatic suspension of rats and mice resulted in a transient inhibition of interferon-alpha/beta production [2,3]. An additional study indicated that such suspension of mice resulted in decreased resistance to encephalomyocarditis-D virus, which correlated with the decreased interferon production [4]. The current grant extended these studies in the following fashion:

1) Effects of suspension on interferon-gamma production,

2) Effects of suspension on interleukin-1 and interleukin-2 production,

3) Effects of suspension on distribution of leukocyte sub-populations,

4) Role of changes in calcium levels in initiating these effects, in an attempt to determine the nature of the interactions of the immune system with other physiological systems when suspension and space flight effects are induced.

METHODS, RESULTS AND DISCUSSION

The work that has been performed as part of this research grant has involved the study of ground-based models for the effects of space flight on immune responses. The model for which most time was spent was the antiorthostatic, hypokinetic, hypodynamic suspension model for rats. From our results, it appears that suspension is useful for modeling the effects of space flight on functional immune responses, such as interferon and interleukin production. It does not appear to be useful for modeling shifts in leukocyte sub-populations. Calcium and 1,25-dihydroxyvitamin D_3 appear to play a pivitol role in regulating shifts in immune responses due to suspension. The macrophage appears to be an important target cell for the effects of suspension on immune responses.

Other studies were carried out to determine the role of various immunological parameters in resistance to infection. Since we have shown that these parameters can be altered after space flight and in the suspension modeling, it was important to determine the role of these immune responses in resistance to infection, which could have impact in the design of future flight and suspension studies to determine if the space-flight-induced cganges in immune response actually alter resistance to infection. We shoewed that both the classII histocompatibility antigens and the cytokines, which can be altered by space flight and/or by suspension, played major roles in resistance to infection with microorganisms.

In addition, we have been carrying out collaborative studies with Drs. Felix Gmunder and Augusto Cogoli to determine the role of the substratum in regulating the effects of microgravity on lymphocyte activation. The results of this study indicate that the interaction of lymphocytes with surfaces plays a central role in regulating lymphocyte activation.

In summary, we have successfully developed models for _in vivo_ effects of space flight on rat functional immune responses, and an _in vitro_ model for functional human immune

responses.

The details of this work are described in papers that have been published as a result of the work performed under this grant. The papers, crediting the support from this grant, are appended to this report. In addition, we expect to publish additional manuscripts based on this work in the future. These manuscripts will be forwarded to you as soon as they are ready.

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Livingston, D.H., Appel, S.H., Welhausen, S.R., Sonnenfeld, G., and Polk, H.C., Jr. Depressed interferon gamma production and monocyte HLA-DR expression after severe injury. **Arch. Surgery**, 123:1309, 1988.

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Depressed Interferon Gamma Production and Monocyte HLA-DR Expression After Severe Injury

David H. Livingston, MD; Sarah H. Appel; Samuel R. Wellhausen, PhD;
Gerald Sonnenfeld, PhD; Hiram C. Polk, Jr, MD

• **Monocyte HLA-DR antigen expression and mitogen-stimulated interferon gamma production were measured sequentially on days 1, 3, 7, 14, and 21 after admission in 20 multiply injured patients (mean Injury Severity Score, 33). Ten patients recovered uneventfully and ten developed a major infection, three of whom died. Trauma resulted in immediate and profound depression of both interferon gamma production and monocyte HLA-DR antigen expression compared with controls. Interferon gamma remained below control levels for all days on which it was measured in all patients. In uninfected patients, interferon gamma production began to recover after day 7 and interferon gamma levels on day 21 were greater than on all other days. Monocyte HLA-DR antigen expression returned to normal between days 7 and 14 in uninfected patients, despite subnormal production of interferon gamma. Failure to increase interferon gamma production and monocyte HLA-DR antigen expression was associated with an episode of major infection. We postulate that stimulation of the immune system early after injury may reverse the defects reported and decrease the incidence of infection after severe trauma.**

(Arch Surg 1988;123:1309-1312)

The recognition that the severely injured patient has an increased susceptibility to infection has led investigators to examine the phenomenon of posttraumatic immune depression as a potential cause.[1-4] Monocytes are involved in multiple facets of cell-mediated immunity, including antigen presentation, phagocytosis, killing of microorganisms, cytokine production, and the induction of cytotoxic effector cells.[5,6] The ability to endocytose and process antigens is an early and critical step in initiating an immune response.[7] Expression of class II major histocompatibility complex (MHC) antigens (HLA-DR and related antigens) are thought to be required for monocytes to present an antigen to lymphocytes and act as accessory cells.[8-11]

Over 80% of circulating peripheral blood monocytes constitutively express HLA-DR antigen on their cell surface.[2,8] The sequential determination of the percent of monocytes that express HLA-DR antigen has been demonstrated to be a useful marker in following the immuno-

logic recovery of patients after injury.[2] Three distinct patterns of monocyte HLA-DR antigen expression were seen after severe trauma that separated patients who recovered uneventfully from patients who developed an episode of major sepsis or died.[2] Modulation of HLA-DR antigen expression is regulated, in part, by interferon gamma and, therefore, differences in monocyte HLA-DR antigen expression observed in the peripheral blood monocytes of injured patients may represent differences in the ability to synthesize interferon gamma.[12] Pure cultures of monocytes lose their HLA-DR expression after incubation but can be induced to reexpress this antigen by the addition of exogenous interferon gamma for T cells.[13,14] Trauma patients who have low monocyte HLA-DR antigen expression will also increase such expression when their monocytes are cultured in vitro with interferon gamma.[15]

Interferon gamma is a glycoprotein produced by activated lymphocytes in response to antigenic or mitogenic challenges and is a potent regulator of monocyte function.[16] Interferon gamma has been shown to enhance expression of MHC class I and class II antigens on monocytes; the production of the monokines interleukin 1 (IL-1), tumor necrosis factor, and granulocyte-monocyte colony-stimulating factor; and monocyte-phagocytosis and bactericidal capability.[17] Decreased interferon gamma production has been reported after thermal and nonthermal injury.[3,18]

In the present study, we investigated monocyte HLA-DR expression and the production of interferon gamma after admission to hospital in 20 multiply injured patients. Correlations between interferon gamma production and monocyte HLA-DR expression were examined, with specific attention given to the relationship between recovery of these factors and infectious morbidity.

PATIENTS AND METHODS
Patient Selection

Twenty multiply injured patients admitted to the University of Louisville Trauma Service from July to December 1987 were studied. There were 17 men and three women, with a mean age of 33 years (range, 16 to 74 years). Injuries included four gunshot wounds, four burns, and 12 blunt injuries (ten motor vehicle accidents, one fall, one airplane crash). The mean Injury Severity Score (ISS) was 33 (range, 16 to 59). Patients were studied serially on days 1, 3, 7, 14, and 21 after admission. All patients underwent operation either immediately or soon after admission. The mean number of operative procedures performed was 3.4 per patient (range, one to ten). Each patient's hospital course was observed for major infection, which was defined as bacteremia, intra-abdominal, or intrathoracic infections that required operative or percutaneous drainage, burn-wound infection, or pneumonia.

Burn-wound infection was defined by the presence of greater than 10^5 organisms per gram of tissue on quantitative culture in the presence of fever and leukocytosis, the histologic presence of organisms invading normal skin on punch biopsy, or progressive graft loss in the face of positive quantitative cultures. Pneumonia was defined by positive sputum cultures for pathogenic organisms, leukocytosis, fever, and the appearance of a new infiltrate on chest roentgenogram.

Twenty healthy volunteers served as control subjects for the determination of the normal range of interferon gamma production and monocyte HLA-DR antigen expression.

Four patients undergoing minor operations (two rhinoplasties, one chin augmentation, and one herniorrhaphy) were studied to assess the effect of general anesthesia in patients with minimal tissue trauma on HLA-DR antigen expression and interferon gamma production. Blood was drawn for these studies just prior to their operative procedure and 24 hours postoperatively.

Statistical analysis was done using a prior analysis of variance followed by a Dunnetts, Tukey, or Student t test. (Significance was set at $P<.05$.) This study was reviewed and approved by the University of Louisville Human Studies Committee.

Interferon Gamma Production

Mitogen-stimulated interferon gamma was produced in cultures of whole, unseparated blood, as described by Kirchner et al.[19] Venous blood was collected in tubes containing lithium heparin (Becton-Dickinson, Rutherford, NJ). The blood was diluted 1:10 with serum-free RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with HEPES buffer (6 mmol/L), tricene buffer (3 mmol/L), gentamicin sulfate (50 mg/L), and glutamine salt (2 mmol/L). One milliliter of the diluted blood was placed in plastic tissue-culture tubes with 5 mg/L of phytohemagglvtimim-P (Sigma, St Louis) and incubated at 37°C in 5% carbon dioxide for 24 hours. Following incubation, the supernatant was collected after centrifugation and frozen at −70°C until assayed. The interferon gamma level was measured in duplicate by a radioimmunoassay (Centocor Inc, Malvern, Pa). This assay is specific for interferon gamma alone and results are expressed in units of interferon gamma per milliliter, based on the National Institutes of Health (Bethesda, Md) reference standard for interferon gamma.

Monocyte HLA-DR Expression

HLA-DR expression on peripheral blood monocytes was determined using fluorochrome-labeled monoclonal antibodies and flow cytometry, as previously described.[2] Forty-microliter aliquots of buffy coat cells prepared from acid-citrate-dextrose–anticoagulated blood were stained for 20 minutes with the manufacturer's specified dilution of antibody in the presence of 0.1% sodium azide. Red blood cells were removed by hypotonic lysis and the samples were preserved in 1% paraformaldehyde solution. Dual staining was used in which monocytes were identified via the labeled monoclonal antibody MO2 (Coulter Immunology, Hialeah, Fla), and the HLA-DR antigen was identified via a second labeled monoclonal antibody (Becton-Dickinson, Sunnyvale, Calif).

Samples were analyzed on a cytofluorograph IIS flow cytometer (Ortho Diagnostic, Westwood, Calif) configured for simultaneous two-color (red and green) fluorescent analysis. The forward-angled and right-angled light-scattering properties of the leukocytes enabled differentiation between lymphocytes, monocytes, and neutrophils. The presence of HLA-DR was measured on monocytes previously identified. A minimum of 200 monocytes were analyzed for each sample. Results are expressed as the percent of monocytes staining positive for HLA-DR.

RESULTS

Half of the patients studied developed an episode of major infection. The sources of infection were the lungs in seven, burn wound in three, thoracic and abdominal cavities in two each, and major soft-tissue wounds in two. Six patients had bacteremia, with organisms recovered from another site of infection. Six of the ten patients had more than one source of sepsis. The mean intensive care unit and total hospital stays were 14 and 31 days, respectively. The mean ISS was significantly greater in the infected patients compared with the noninfected patients (43 vs 25; $P<.05$). Three patients died 30, 37, and 42 days, respectively, after injury secondary to ongoing sepsis and multiple-system organ failure.

The normal range for interferon gamma production was 28 ± 13 μm/mL. General anesthesia without significant tissue trauma was associated with minimal change in interferon gamma production and monocyte HLA-DR expression. The mean preoperative value for interferon gamma was 25 μm/mL compared with the mean postoperative value of 30 μm/mL.

Trauma had a profound and immediate effect on interferon gamma production. In all injured patients, interferon gamma levels remain below control levels for all days studied ($P<.05$) (Table). In noninfected patients, interferon gamma levels began to increase by day 14. Interferon gamma production was significantly greater on day 21 than on days 1, 3, 7, and 14 but remained well below that of the controls. In infected patients, interferon gamma levels were depressed markedly throughout the study period. Inclusion of the three patients who died with the infected patients did not significantly alter the pattern of interferon gamma production.

The normal range for monocyte HLA-DR expression was $89\%\pm4\%$ and is comparable with previously reported normal values.[2,7] Monocyte HLA-DR expression fell significantly after injury in both groups (Figure). Patients who made an uneventful recovery exhibited return of HLA-DR expression by day 14. In contrast, in patients who experienced an episode of major infection, HLA-DR expression never returned to normal. When the patients who died are excluded, HLA-DR expression in the patients with major infection increased only on day 21 compared with all infected patients. This is similar to previously described patterns of the recovery[2] of monocyte HLA-DR expression and indicates that inclusion of the three patients who died did not skew the results.

COMMENT

Monocytes express both class I (A, B, and C loci) and class II (D locus) major histocompatibility antigens. Human MHC class II antigens are composed of DP, DR, DQ, and DS subunits and are the equivalent of the immune-response antigens (IA and IE) in the murine system.[5] Expression of MHC class II antigens is a necessary condition for the monocyte to act as an accessory cell and present antigen, although which specific subgroup or groups of antigens are important remains unknown.[8-11] Gonwa et al[8] demonstrated that in a population of HLA-DR–positive monocytes, cells that expressed HLA-DS could present antigen and stimulate an autologous mixed lymphocyte reaction better than HLA-DS–negative cells. Expression of HLA-D antigens is a dynamic process, and each subgroup has its own normal constitutive level of expression. One study of freshly isolated human monocytes demonstrated that HLA-DR, HLA-DQ, and HLA-DP were expressed by 90%, 31%, and 19% of cells, respectively.[20] Since HLA-DR is expressed with a greater frequency than the other HLA-D antigens, down-regulation and recovery of monocyte function may be best assessed by changes in this antigen. The three patterns of HLA-DR antigen recovery seen after injury in this study demonstrate this hypothesis and explain why we chose to measure this antigen. Monocyte cultures will lose their HLA-DR antigen expression on incubation, which can be restored by the addition of interferon gamma.[13,14] Interferon gamma will also increase the expression of HLA-DQ and HLA-DP antigens.[20] Monocyte function, as measured

Interferon Gamma Production After Injury*					
Patient Groups	Interferon Gamma Production by Day After Injury				
	1	3	7	14	21
Noninfected	5.0 ± 1.1	3.4 ± 1.4	3.5 ± 0.7	7.2 ± 3.0†	16.1 ± 4.3‡
Infected (all patients)	2.1 ± 0.8	1.4 ± 0.4	2.1 ± 0.7	3.2 ± 1.0	3.9 ± 1.4
Infected (survivors only)	1.2 ± 0.3	0.9 ± 0.4	2.1 ± 1.2	3.3 ± 1.6	5.1 ± 2.7

*All values are mean ± SEM. Controls produced 27.6 ± 3.4 U/mL of interferon gamma.
†$P<.05$ vs infected patients.
‡$P<.05$ vs days 1, 3, 5, 7, and 14.
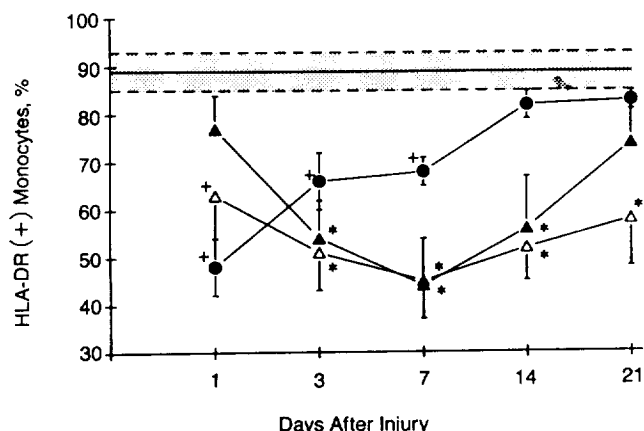
Days After Injury

Monocyte HLA-DR expression measured sequentially after injury in uninfected patients (circles), all infected patients (open triangles), infected patients exclusive of the three patients who died (solid triangles), and controls (shaded area). Values are mean of percent HLA-DR–positive monocytes (± SEM). Dagger indicates $P<.05$, infected patients vs controls; asterisk, $P<.05$, infected patients vs controls and uninfected patients.

by IL-1 production, declines simultaneously with HLA-DR expression after overnight culture and can also be restored by the addition of exogenous interferon gamma.[21] The close relationship between monocyte function, as measured by IL-1 production, and MHC class II expression, as measured by HLA-DR expression, is further evidence that cell-surface antigen expression is an important marker of the ability of monocytes to function as accessory cells. Although the correlation between monocyte MHC class II expression and the presentation of a class II restricted antigen is far from simple, it is clear that the lack of class II antigen expression indicates that the monocyte is probably impaired in its ability to act as an antigen-presenting cell.

Interferon gamma has numerous functions other than the regulation of cell-surface expression of MHC antigens.[16,17] Endogenous production of interferon gamma has been linked to protection from viral infection. Mice that are resistant to herpes simplex type I virus demonstrate elevated interferon gamma titers compared with susceptible mice.[22] The inability to produce interferon gamma may reflect an increased susceptibility to infection. Prophylactic administration of interferon gamma has been shown to decrease mortality from bacterial and protozoan infections.[23] Interferon gamma given even after bacterial contamination decreased the incidence of wound infection after hemorrhagic shock[24] and increased the survival from gram-negative infection after laparotomy.[25] The patients in this series who developed a major infection had consistently lower interferon gamma levels than those who made an uneventful recovery.

Both interferon gamma and monocyte HLA-DR expression were measured by assays using whole blood. Previous studies of monocyte MHC class II expression have used monocytes separated by adherence to glass or plastic.[8] These maneuvers select a subset of the monocyte population or cause activation of the cells resulting in changes of HLA-DR antigen expression.[26] The use of flow cytometric analysis allows for the study of freshly isolated monocytes that are subjected to minimal handling and the ability to count thousands of cells, which decrease any potential sampling errors. Although the use of whole blood makes it impossible to dissect the mechanism of interferon gamma depression after injury, we feel that this disadvantage is outweighed by the fact that the assays were done in a more physiologic situation. Serum from trauma patients has been suggested as a factor in immunosuppression[27] and, therefore, a whole-blood assay may be more clinically relevant.

It is unknown whether prolonged depression of interferon gamma production and HLA-DR antigen expression causes an increased susceptibility to infection or if secondary infection after injury results in sustained depression of the variables measured. Mean ISS, as previously reported, was higher in the group of patients who had prolonged immune depression and who developed a major infection.[3] Our data show that normal monocyte HLA-DR antigen expression returns between seven and ten days after injury in uninfected patients. Return to normal HLA-DR antigen expression was not predicated by the ability to produce normal levels of interferon gamma in culture, since the highest levels of interferon gamma produced in infected patients in this series were significantly lower than those of the controls. Small amounts of interferon gamma can increase HLA-DR antigen expression on monocytes, and "subnormal" levels of interferon gamma may be sufficient to return HLA-DR antigen levels to normal.[18] It is also recognized that regulation of monocyte HLA-DR antigen expression is not regulated by interferon gamma alone.[11] The failure to increase monocyte HLA-DR antigen expression to the normal range by day 14 was associated with an episode of major infection. Polk and associates[2] demonstrated that recovery of monocyte HLA-DR antigen expression in patients who made an uneventful recovery was significantly different from that in patients who developed major infection. Our study confirms that finding and demonstrates that the recovery of interferon gamma production also shows a difference between infected and noninfected patients. Increased production of interleukin 2[3] and colony-stimulating factor[27] have also been demonstrated to occur earlier in patients who experience an uneventful recovery after injury than in patients who develop infections.

In conclusion, we have demonstrated that severe injury results in an alteration of monocyte HLA-DR antigen expression and interferon gamma production. Rapid recov-

ery of monocyte HLA-DR antigen expression was associated with an uneventful recovery. Although interferon gamma production remained below control levels for all periods studied, uninfected patients showed a parallel recovery of interferon gamma production with monocyte HLA-DR antigen expression. The failure to increase interferon gamma production and monocyte HLA-DR antigen expression was associated with an episode of major sepsis or death. These data lend support to the concept that stimulation of the immune system early after injury, possibly with interferon gamma, may be beneficial and decrease the incidence of infection after severe trauma.

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Discussion

GEORGE W. MACHIEDO, MD, Newark, NJ: In light of the continued problems that we face in the clinical outcome of patients with sepsis, particularly when septic shock or multiple-system organ failure develops, it is necessary to investigate methods of prophylaxis as well as treatment. As we enter the last decade of the 20th century, it is evident that new antibiotics and improved organ-support systems will not provide the answers. It is also evident from the plethora of studies that have been presented at this meeting on the function and dysfunction of the monocyte-macrophage system that this cell line is assuming a central position in the problems of sepsis. It follows logically, then, that attempting to define these changes and modulate them is a promising mechanism for therapy.

This article is one of a series by Dr Livingston and his associates clarifying the changes that occur in interferon gamma levels and in the expression of the HLA-DR antigen on monocytes following trauma. They clearly showed an association between an impaired return to normal parameters and the development of subsequent infection; however, I think that the measure of that relationship still needs to be defined.

Dr Livingston commented that patients who were subsequently infected were more severely injured. Could he comment on the incidence or level of hypotension, since we have recently reported (*Ann Surg* 1988;207:549-554) a high incidence of early bacteremia within the first hour or two following trauma in patients who were admitted with blood pressures below 80 mm Hg? Why should he not be looking at the effect of this early bacteremia rather than a predicted cause? Has he looked at the data in a different way? In other words, has he looked at it not by outcome, as he presented, but by the actual interferon levels, with the percent of HLA-DR–positive cells to determine, particularly with the interferon, that there is a critical level above which no subsequent infection occurs?

KENNETH L. BRAYMAN, MD, Philadelphia: I would be interested in knowing whether there was a difference in blood transfusion or the initial septic insult in the patients who went on to suffer more severe degrees of sepsis vs those who got better and whether there was any particular value in relationship to HLA-DR expression of the monocytes.

We know that activated T cells are the ones thought to produce interferon gamma, which in turn stimulates the macrophage HLA-DR expression, and I was wondering whether you looked at HLA-DR expression on T cells relative to other data.

DR LIVINGSTON: I will answer the questions in reverse order. We did look at the impact of blood transfusions in this study and it appeared that the volume of blood transfusions really made no difference, ie, there was no difference among the groups. We do recognize that T cells do express HLA-DR antigens; however, their influence on interferon gamma production is not known at this time. It may be interesting to look at that in the future.

Dr Machiedo recognized the fact that perhaps the more severely injured patients did, in fact, get infected; however, the group as a whole was fairly injured. The levels of initial contamination from the outside were equal. We do recognize that bacterial translocation occurs most often in the more severely injured patients, and I really cannot comment about the possible theoretical basis of the increased levels of internal contamination.

The critical levels of endogenous interferon gamma production is unknown, and we did not try to answer that question, although it is interesting. How much interferon gamma is sufficient? It was interesting to us that HLA-DR expression returned to normal long before interferon gamma production was normal. It appeared that the body could definitely regulate these cell-surface antigens while producing very small amounts of interferon gamma, as observed in culture.

Depressed Interferon Gamma Production—Livingston et al

Effects of Interferon-γ and Tumor Necrosis Factor-α on Macrophage Enzyme Levels

SILVIA S. PIERANGELI and GERALD SONNENFELD

ABSTRACT

Murine peritoneal macrophages were treated with interferon-γ (IFN-γ) or tumor necrosis factor-α (TNF). Measurements of changes in acid phosphatase and β-glucuronidase levels were made as an indication of activation by cytokine treatment. IFN-γ or TNF-γ treatment resulted in a significant increase in the activities of both enzymes measured in the cell lysates. This increase was observable after 6 h of incubation, but reached its maximum level after 24 h of incubation. The effect of the treatment of the cells with both cytokines together was additive. No synergistic effect of addition of both cytokines on the enzyme levels was observed.

INTRODUCTION

INTERFERONS (IFNs) ARE NATURALLY OCCURRING PROTEINS whose originally recognized function was antiviral activity.[1] However, considerable evidence has accumulated for a broader biological role for IFNs. This includes: inhibition of proliferation of normal and malignant cells,[2,3] enhancement of numerous macrophage functions including phagocytosis[4] and nonspecific tumoricidal activity, augmentation of natural killer cell activity,[5] antiproliferative and immunomodulatory activities,[6,7] and inhibition of multiplication of nonviral intracellular parasites.[8-11]

Another cytokine of interest is tumor necrosis factor-α (TNF). TNF was recognized by Carswell et al.[12] in 1975. It is characterized by its ability to produce hemorrhagic necrosis of certain mouse transplanted sarcomas in vivo and has cytostatic/cytotoxic effects on mouse and human tumor cells in vitro.[13-16] In addition, sera containing TNF have been reported to inactivate certain species of malaria parasites in vitro and to protect mice against Klebsiella pneumoniae and Listeria monocytogenes.[17] Lately it has been demonstrated that TNF inhibits intracellular multiplication of Trypanosoma brucei in murine peritoneal macrophages.[18] TNF can also act as an immunomodulator because it increases the cytotoxic and phagocytic activities of polymorphonuclear cells.[19] TNF and IFN-γ have been shown to act in a synergistic fashion in some cases. A strong synergistic interac-

Department of Microbiology and Immunology and Department of Oral Health, Schools of Medicine and Dentistry, University of Louisville, Louisville, KY 40292.

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tion between both cytokines has also been found when their cytotoxic activity was measured on cervical, breast, and ovarian carcinoma cell lines.[20-24]

This study was designed to determine the effects of IFN-γ and TNF treatment on levels of activity of acid phosphatase and β-glucuronidase in murine peritoneal macrophages. These enzymes can serve as markers of macrophage activation.[25]

MATERIALS AND METHODS

Preparation of Mouse Peritoneal Macrophages: Four- to six-week-old C57Bl/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were maintained in an AAALAC-accredited facility according to NIH guidelines under the direct supervision of a veterinarian. The abdomen of each animal was thoroughly swabbed with alcohol and the skin deflected so as to expose fully the peritoneal wall. The peritoneum was lavaged by injection of 5 ml of sterile RPMI 1640 medium (GIBCO Labs, Grand Island, NY), supplemented with 10% heat-inactivated fetal bovine serum (GIBCO), 100 IU/ml penicillin, and 100 μg/ml streptomycin. After gentle abdominal massage, the peritoneal fluids were collected aseptically.[26] The cells were washed three times with medium for 10 min, and resuspended in the same medium. The nucleated cells were counted microscopically by using an hemacytometer and were adjusted to 10 viable cells/ml. Viability was assayed by the trypan blue dye exclusion test. Aliquots (0.8 ml) were placed in Lab-tek tissue culture chamber (Becton-Dickinson, Inc., Oxnard, CA) and incubated for 24 h at 37°C in 5% CO_2. Nonadherent cells were then removed by flushing the culture chambers vigorously with fresh medium several times.

Lysis of Macrophages: The cell monolayers were treated for 10 min with 0.8 ml of 0.1% Triton-X100 (Sigma Chemical Co., St. Louis, MO) in 0.05 M HCl and 0.15 M NaCl.[27]

Cytokines: Murine IFN-γ, produced by recombinant DNA technology, was a gift of Genentech, Inc. (South San Francisco, CA), and supplied at a specific activity of 0.68 × 10^7 U/mg of protein. A stock solution was made in culture medium containing 1,000 U/ml. Dilutions were made up immediately before use in the experiments (30, 100, and 1,000 U/ml). Human TNF, produced by recombinant DNA technology, was also kindly provided by Genentech, Inc. South San Francisco, CA) at a specific activity of 5.02 × 10^7 U/mg of protein). A stock solution of 10,000 U/ml was made in culture medium and kept in the refrigerator until use. Solutions of 30, 100, and 1,000 U/ml were prepared immediately before use. Both IFN-γ and TNF were assayed for endotoxin by Associates of Cape Cod, Inc. (Woods Hole, MA) and no inhibition was observed.

Enzymatic Assays: Acid phosphatase (E.C. 3.1.3.2.) and β-glucuronidase (E.C. 3.2.1.31) were assayed in lysates using commercially available kits from Sigma Chemical Co. (St. Louis, MO). Acid phosphatase was expressed in IU/liter and β-glucuronidase in modified U/ml.

General Assay Procedure: After 24 h of incubation in the plastic plates, nonadherent cells were removed by flushing the plates vigorously. At this point the cells were ready for use. The macrophages were then incubated for 6 or 24 h with medium or with cytokines in three different concentrations, either alone or together or with the supernatant from the absorption with specific antibody anti-IFN. After this incubation at 37°C 5% CO_2, the cells were washed twice with medium and once with sterile phosphate-buffered saline (PBS) and lysed as described previously. Lysates were frozen at −70°C until the enzymatic assays were done.

Neutralization of IFN-γ with Monoclonal Antibody against IFN-γ: Monoclonal antibody was obtained from the hybridoma cell line R4-6A2, kindly provided by Dr. Edward E. Havell (Trudeau Institute, Inc., Saranac Lake, NY).[28] Hybridoma cells were cultured in minimal essential me-

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dium (GIBCO Labs, Grand Island, NY), supplemented with 10% fetal bovine serum, antibiotics, glutamine, and 1 mM sodium pyruvate in a 7% CO_2 atmosphere. Supernatants from culture were concentrated to 15 mg/ml, determined by spectrophotometric readings. The antibody was dialyzed exhaustively in phosphate buffer (0.2 M, pH 7.6). The following standard immunoadsorbent procedure was used for inactivation of IFN-γ.[29] The antibody was polymerized with glutaraldehyde (Sigma Chemical Co., St. Louis, MO) in a proportion protein/glutaraldehyde of 1:5. After 24 h the gel was broken in a tissue homogeneizer and washed exhaustively. The broken gel was then incubated with continuous rotation for 24 h at 4°C with an equal volume of IFN-γ at a concentration of 30 U/ml. The mixture was centrifuged at 3,000 rpm for 10 min and the supernatant was kept at 4°C and then assayed for IFN-γ activity.[29]

Statistics: The results were subjected to Student's t test to determine whether or not there was a significant difference between the averages of enzymatic determinations with cytokines and the controls.[26] Alpha was set *a priori* at $p < 0.05$.

RESULTS

Effect of the treatment of mouse peritoneal macrophages with IFN-γ on the activities of β-glucuronidase and acid phosphatase in lysates

Mouse peritoneal macrophages were treated with different concentrations of IFN-γ (30, 100, and 1,000 U/ml), and after 24 h of incubation they were washed and treated with medium, according to procedure described in Materials and Methods. The enzymatic activities were measured in lysates (Table 1). When IFN-γ-treated cells were compared with medium-treated cells, there was an increase of 4.4-, 5.7-, and 7.1-fold in the activity of β-glucuronidase for 30, 100, and 1,000 U/ml, respectively. When acid phosphatase was measured, cells treated with IFN-γ showed an increase of 2.5-, 2.6-, and 2.8-fold over the cells treated only with medium.

Effect of the treatment of murine peritoneal macrophages with TNF on the activities of β-glucuronidase and acid phosphatase in cell lysates

In this case, cells were treated with three different concentrations of TNF, 30, 100, and 1,000 U/ml, as described in Materials and Methods. Enzymatic activities were determined in lysates (Table 2). When cells treated with TNF were compared with cells treated with medium, there was an enhancement of β-glucuronidase activity of 4.5-, 5.6-, and 5.4-fold for 30, 100, and 1,000 U/ml of TNF, respectively. TNF-treated cells showed an increase of 1.6-, 2.0-, and 1.5-fold increase in the activity of acid phosphatase, when compared with medium-treated cells, at the three different concentrations mentioned above.

TABLE 1. EFFECT OF THE TREATMENT OF MOUSE PERITONEAL MACROPHAGES WITH IFN-γ ON THE ACTIVITY OF β-GLUCURONIDASE AND ACID PHOSPHATASE[a]

Treatment	β-Glucuronidase activity (U/ml)	Acid phosphatase activity (IU/liter)
Medium	12.7 ± 2.2	0.70 ± 0.06
IFN-γ 30 U/ml	70.5 ± 5.6[b]	1.75 ± 0.10[b]
IFN-γ 100 U/ml	72.7 ± 5.2[b]	1.79 ± 0.11[b]
IFN-γ 1,000 U/ml	90.2 ± 2.7[b]	1.89 ± 0.03[b]

[a]Results are expressed as the mean of five experiments ± standard error of the mean.
[b]$p < 0.05$.

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TABLE 2. EFFECT OF TREATMENT OF MOUSE PERITONEAL MACROPHAGES WITH TNF ON THE ACTIVITY OF β-GLUCURONIDASE AND ACID PHOSPHATASE IN CELL LYSATES[a]

Treatment	β-Glucuronidase activity (U/ml)	Acid phosphatase activity (IU/liter)
Medium	15.75 ± 3.7	0.68 ± 0.16
TNF 30 U/ml	70.50 ± 1.5[b]	1.11 ± 0.19[b]
TNF 100 U/ml	88.50 ± 1.5[b]	1.37 ± 0.33[b]
TNF 1,000 U/ml	85.50 ± 1.6[b]	1.02 ± 0.02[b]

[a]Results are expressed as the mean of five experiments ± standard error of the mean.
[b]$p < 0.05$.

Effect of combined treatment of mouse peritoneal macrophages with IFN-γ and TNF on the activities of β-glucuronidase and acid phosphatase

Murine peritoneal macrophages were treated with medium or with a mixture of both cytokines at final concentrations of 30, 100, and 1,000 U/ml of each cytokine for 24 h. The enzymatic activities were determined in lysates (Table 3). Macrophages treated with both cytokines at the three different concentrations showed a pattern of increase in β-glucuronidase of 8.2-, 10.7-, and 13.0-fold. When acid phosphatase was measured in lysates, cells treated with both cytokines simultaneously, the enhancement was 4.1-, 4.2-, and 4.4-fold at concentrations of each cytokine of 30, 100, and 1,000 U/ml.

Effect of anti-IFN-γ on the enhancement of activities of β-glucuronidase and acid phosphatase induced by IFN-γ

IFN-γ was mixed with anti-IFN-γ, and then cells were treated with antibody-neutralized-IFN or with medium and/or TNF (30 U/ml) for 24 h. The enzymatic activities were analyzed in lysates (Table 4). In both experiments enhancement of β-glucuronidase and acid phosphatase by IFN-γ was abrogated totally by antibody treatment. Furthermore, cells treated with TNF retained the enzyme activation that was due to TNF. Effect of time of incubation of cytokines on the activity of β-glucuronidase in mouse peritoneal macrophages.

Macrophages were incubated with IFN-γ or TNF for 6 or 24 h, and then washed and lysed for enzymatic analysis. At 6 h, the activity of β-glucuronidase induced by IFN-γ was loser in all samples than in the corresponding sample at 24 h of incubation (Fig. 1). After 6 h of treatment with TNF the values were 80–90% of the ones obtained with 24 h of incubation (Fig. 1).

TABLE 3. EFFECT OF COMBINED TREATMENT OF MOUSE PERITONEAL MACROPHAGES WITH IFN-γ AND TNF ON THE ACTIVITY OF β-GLUCURONIDASE AND ACID PHOSPHATASE IN LYSATES[a]

Treatment	β-Glucuronidase activity (U/ml)	Acid phosphatase activity (IU/liter)
Medium	15.75 ± 3.8	0.68 ± 0.16
IFN 30 units + TNF 30 units	129.00 ± 30.0[b]	2.99 ± 0.43[b]
IFN 100 units + TNF 100 units	168.00 ± 22.5[b]	2.90 ± 0.20[b]
IFN 1,000 units + TNF 1,000 units	206.25 ± 36.6[b]	2.14 ± 0.36[b]

[a]Results are expressed as the mean of five experiments ± standard error of the mean.
[b]$p < 0.05$.

TABLE 4. EFFECT OF INACTIVATION OF IFN-γ BY TREATMENT WITH ANTI-IFN-γ ON THE ACTIVITY OF β-GLUCURONIDASE AND ACID PHOSPHATASE IN LYSATES OF MOUSE PERITONEAL MACROPHAGES[a]

Treatment	β-Glucuronidase activity (U/ml)	Acid phosphatase activity (IU/liter)
Medium	15.75 ± 3.7	0.84 ± 0.03
IFN-γ 30 units + anti-IFN-γ	12.75 ± 2.2	0.85 ± 0.09
IFN-γ 30 units + anti-IFN-γ + TNF 30 units	56.50 ± 6.5[b]	1.44 ± 0.30[b]

[a]Results are expressed as the mean of five experiments ± standard error of the mean.
[b]$p < 0.05$.

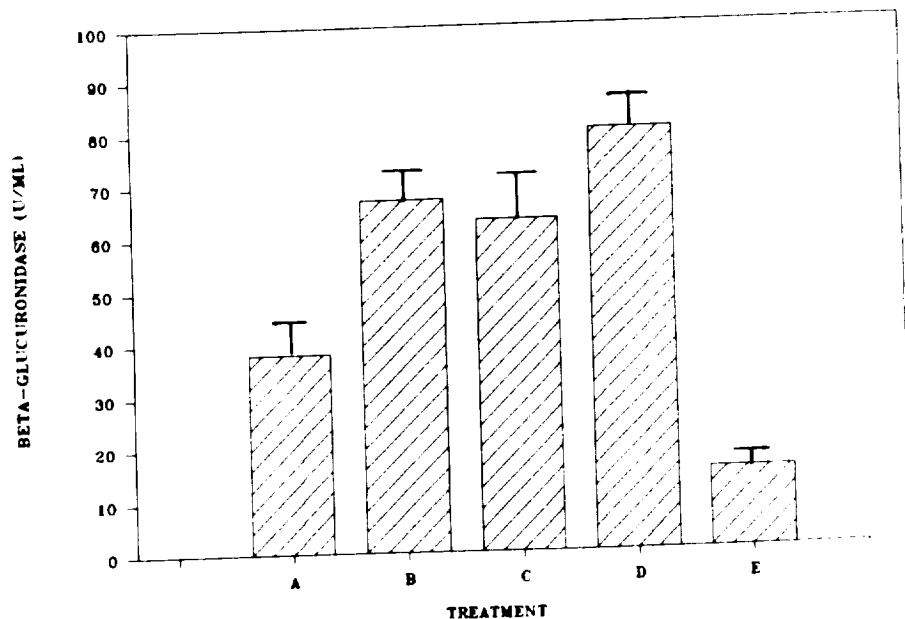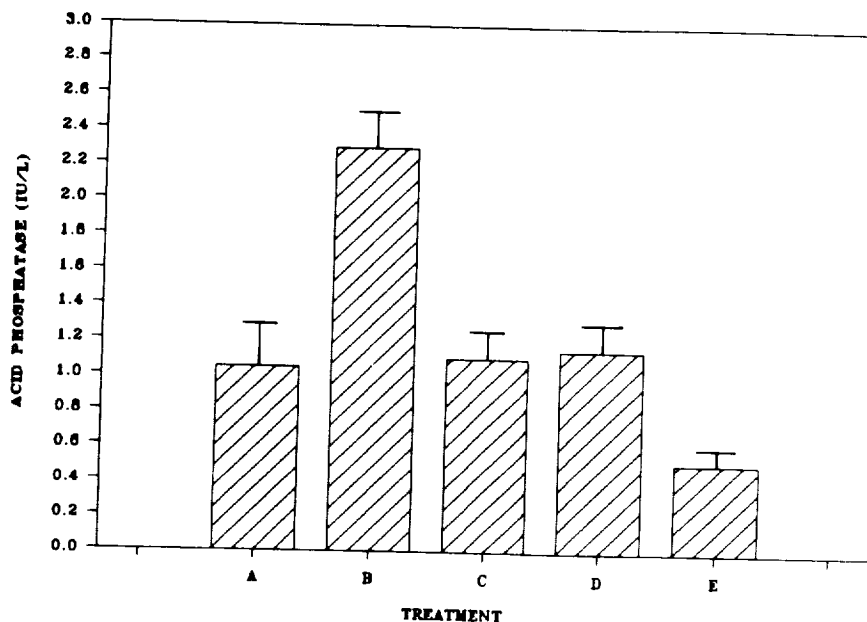FIG. 1. Effect of time of incubation with IFN-γ and TNF on the activity of β-glucuronidase in lysates of mouse peritoneal macrophages. Treatment A, IFN-γ 30 U/ml (6 h); treatment B, IFN-γ 30 U/ml (24 h); treatment C, TNF 30 U/ml (6 h); treatment D, TNF 30 U/ml (24 h); treatment E, medium.

Effect of time of incubation of cytokines on the activity of acid phosphatase in lysates

Macrophages were incubated with IFN-γ or TNF for 6 or 24 h followed by washing and lysis. Acid phosphatase activity was tested in lysates. When IFN-γ was used as the stimulating cytokine, the values obtained at 6 h of incubation were lower (40–60%) than the ones obtained with 24 h of incubation (Fig. 2). With TNF, there was no difference between 6 h and 24 h of incubation (Fig. 2).

DISCUSSION

Activated macrophages possess properties exceeding, qualitatively or quantitatively, the baseline values exhibited by resident macrophages. Although there seem to be a variety of biochemical criteria to describe the state of activation in mouse peritoneal macrophages, several parameters have

FIG. 2. Effect of time of incubation with IFN-γ and TNF on the activity of acid phosphatase in lysates of mouse peritoneal macrophages. Treatment A, IFN-γ 30 U/ml (6 h); treatment B, IFN-γ 30 U/ml (24 h); treatment C, TNF 30 U/ml (6 h); treatment D, TNF 30 U/ml (24 h); treatment E, medium.

been used to describe this particular status. These include enzyme synthesis and secretion, as well as functional capacities.[31]

In the present study, the enhancement of the activity of two macrophage hydrolases by cytokine treatment was used as a method to determine the *in vitro* activation of such cells. IFN-γ or TNF treatment produced a significant increase in levels of β-glucuronidase and acid phosphatase. Furthermore, a dose of 100 U/ml of TNF appeared to be most effective in raising enzyme levels. This dose could have been most effective due to a cytotoxic effect of TNF at higher concentrations.

It has been demonstrated that lipopolysaccharide (LPS) by itself is a potent stimulator of macro-phages.[32] LPS can be present in some preparations of recombinant cytokines as a product of the DNA technology, when the gene is cloned in bacteria. However, the possibility of LPS playing a role in the present study was minimized, as the activity of the IFN was completely abrogated by treatment with specific monoclonal antibody, and the IFN-γ and TNF preparations appeared to be free of LPS. Control experiments were carried out with the same batches of medium in which the cytokines were prepared. These results suggest that the activity observed was due to IFN-γ, and not LPS.

Macrophages treated for 6 h with IFN-γ were activated up to 40-60% of maximum for β-glucu-ronidase and acid phosphatase production. However, maximum effect was not observed until 24 h of incubation. Morland *et al.* have demonstrated similar results, and they also found that pro-longed treatment for 48-72 h could produce a decrease in the enzymatic levels.[25] However, in the present study when the cells were incubated with TNF, maximum acid phosphatase activity and 80% of the maximum β-glucuronidase activity was reached by 6 h of incubation. Therefore, it is possible that each cytokine may utilize a different mechanism of activation.

It is becoming apparent that both IFN-γ and TNF have a complex pattern of interaction. Several

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synergistic interactions between IFN-γ and TNF have been reported. For example, the B16 melanoma cell line is not sensitive to TNF unless it is applied in combination with IFN-γ.[33] Synergistic effects between both cytokines have been described regarding the antiproliferative response *in vivo* and *in vitro* systems, and in the production of Class I and Class II histocompatibility antigens.[34] The molecular mechanism of the synergistic effect is not well understood. However, some investigators[35] have demonstrated that in several target cell types, short-term inhibition of target cell protein or energy metabolism caused a highly accelerated cytolisis. It has also been postulated[36] that IFN-γ renders target cells more sensitive to TNF lysis by some form of alteration in the cell membrane structure, perhaps through the exposure of a TNF-binding receptor. In the present study, the effect of incubation of both cytokines together was additive rather than synergistic regarding the enzymatic activities in the lysates. Although the use of additional cytokine concentrations could possibly yield different results, the present data suggest that a generalization that TNF and IFN-γ will act synergistically in all systems cannot be made. This is supported by the recent observation that TNF is associated with macrophage killing of *Mycobacterium avium* complex, but that IFN-γ plays no role in this act.[37]

It should be noted that the TNF available to be used in this study was human. We chose to use the human TNF and murine IFN-γ because the bulk of studies showing synergy have used this combination.[19,20,33,34] Even though human TNF is cross-reactive in mouse cells, it is still a foreign protein and it is possible that different results could be obtained using murine TNF in the system used in the present study.

The results of the present study indicate that both IFN-γ and TNF can activate macrophages as indicated by enhancement of β-glucuronidase and acid phosphatase activities. The significance of this activation and possible interactions between IFN-γ and TNF in an *in vivo* model of infection remains to be established.

ACKNOWLEDGMENTS

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Address reprint requests to:
Dr. Gerald Sonnenfeld
Department of Microbiology and Immunology
School of Medicine
University of Louisville
Louisville, KY 40292

INTERFERONS AND MICROBIAL INFECTIONS

Gerald Sonnenfeld

Running Title : IFNs and Microbes

Department of Microbiology and Immunology
and Department of Oral Health,
Schools of Medicine and Dentistry, University of Louisville,
Louisville, Kentucky 40292, USA

ABSTRACT

Interferons have been shown to be involved in several non-viral, microbial infections. These include both protozoan parasite and bacterial infections. Interferons have been shown to be induced during those infections and to alter the course of the infections. The mechanism for the effects of interferons on non-viral microbial infections may involve the modulation of immune responses. Many different types of immune responses can be affected by interferons, and this may affect host defenses against microbial infections. Many exciting research areas are developing for the study of this intriguing property of interferons.

INTRODUCTION

Interferons (IFNs) were described originally as antivirals (1). However, in recent years it has become clear that IFNs can affect the outcome of several different non-viral microbial pathogens. Recent studies have shown that IFNs have several activities in addition to the antiviral activity, and it is possible that these additional activities could contribute to the anti-microbial effects of IFNs. One of the most significant of those activities is modulation of immune responses (2). As natural IFNs have been purified and pure IFNs have been produced by recombinant DNA technology, it has become apparent that the IFNs themselves are responsible for the immunomodulatory activities that have been observed (2). As a result of its immunomodulatory properties, IFNs could play major roles in resistance to various microbial infections.

TABLE 1   TYPES OF INTERFERONS

IFN-ALPHA	leukocyte type I
IFN-BETA	fibroblast type I
IFN-GAMMA	immune type II

It is now clear that all three types of IFNs (IFN-alpha, IFN-beta, and IFN-gamma) have immunomodulatory properties (Table 1). IFN-alpha is produced primarily by lymphocytes in response to viruses, double-stranded RNAs, lipopolysaccharide and other non-specific inducers (3). Several sub-species of IFN-alpha have been shown to exist as a results of cloning of the genes (3). IFN-beta is produced by fibroblasts in response

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to the same types of inducers as IFN-alpha (3). In rodents, it is difficult to separate these two types of IFN, and they are often referred to as IFN-alpha/beta. The third type of IFN, IFN-gamma, is an actual product of cell-mediated immune responses. IFN-gamma is produced by sensitized T-lymphocytes in response to specific antigen, or by T-lymphocytes in response to a mitogen such as phytohemagglutinin or con-canavalin-A (3). These IFNs differ from each other with regard to antigenicity, structure, and the genetic information coding for the proteins (3).

The IFNs have all been shown to have a variety of immunomodulatory activities (Table 2). In both human and animal models, IFNs have been shown to modulate antibody production (4, 5). When IFN-alpha/beta or inducers of IFN-alpha/beta were administered to mice at the time of or prior to sensitization of the mice with sheep red blood cells, the plaque forming cell response was suppressed (6 – 15). This type of response was found to occur both in vitro and in vivo, and when the dose of IFN-alpha/beta was reduced or when IFN was administered after sensitization with antigen, antibody production was enhanced (7 – 9). Similar results were observed using human in vitro systems (16, 17). Therefore, IFN-alpha/beta had a time and dosage dependent effect on antibody production. This would suggest a truly immunoregulatory role for IFNs, as the same substance in the same system can enhance or suppress an immune response.

TABLE 2   IMMUNOMODULATORY ACTIVITIES OF INTERFERONS

1. Modulation of antibody production
2. Modulation of delayed hypersensitivity/cell – mediated immunity
3. Regulation of cell surface antigen expression
4. Activation of Natural Killer cells
5. Activation of macrophages
6. Interaction with other cytokines
   (interleukin – 2, tumor necrosis factor)

Similar results were observed using IFN-gamma or inducers of IFN-gamma (18 – 20). In the case of IFN-gamma, much smaller doses of IFN-gamma were required to observe the same effects as with IFN-alpha/beta, suggesting that IFN-gamma might be a more potent immunoregulatory agent. Modulation of antibody responses by IFN-gamma was also time and dosage dependent in a similar fashion to what has been reported for IFN-alpha/beta (18 – 20). B cells might have been affected directly by the IFN-gamma preparation (20).

IFNs can also affect the cell-mediated immune response. Administration of IFN-alpha/beta resulted in an inhibited delayed hypersensitivity response to sheep red blood cells in mice (21).

In addition, IFNs have been shown to play a crucial role in regulation of the response of natural killer cells (22 – 28) in humans and rodents (Table 2). Natural killer cells are naturally cytotoxic to infected or tumor cells. The presence of IFNs augments dramatically the activity of natural killer cells (22 – 28). IFNs-alpha and -beta may be most effective in activating natural killer cells. It should be noted that treatment of tumor target cells with IFN results in protection of those cells from natural killer cells

(22). This is additional evidence for a natural immunoregulatory role for IFN where presence of the IFN at a specific time and place during the development of an immune response could result in a differing end result.

IFNs have also been shown to regulate macrophage activation (29 – 35). All IFNs have been shown to be able to regulate macrophage activation in rodent and human systems, but IFN-gamma appears to be most effective in activating macrophages. The activation appears to result in enhanced phagocytosis, and in some cases, enhanced killing of tumors or microbial pathogens (29 – 35).

There are several possible mechanisms for modulation of immune responses by IFNs. There can be direct effects of IFNs on cells, or interactions of IFNs with other cytokines.

IFNs could affect directly the maturation, activation and development of cells important in immune responses. As stated above, IFNs can regulate the maturation of B cells, thereby resulting in the regulation of antibody responses (36).

One of the most fascinating potential mechanisms for IFNs to regulate immune responses is through the expression of cell surface antigens (Table 2). In a series of experiments first carried out by Gresser and his associates (37 – 41), IFN-alpha/ beta was shown to be able to enhance expression of class I histocompatibility antigens on the surface of cells. Later, IFN-gamma was shown to have a similar effect (42). Regulation of expression of class I histocompatibility antigens could affect the efficiency by which cytotoxic T-lymphocytes interact with targets such as tumor cells or infected cells. IFNs can also modulate levels of expression of Fc receptors on cells (43). Alterations in these receptors could affect the ability of a host to mount several immune responses involving these receptors such as allergic responses involving IgE.

IFN-gamma can also regulate the expression of class II histocompatibility antigens on a variety of cell types (42, 44 – 48). The class II histocompatibility antigens play a fundamental role in foreign antigen presentation from macrophages to lymphocytes. Therefore, the ability of IFN-gamma to regulate their level could result in a fundamental immunoregulatory role for IFN in a wide variety of immune responses.

In addition IFNs have been shown to interact and regulate production of a wide variety of other cytokines. These include interleukin-2 and tumor necrosis factor-alpha (49 – 51). As a result of this interaction, IFNs could play a major role in the regulation of immune responses by regulating the duration and extent of such responses.

It is, therefore, possible that IFNs could be major regulators of native immune responses in a host. If IFNs do play such a natural role, then it is possible that IFNs could regulate resistance to infection with a wide variety of pathogens. These pathogens could include a range of organisms far beyond the viruses for which IFNs were originally thought to be active. I shall now describe experiments using mouse models of infection carried out in conjunction with my laboratory to explore the role of IFNs in resistance to protozoan parasite and bacterial infections (Table 3).

TABLE 3 MICROBIAL INFECTIONS AFFECTED BY INTERFERONS
STUDIED IN THE AUTHOR'S LABORATORY

BACTERIAL :

    Salmonella typhimurium

    Escherichia coli

    Staphylococcus aureus

    Klebsiella pneumoniae

PARASITIC :

    Trypanosoma cruzi

    Trypanosoma brucei

    Naegleria fowleri

INTERFERONS AND MICROBIAL INFECTIONS

Studies in my laboratory have been directed towards two protozoan parasites. Trypanosoma cruzi, the intracellular South American trypanosome, and Trypanosoma brucei rhodesiense, an extracellular African trypanosome. IFNs appear to be able to be involved in the course of both the intracellular and extracellular infections.

T. cruzi is the cause of Chagas' disease, and early reports indicated that a substance similar to IFN was induced during the course of infection of mice (52, 53). We were able to show that mice infected with T. cruzi actually did produce IFN-alpha/beta during the early stage of infection (54, 55). Others showed that interferon-gamma was produced late in the course of infection (56). Mice treated with IFN-alpha/beta beginning at the day of infection showed a transient decrease in parasitemia, compared to controls (55). Reed and associates later showed that treatment of mice with massive does of IFN-gamma resulted in improved survival after infection with T. cruzi (57).

During in vitro experiments, when macrophage or heart cell cultures were treated with IFN-beta, there was no effect on the uptake of T. cruzi (58). However, when the parasites were treated directly with IFN-beta, decreased uptake of the trypanosomes by cells was observed (58). Therefore, IFN-beta may be able to interact directly with the parasite, resulting in decreased uptake of the parasite. When IFN-gamma was applied to cultures of macrophages, increased uptake and oxidative killing was observed (59). Therefore, a two-step role for IFNs could be envisioned in resistance to Chagas' disease. First IFN-beta could protect by limiting the uptake of the parasite by cells. Then, IFN-gamma could enhance the destruction of the parasite in cells that had been invaded.

Additional studies were carried out with T.b. rhodesiense, one causative agent of African sleeping sickness. Many different groups have shown that IFNs are produced in the course of African trypanosome infections (60, 61). In our laboratory, we were able to show that IFN production correlated with resistance of inbred strains of mice to trypanosme infection (62). Mouse strains that were genetically susceptible to infection produced no IFN, intermediately resistant strains produced only IFN-alpha/beta, and resistant strains of mouse produced both IFN-alpha/beta and IFN-gamma during the course of infection with T.b. rhodesiense (62).

We have not been able to conclusively demonstrate that the IFN produced during infection contributes to resistance to T.b. rhodesiense. Treatment of mice with IFNs, IFN-inducers, or anti-IFNs has not altered the course of infection with T.b. rhodesiense (63). The course of infection is so complex that it may require several different protocols and concentrations of IFNs to show direct protective effects on the primary infection. However, we have shown effects on secondary infections. Mice infected with African trypanosomes are immunosuppressed, and often succumb to secondary infections (64). We were able to show that mice that were infected with T.b. rhodesiense and were producing IFNs were resistant to superinfection with the D variant of encephalomyocarditis virus (65). This indicates that a mouse strain resistant to infection to T.b. rhodesiense and producing IFN could have reduced morbidity and mortality as a result of not being subject to secondary viral infections.

Additional studies were carried out with the amoeba Naegleria fowleri (66). This is a free-living amoeba, but in thermally polluted lakes, can infect children and cause a primary amoebic meningoencephalitis (66). When N. fowleri were treated in vitro with IFN-alpha/beta, they were no longer infectious for mice (66).

We have also carried out studies with bacteria. We have been able to show that IFN-alpha/beta activated mouse macrophages to ingest Escherichia coli and increase levels of phagocytic enzymes, but IFN-gamma was not as effective in this model (67, 68). In this case, IFN-alpha/beta might be more effective than IFN-gamma.

In additional studies, models of surgical wound infection were used. Individuals who survive trauma, such as an automobile accident or a gunshot wound, are immuno-suppressed (69). If these individuals survive the initial trauma, the leading cause of death is infection due to bacterial sepsis, and antibiotics are usually ineffective (69). We were able to show using four models of wound infection in mice similar to what occurs in trauma, that treatment with recombinant IFN-gamma, provided by Genentech, resulted in inceased survival of the animals (70 – 73). The bacteria used included Klebsiella pneumoniae and Escehrichia coli, which are common surgical wound contaminants. There have been no studies in these models with IFN-alpha to date, but since IFN-alpha/beta may activate phagocytic cells differently from IFN-gamma, there are exciting research possibilities with IFN-alpha in this area.

The studies described above indicate that IFNs can demonstrate many immunoregulatory activities. As a result of these activities, it appears that IFNs can play a role in resistance to a wide variety of pathogens. IFNs may play a role in modulating natural resistance to these infections as a result of immunoregulation and other activities. It remains to be seen whether the use of exogenous IFNs, and IFN-alpha in particular, can have practical clinical efficacy in reducing morbidity and mortality of infections for which no effective chemotherapy exists and treatable infections in hosts that are immuno-suppressed. The door is just beginning to open for experiments of this nature to be carried out.

ACKNOWLEDGEMENTS

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INHIBITION BY *TRYPANOSOMA CRUZI* OF INTERFERON-GAMMA PRODUCTION BY MITOGEN-STIMULATED MOUSE SPLEEN CELLS

LISA A. BELTZ,* G. SONNENFELD† and F. KIERSZENBAUM*‡

* Department of Microbiology and Public Health, Michigan State University, East Lansing, MI 48824, U.S.A.
† Department of Microbiology and Immunology, University of Louisville School of Medicine, Louisville, KY 40292, U.S.A.

Abstract—BELTZ L. A., SONNENFELD G. and KIERSZENBAUM F. 1989. Inhibition by *Trypanosoma cruzi* of interferon-gamma production by mitogen-stimulated mouse spleen cells. *International Journal for Parasitology* 19: 555–559. Infection by *Trypanosoma cruzi* is accompanied by severe immunosuppression during the acute period. As part of our studies, to define the alterations caused by *Trypanosoma cruzi* in lymphocyte function, we examined in this work the interferon-gamma (IFN-γ)-producing capacity of mitogen-stimulated mouse spleen and human peripheral blood mononuclear cells in the presence or absence of blood forms of the parasite. Co-culture of phytohaemagglutinin- or concanavalin A-stimulated spleen cells from normal mice with *T. cruzi* significantly decreased the levels of IFN-γ activity found in the supernatants at 48 or 72 h. In contrast, human peripheral blood mononuclear cells, though suppressed by *T. cruzi* in their capacity to proliferate upon mitogenic stimulation, showed no significant decrease in IFN-γ production. The addition of exogenous IFN-γ did not reverse the suppressive effect of *T. cruzi* on either mouse or human cells. These results revealed, for the first time, the ability of *T. cruzi* to impair IFN-γ production by activated mouse lymphocytes. The lack of restoration by exogenous IFN-γ suggested that the reduced levels of this lymphokine were not, at least by themselves, the causative factor of reduced lymphoproliferation.

INDEX KEY WORDS: *Trypanosoma cruzi*; Chagas' disease; interferon gamma; immunosuppression.

INTRODUCTION

THE acute phase of *Trypanosoma cruzi* infection in mice is accompanied by several manifestations of immunosuppression, including decreased interleukin 2 (IL2) production and reduced mitogen-induced lymphocyte proliferation (Hayes & Kierszenbaum, 1981; Harel-Bellan, Joskowicz, Fradelizi & Eisen, 1983; Reed, Inverso & Roters, 1984; Tarleton & Kuhn, 1984). The parasite also suppresses the proliferation of normal mouse spleen and human peripheral blood mononuclear cells and *in vitro* following stimulation with mitogens or a monoclonal antibody specific for an epitope of the T cell receptor complex (Maleckar & Kierszenbaum, 1983; Beltz & Kierszenbaum, 1987; Beltz, Sztein & Kierszenbaum, 1988). The binding of IL2 to its receptor on T lymphocytes triggers both IFN-γ production (Farrar, Johnson & Farrar, 1981) and cell division (reviewed by Smith, 1984). Since IL2 production by mouse spleen cells is decreased by *T. cruzi*, it is possible that IFN-γ synthesis may also be impaired.

IFN-γ plays an important role in host defence against intracellular parasites, enhancing the *in vitro* killing of *Toxoplasma gondii, Leishmania donovani* and

‡ To whom all correspondence should be addressed.

T. cruzi by macrophages (Pfefferkorn & Guyre, 1983; Murray, Rubin & Rothermel, 1983; Plata, Wietzerbin, Garcia-Pons, Falcoff & Eisen, 1984). *In vivo*, IFN-γ acts synergistically with anti-*T. cruzi* antibodies to decrease parasitemia and prolong the survival of infected mice (Plata, Garcia-Pons & Wietzerbin, 1987). This lymphokine also affects mitogen-induced lymphocyte proliferation by either inhibiting or enhancing T cell activity, depending on the dosage and time of administration (Sonnenfeld, Mandel & Merigan, 1978; Friedman & Vogel, 1983). Given the roles of IFN-γ in host defense and lymphoproliferation, we looked into whether *T. cruzi* can alter production of this important lymphokine by normal murine and human lymphocytes and, if so, whether this alteration plays a role in the suppression of lymphocyte proliferation.

MATERIALS AND METHODS

Parasites. Trypomastigotes of *T. cruzi* (Tulahuen isolate) were purified from the blood of Crl-CDl(ICR)BR Swiss mice (Charles River Laboratory, Portage, MI) infected intraperitoneally 2 weeks previously with 2 × 10⁵ blood forms. The parasites were purified by density gradient centrifugation over a mixture of Ficoll–Hypaque of density 1.077 (Budzko & Kierszenbaum, 1974) followed by diethylaminoethyl-cellulose chromatography (Villalta & Leon, 1979), washed twice by centrifugation (800 × *g*, 20

min, 4°C) and resuspended in RPMI 1640 medium (Gibco, Grand Island, NY) containing 100 i.u. penicillin and 100 μg streptomycin per ml plus either 2.5 or 5% heat-inactivated (56°C, 20 min) fetal bovine serum (RPMI + 2.5%FBS or RPMI + 5%FBS, respectively).

Mouse spleen cells (mSC). Spleens were removed from ether-anesthetized inbred CBA/J mice (Jackson Laboratories, Bar Harbor, ME). Cell suspensions were prepared as described previously in detail (Maleckar & Kierszenbaum, 1983); the nucleated cells were counted and working suspensions were made in RPMI + 2.5%FBS. Cell viability, determined by trypan-blue dye exclusion, was always >99%.

Human peripheral blood mononuclear cells (hPBMC). The hPBMC were isolated from the blood of healthy donors by centrifugation over a mixture of Ficoll–Hypaque of density 1.077 (350 × *g*, 45 min, 20°C). The cells collected at the interface were washed three times by centrifugation (350 × *g*, 10 min, 4°C) with serum-free RPMI 1640 medium. The hPBMC were then resuspended in RPMI + 2.5%FBS. Cell viability was determined by trypan-blue exclusion and was always >99%.

Lymphoproliferation assay. Mouse spleen cells at 2.5×10^6 cells ml^{-1} were incubated in 96-well plates (final culture volume = 0.1 ml) in the presence or absence of 5 μg phytohaemagglutinin P ml^{-1} (PHA; Sigma Chemical Co., St. Louis, MO), together with or without 2.5×10^6 parasites ml^{-1}. In some experiments, exogenous recombinant murine IFN-γ (specific activity 2.3×10^7 units per mg protein, Genentech, South San Francisco, CA) was added, keeping the final volume at 0.1 ml. Each condition was tested in triplicate. The cultures were incubated at 37°C (5% CO_2) for 72 h and 37 kBq ^{3}H-thymidine (Amersham, Arlington Heights, IL) was present during the last 24 h. The hPBMC cultures were performed similarly, except that the parasite concentration was 5×10^6 organisms ml^{-1}. When exogenous IFN-γ was added to human cell cultures, we used partially purified human IFN-γ (Meloy Laboratories, Springfield, VA). All cultures were performed in triplicate and were terminated by automated harvesting using a MASH II apparatus (M. A. Bioproducts, Walkersville, MD). The results were expressed as counts per minute (c.p.m.) representing ^{3}H-thymidine uptake during the last 24 h of culture.

Measurement of IFN-γ. To determine IFN-γ activity, suspensions of mSC or hPBMC were incubated in sterile 24-well plates (final culture volume = 1 ml) at 37°C (5% CO_2) for 48 or 72 h in the presence or absence of 5 μg PHA ml^{-1} with or without 2.5×10^6 (for mSC) or 5×10^6 (for hPBMC) *T. cruzi* ml^{-1}. The latter concentration of parasites was that found to be the minimal level to consistently decrease the proliferation of PHA-stimulated hPBMC. Following passage through 0.22-μm-pore-size filters, the supernatants were assayed for IFN-γ activity by a plaque reduction assay using mouse L-929 cells and the Indiana strain of bovine vesicular stomatitis virus (Sonnenfeld, Mandel & Merigan, 1977). The titer was expressed as units ml^{-1} corresponding to the reciprocal of the highest dilution that reduced plaques by 50%. In this assay, one unit was equivalent to 0.88 NIH G-002-904511 reference units and the lower sensitivity limit was 30 units IFN-γ ml^{-1}. Identification of anti-viral activity as that of IFN-γ was provided by its lability at pH 2 and inhibition with anti-murine IFN-γ antibodies (a gift from Dr E. Havell, Trudeau Institute, Saranac Lake, NY). In the experiments in which we measured IFN-γ activity in supernatants from hPBMC co-cultures with *T. cruzi*, we used

a radioimmunoassay kit (Centocor, Malvern, MY) which utilizes two monoclonal antibodies directed at different epitopes of human IFN-γ and is designed to detect only biologically active material. The lower limit of sensitivity of this assay was 5 units IFN-γ ml^{-1}.

Absorption of murine IFN-γ. Aliquots of a solution of recombinant murine IFN-γ (Genentech) were incubated in 24-well plates with or without 5×10^6 to 1×10^7 *T. cruzi* ml^{-1} at 37°C for 48 h. After filtration through 0.22-μm-pore-size filters, residual IFN-γ activity was determined in the filtrates by the biological assay described above.

RESULTS

Effects of T. cruzi *on IFN-γ production* in vitro

The levels of IFN-γ activity in supernatants of PHA-stimulated mSC cultures which contained *T. cruzi* were significantly lower than those of control, parasite-free cultures (Table 1). A relatively small but nevertheless significant reduction was demonstrable 48 h after the initiation of the cultures and a more pronounced effect was seen at 72 h. At these times, the decreases in ^{3}H-thymidine incorporation due to the presence of parasites (recorded in parallel cultures) were approximately 50% and >59%, respectively, in the two repeat experiments (data not shown). Similar results were obtained when another T cell-specific mitogen, concanavalin A (2 μg ml^{-1}), was used in experiments of identical design (data not shown). No detectable IFN-γ-like activity was found in the supernatants of *T. cruzi* suspensions at concentrations which suppressed mSC responses, nor did the parasite induce unstimulated mSC to secrete measurable levels of IFN-γ.

TABLE 1—*T. cruzi*-INDUCED INHIBITION OF IFN-γ PRODUCTION BY PHA-STIMULATED MOUSE SPLEEN CELLS*

Material tested	IFN-γ (units ml^{-1}) measured after	
	48 h	72 h
mSC	≤30	≤30
T. cruzi	≤30	≤30
mSC + *T. cruzi*	≤30	≤30
T. cruzi + PHA	≤30	≤30
mSC + PHA	58	67
mSC + PHA + *T. cruzi*	31†	≤30†

* The tested materials consisted of the culture supernatants of mSC (2.5×10^6 cells ml^{-1}) and/or *T. cruzi* (2.5×10^6 organisms ml^{-1}) in the presence or absence of 5 μg PHA ml^{-1}. The supernatants were collected at the indicated times after initiation of the cultures. This set of results was obtained with individual cultures and was representative of two separate repeat experiments.

† This reduction in IFN-γ activity with respect to the corresponding positive control value was statistically significant ($P < 0.05$).

The noted decrease in IFN-γ levels might have resulted from either removal of the lymphokine by the parasite or reduced production or secretion. This was tested by incubating aliquots of a solution of

recombinant murine IFN-γ with or without *T. cruzi* for a 48-h period. The IFN-γ activities remaining after absorption with parasites or mock absorption did not differ significantly ($P>0.1$). Thus, for example, in one of the experiments aliquots absorbed with 5×10^6 and 1×10^7 parasites ml^{-1} had 357 ± 140 and 303 ± 51 units IFN-γ ml^{-1}, respectively whereas the mock-absorbed control had 284 ± 74 units IFN-γ ml^{-1}. It is noteworthy that the concentrations of parasites used for absorption represented two and four times, respectively, the level which reduced IFN-γ production in mitogen-stimulated mSC cultures.

Attempts to reverse the suppressive effect of T. cruzi *with exogenous IFN-γ*

The lymphoproliferative capacity of mitogen-stimulated lymphocytes from either infected mice (Hayes & Kierszenbaum, 1981; Harel-Bellan *et al.*, 1983) or humans (Teixeira, Teixeira, Macedo & Prata, 1978) has been shown to be impaired. Furthermore, co-culture of either mSC or hPBMC from normal individuals with *T. cruzi* results in significant suppression of lymphoproliferation (Maleckar & Kierszenbaum, 1983; Beltz & Kierszenbaum, 1987). Because IFN-γ can enhance T cell proliferation at certain dosages and times of administration (Sonnenfeld *et al.*, 1978), the possibility that the observed suppression in lymphocyte growth resulted from the inhibition of IFN-γ production by *T. cruzi* was explored. To this end, IFN-γ was added to the cultures so as to attain final concentrations ranging from 8 to 250 units ml^{-1} and the level of ^{3}H-thymidine incorporation was monitored. None of these IFN-γ concentrations afforded a significant restorative effect (Table 2).

TABLE 2—LACK OF RESTORATION BY EXOGENOUS IFN-γ OF THE CAPACITY OF PHA-STIMULATED MOUSE SPLEEN CELLS TO PROLIFERATE AFTER *T. cruzi*-INDUCED SUPPRESSION*

IFN-γ	^{3}H-thymidine uptake (in thousand c.p.m.)		% Decrease
(units ml^{-1})	mSC + PHA	mSC+PHA+ *T. cruzi*	
0	15.2 ± 1.0	0.8 ± 0.0†	95
8	14.6 ± 1.0	0.8 ± 0.2†	95
16	15.9 ± 0.8	0.7 ± 0.2†	95
32	14.6 ± 0.4	1.0 ± 0.4†	93
64	14.7 ± 1.7	0.9 ± 0.5†	94
125	13.9 ± 0.5	0.9 ± 0.1†	94
250	4.6 ± 0.7	0.9 ± 0.0†	94

* Recombinant murine IFN-γ was added at the indicated concentrations to cultures of mSC (2.5×10^6 cells ml^{-1}) containing 5 μg pHA ml^{-1} in the presence or absence of *T. cruzi* (2.5×10^6 organisms ml^{-1}). Results are the means of triplicate determinations made with 72-h cultures pulsed with 37 kBq ^{3}H-thymidine during the last 24 h. This set of results is representative of three separate repeat experiments.

† $P<0.05$ (Student's '*t*' test) for the reduction in c.p.m. with respect to either control, i.e. mSC + PHA with or without IFN-γ.

The capacity of mSC to produce IL2 is decreased following exposure to *T. cruzi* either *in vivo* or *in vitro* (Harel-Bellan *et al.*, 1983; Beltz & Kierszenbaum, unpublished results). The addition of IL2 to these supposed cultures restores their ability to secrete immunoglobulin (Reed *et al.*, 1984; Tarleton & Kuhn, 1984) and to proliferate following mitogen stimulation (Beltz *et al.*, 1988). Because IFN-γ has been reported to increase the expression of IL2 receptors on both T cells (Johnson & Farrar, 1983) and monocytes (Herrman, Canistra, Levine & Griffin, 1985), and higher levels of the IL2 receptor allow cells to respond to lower concentrations of IL2 (Cantrell & Smith, 1984), we tested whether IFN-γ would enhance the restorative capacity of IL2. The presence of 16 or 125 units IFN-γ ml^{-1} did not overcome *T. cruzi*-induced suppression of mSC lymphoproliferation whether or not added together with a suboptimal level of IL2 (50 units ml^{-1}). Furthermore, IFN-γ did not act synergistically with 100 units IL2 ml^{-1} (data not shown), a concentration which does restore suppressed lymphoproliferative mSC responses in our system (Beltz *et al.*, 1988).

TABLE 3—LACK OF EFFECT OF *T. cruzi* ON IFN-γ PRODUCTION BY PHA-STIMULATED hPBMC*

Material tested	IFN-γ (units ml^{-1}) measured after	
	48 h	72 h
hPBMC	≤ 5	≤ 5
T. cruzi	≤ 5	≤ 5
hPBMC + *T. cruzi*	≤ 5	≤ 5
T. cruzi + PHA	≤ 5	≤ 5
hPBMC + PHA	120	125
hPBMC + PHA + *T. cruzi*	96	119

* The tested materials consisted of the culture supernatants of hPBMC 1.25×10^6 cells ml^{-1}) and/or *T. cruzi* (5×10^6 organisms ml^{-1}) in the presence or absence of 5 μg PHA ml^{-1}. The supernatants were collected at the indicated times after initiation of the cultures, filtered and tested for IFN-γ activity by radioimmunoassay. This set of results was obtained with individual cultures and was representative of two separate repeat experiments. None of the noted reductions in IFN-γ activity due to the presence of *T. cruzi* was statistically significant.

We next examined whether *T. cruzi* would also inhibit IFN-γ production or secretion by human lymphocytes. As can be seen in Table 3, the presence of 5×10^6 *T. cruzi* ml^{-1} in cultures of PHA-stimulated hPBMC did not lead to a significant reduction in IFN-γ levels in the supernatants. The addition of exogenous human IFN-γ did not restore the suppressed lymphoproliferative response of hPBMC exposed to *T. cruzi* whether or not IL2 was present (data not shown).

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DISCUSSION

These results show, for the first time, that *T. cruzi* can diminish the capacity of mouse lymphocytes to produce IFN-γ upon activation by mitogenic stimuli. Immunosuppression of *T. cruzi in vitro* has been shown not to be due to nutrient consumption by the parasite (Maleckar & Kierszenbaum, 1983; Beltz & Kierszenbaum, 1987) and the present data showed that the flagellate does not absorb, consume or inactivate IFN-γ. Furthermore, in our culture system the numbers and the viability of mouse cells co-cultured with *T. cruzi* are comparable at 48 and 72 h with those of parasite-free cultures (Maleckar & Kierszenbaum, 1983). Thus, the noted reduction in IFN-γ activity was probably the result of inhibited production and/or secretion.

Decreased proliferation by spleen cells from infected mice or by normal mSC incubated with *T. cruzi in vitro* is paralleled by decreases in IL2 (Harel-Bellan *et al.*, 1983; Beltz & Kierszenbaum, unpublished results) and IFN-γ (Table 1) production. In contrast, *T. cruzi* is unable to decrease IL2 production by hPBMC under optimal culture conditions (Beltz *et al.*, 1988) and, as reported herein, also had no significant effect on IFN-γ production by hPBMC (Table 2). Thus, there would appear to exist a notable difference in how the parasite affects mSC and hPBMC responses to PHA. However, the present data do not clarify whether this difference in the suppressive activities of *T. cruzi* toward mSC and hPBMC stems from the use of different populations of lymphocytes or from an actual disparity in mouse and human lymphocyte responses to the parasite.

Although reduced IFN-γ production/secretion is a major consequence of *T. cruzi*-induced suppression of mSC, it is not, at least by itself, the explanation for such suppression, since exogenous IFN-γ failed to restore responsiveness in terms of lymphoprolifer-ation measured by ^{3}H-thymidine uptake.

T. cruzi suppresses proliferation of hPBMC (Beltz & Kierszenbaum, 1987). Insufficient levels of IFN-γ or IFN-γ plus IL2 do not seem to be the cause for this suppression since no significant drop in the level of IFN-γ was observed in these cultures and neither IFN-γ nor IL2, added to the cultures, separately or together, corrected the suppression caused by the presence of the parasite. IFN-γ and IL2 are elements of a complex regulatory network and are able to affect each other's synthesis and utilization (Farrar *et al.*, 1981; Johnson & Farrar, 1983; Herrman *et al.*, 1985), with IFN-γ production being upregulated by IL2 (Farrar *et al.*, 1981). Since *T. cruzi* decreases the production of both IL2 and IFN-γ by activated mSC, it is possible that the parasite's ability to inhibit synthesis or secretion of IL2 is at least partially responsible for the decrease in IFN-γ production/secretion. It should be noted, however, that lymphocytes from mice infected with *Trypanosoma brucei* have an impairment in the secretion of IL2 but not IFN-γ (Sileghem, Hamers & de Baetselier, 1987), demonstrating that normal IL2 levels may not be an absolute requirement for optimal IFN-γ synthesis. Conceivably, *T. cruzi* could exert independent suppressive effects on T cells; this remains to be studied.

Although the deficient capacity of mSC to produce or secrete IFN-γ after exposure to *T. cruzi* does not appear to determine suppressed lymphocyte proliferation, occurrence of this phenomenon *in vivo* could alter the capacity of other host cells to interact with the parasite. For example, macrophages take up and destroy the parasite, and IFN-γ has been shown to enhance these functions (Wirth, Kierszenbaum, Sonnenfeld & Zlotnik, 1985). Furthermore, IFN-γ appears to increase the resistance of fibroblasts to *T. cruzi* infection *in vitro* (Plata *et al.*, 1984) and, synergistically with anti-*T. cruzi* antibodies, to decrease the parasitaemia and increase the survival time of infected mice (Plata *et al.*, 1987).

In closing, it should be noted that the present data do not clarify whether reduced IFN-γ production by mSC in the presence of *T. cruzi* is an indirect consequence of infection of accessory cells, e.g. macrophages, by the parasite.

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RESPONSE OF LYMPHOCYTES TO A MITOGENIC STIMULUS DURING SPACEFLIGHT

Gerald Sonnenfeld
Department of Microbiology and Immunology
and
Department of Oral Health,
Schools of Medicine and Dentistry
University of Louisville
Louisville, KY 40292

ABSTRACT

Several studies have been carried out that demonstrate that immunological activities of lymphocytes can be affected by spaceflight or by models that attempt to simulate some aspects of weightlessness. Included among these are the responses of lymphocytes to external stimuli such as mitogens and viruses. When cultures of lymphocytes were flown in space, the ability of the lymphocytes to respond to mitogens was inhibited. Similar results were obtained when lymphocytes from astronauts or animals just returned from space were placed into culture immediately upon return to earth, and when models of hypogravity were used. Lymphocytes placed in culture during spaceflights produced enhanced levels of interferon compared to control cultures. When cultures of lymphocytes were prepared from cosmonauts or rodents immediately upon return to earth, interferon production was inhibited. These results suggest that space flight can have profound effects on lymphocyte function, and that effects on isolated cells may be different from that on cells in the whole organism.

INTRODUCTION

Over the years, it has become apparent that spaceflight can have profound effects on biological systems. Included among those systems is the immune system of mammals (Barone and Caren, 1984; Jackson and Warner, 1986). In most cases, suppression of immune responses has occured, but there have been occasional reports of immune enhancement (Barone and Caren, 1984; Jackson and Warner, 1986). Similar results have occurred when ground-based models of weightlessness have been utilized.

The mechanism of the effects of spaceflight on immune responses remains to be established. Weightlessness, stress, and low-level radiation could all contribute to alterations in immune responses. Although studies on the effects of spaceflight on immune responses have been limited, some interesting observations have been made. In this monograph, I will review the effects of spaceflight and modeling of weightlessness on lymphocyte function as determined by the response of the lymphocytes to external stimuli such as mitogens.

EFFECTS OF SPACEFLIGHT AND MODELING ON THE BLASTOGENIC RESPONSE OF LYMPHOCYTES

Several studies have been carried out by obtaining the blood of astronauts/cosmonauts immediately after return from spaceflight. Blood was

also obtained from astronauts and cosmonauts before flight, and in some cases, during flight, to allow for the determination of the kinetics of changes in immune responsiveness. In these experiments, white blood cells were separated from the blood and placed in tissue culture. Mitogens, such as phytohemmaglutinin or concanavalin-A were added to the cultures. Over time, lymphocytes from normal individuals would divide and incorporate ^{3}[H]-thymidine, indicating a blastogenic response of the lymphocytes to the mitogen. The blastogenic response to lymphocytes requires interaction with another cell type, the macrophage, as well as interaction with soluble regulatory factors known as cytokines. The blastogenic response and the production of cytokines are indications of a normal functioning immune system.

Several experiments were carried out to determine the effects of spaceflight on lymphocyte blastogenesis. In most cases (Table 1), the blastogenic response of lymphocytes to mitogens was inhibited severely in cells obtained from individuals immediately after return to earth (Fischer et al., 1972; Kimzey et al., 1975 and 1976; Criswell and Cobb, 1977; Lesnyak and Tashputalov, 1981; Taylor, 1983; Taylor and Dardano, 1983; Konstantinova et al., 1985; Taylor and Neale, 1986). The duration of the flights was from several days to several months. Recent reports (Taylor, 1983; Taylor and Dardano, 1983; Taylor and Neale, 1986) have also indicated decreased levels of circulating monocytes in astronauts after spaceflight (Table 1). Since the monocyte is an important accesory cell for the blastogenic response of lymphocytes, this could have contributed to the suppression observed.

While the results described above indicate that blastogenesis of lymphocytes in response to mitogens was inhibited when the cells were taken from individuals immediately after return from space, the question still remained whether spaceflight could affect blastogenesis of lymphocytes actually held in tissue culture during spaceflight. This question was addressed by a series of experiments using simulation and actual flight studies carried out by Cogoli and his associates.

Human peripheral blood leukocytes were placed in culture in a fast-rotating clinostat. This clinostat has constantly changing gravity vectors, and has been used as a technique for simulating microgravity conditions (Cogoli et al., 1980). Lymphocyte blastogenesis was inhibited greatly when the cells were maintained in this clinostat (Table 2) (Cogoli et al., 1980).

In addition, an incubator was developed that allowed the performance of similar experiments during spaceflight. A drastic inhibition of lymphocyte blastogenesis was observed when human peripheral blood leukocytes were placed in culture and challenged with mitogen during space flight (Table 2) (Cogoli and Tschopp, 1984 and 1985; Tschopp and Cogoli, 1984). When the cells were incubated in a 1 G centrifuge during spaceflight, much of the blastogenic capacity was retained (Table 2), indicating that the microgravity conditions of spaceflight contributed to the inhibited blastogenesis that was observed during spaceflight (Cogoli and Tschopp, 1984 and 1985; Tschopp and Cogoli, 1984).

EFFECTS OF SPACEFLIGHT AND MODELING ON THE PRODUCTION OF INTERFERON AND OTHER CYTOKINES BY LYMPHOCYTES

Several experiments were also carried out to determine the effects of spaceflight on cytokine production by lymphocytes after mitogenic or antigenic stimulus. Cytokines are molecules that are produced by cells that are

important messengers for the development of immune responses. Without them, lymphocytes and monocytes cannot communicate effectively with each other and immune responses cannot be mounted. The cytokines that have been utilized for space studies are the interferons, important antiviral, anti-cancer and immunoregulatory molecules, and interleukin-3, an important immunoregulatory molecule.

In an Hungarian-Soviet study, blood was removed from cosmonauts and peripheral blood leukocytes were placed in culture during spaceflight (Talas et al., 1983 and 1984). When the cells were challenged with a variety of mitogens and other interferon inducers such as purified protein derivative of Mycobacterium tuberculosis, Newcastle disease virus, and polyriboinosinic-polyribocytidylic acid, interferon-alpha production was enhanced compared to ground controls (Table 3). However, when peripheral blood leukocytes were harvested from cosmonauts immediately upon return to earth after spaceflight, interferon-alpha production in response to Newcastle disease virus challenge of leukocytes was inhibited severely (Table 3) (Talas et al., 1983 and 1984). The number of replicates in this series of experiments was small, and extensive time course experiments to determine how interferon production would have varied in cell cultures from the same individuals on the ground were not carried out. Nevertheless, these experiments suggest that the in vitro response of lymphocytes to spaceflight may differ from the effects of spaceflight on lymphocytes of the intact host.

Inhibited interferon production after simulated weightlessness and spaceflight of animals was also observed. In the first set of experiments, rats and mice were maintained in an antiorthostatic, hypokinetic, hypodynamic supension system that models some aspects of weightlessness (Morey-Holton and Wronski, 1981; Musacchia et al., 1980; Steffen et al., 1984). In this model, the rodents are suspended with a head-down tilt and no load bearing on the hind limbs. This results in simulation of some of the effects of microgravity. When the mice or rats were challenged with polyriboinosinic-polyribocytidylic acid, there was inhibited interferon-alpha/beta production in antiorthostatically suspended rodents compared to normally housed controls (Table 4) (Sonnenfeld et al., 1982; Rose et al., 1984). The inhibition was transient, as a return to normal caging after suspension resulted in recovered ability to produce interferon. Suspension in an orthostatic fashion (no-head down tilt), which does not simulate the effects of microgravity, had no effect on the capacity of mice to produce interferon-alpha/beta (Table 4) (Rose et al., 1984). It must be noted that when animals are challenged systemically with an interferon inducer such as polyriboinosinic-polyribocytidylic acid, many cell types other than lymphocytes can be induced to produce interferon-alpha/beta. Therefore, these experiments went beyond just measuring the effects of suspension on lymphocyte responses to mitogenic stimuli.

In a second series of experiments, rats were flown in Space Shuttle SL-3. Upon return to earth, spleen cells containing lymphocytes were harvested, placed in culture, and challenged with the mitogen concanavalin-A (Gould et al., 1987). After the appropriate period of incubation, the cell culture supernatant fluids were harvested and assayed for production of two cytokines, interferon-gamma and interleukin-3. Interleukin-3 is another important messenger produced by lymphocytes after mitogenic challenge, providing immunologically significant signals to cells (Gould et al., 1987). Cells from rats that had been flown for one week showed very significant inhibition of the production of interferon-gamma, but no effect on

interleukin-3 production (Table 5) (Gould et al., 1987). The results with the interferon-gamma supported previous findings in human flight and rodent suspension studies indicating that interferon-alpha/beta was inhibited. However, the lack of effect of spaceflight on interleukin-3 production indicates that all responses of lymphocytes to mitogens are not affected in the same fashion by spaceflight.

CONCLUSIONS

The studies described above indicate that spaceflight and models that simulate microgravity can have profound effects on the response of lymphocytes to mitogens. The effects of spaceflight appear to be selective, in that all responses of lymphocytes to mitogens are not affected in a similar fashion. In addition, the effects of spaceflight on isolated lymphocytes in culture may differ from effects when lymphocytes are in vivo in a whole animal surrounded by other cells, soluble messengers and interact with systems other than the immune system.

The mechanism of the effects of spaceflight on immune responses remains to be established. Several possiblities exist. Among them are: 1) direct effects of microgravity on lymphocytes, 2) inability of lymphocytes to interact directly with other cell types such as monocytes/macrophages, 3) inability of lymphocytes to produce cytokines, 4) inability of lymphocytes to respond to signals from cytokines, 5) inability of antigenic or mitogenic signals to reach lymphocytes because of fluid-shifts induced during spaceflight, and 6) impaired function of lymphocytes because of faulty interaction with other non-immunological systems such as the neuroendocrine system. Other potential mechanisms surely exist. The study of these mechanisms should progress with time.

Determination of the effects of spaceflight on lymphocytes should yield other fascinating information. Since the immune system is responsible for resistance to infection, the study of lymphocytes should help to determine if long-term exposure to spaceflight conditions could compromise resistance. The ability to produce large amounts of cytokines as a result of genetic engineering probably indicates that enhanced production of cytokines as a result of spaceflight will not be an effective technique for mass production of cytokines. However, studying the response of lymphocytes to spaceflight may aid in our understanding of how the immune response is regulated and may allow the discovery of new cytokines whose actions are masked in normal ground conditions.

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TABLE 1

EFFECTS OF SPACEFLIGHT ON THE ABILITY OF SUBJECTS' CELLS TO RESPOND TO
MITOGENS UPON RETURN TO EARTH

Effect on Blastogenesis	Effect on Monocyte Number	Reference
None	Not Tested	Fischer, 1972
Inhibited	Not Tested	Kimzey, 1975-6
		Criswell, 1977
		Lesnyak, 1981
		Konstantinova 1985
Inhibited	Decreased	Taylor, 1983, 1983, and 1986

TABLE 2

EFFECTS OF SPACEFLIGHT ON IN VITRO BLASTOGENESIS

Effect on Blastogenesis	Effect of Centrifugation	References
Inhibited	Restored	Cogoli, 1984 and 1985
		Tschopp, 1984
HYPOGRAVITY DUE TO CLINOSTAT ON THE GROUND		
Inhibited		Cogoli, 1980

TABLE 3

EFFECT OF SPACEFLIGHT ON HUMAN INTERFERON PRODUCTION

Situation	Effect on Interferon-Alpha	Reference
Leukocytes in Culture in Space	Enhanced	Talas, 1983 and 1984
Leukocytes Harvested after Return from Space	Inhibited	Talas, 1983 and 1984

TABLE 4

EFFECTS OF ANTIORTHOSTATIC SUSPENSION ON INTERFERON PRODUCTION

Treatment	Effect on Interferon-Alpha/Beta	Reference
Rat - 2 week	Inhibited	Sonnenfeld, 1982
Mouse - 1 week	Inhibited	Rose, 1984
Mouse - 1 week + 1 week normal cage	Recovered	Rose, 1984
Mouse - 1 week orthostatic suspension	None	Rose, 1984

TABLE 5

EFFECT OF SPACEFLIGHT ON RAT CYTOKINE PRODUCTION

Duration of Flight	Cytokine	Effect	Reference
1 week	Interferon-gamma	Inhibited	Gould, 1987
1 week	Interleukin-3	Normal	Gould, 1987

A ground-based model to study the effects of weightlessness on lymphocytes

Felix K Gmünder [1], Markus Kiess [1], Gerry Sonnefeld [2], Juliet Lee [1], Augusto Cogoli [1]

[1] *Institute of Biotechnology, Space Biology Group, ETH-Zürich, CH-8093 Zurich, Switzerland;*
[2] *Dept of Microbiology and Immunology, University of Louisville, K 40292, USA*

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Summary — The mitogenic response of human lymphocytes was found to be markedly reduced in weightlessness conditions as compared to normal gravity. One possible explanation is that due to the non-existent sedimentation in space the lymphocytes could not adhere and spread on a substratum. Thus, we investigated the effect of substratum adhesiveness on lymphocyte responsiveness by reducing and blocking cell adhesion with poly-HEMA in a simple on-ground system. Lymphocyte adhesiveness was assessed by measuring the proportion of non-adhesive, slightly, and strongly adhesive ^{51}Cr-radiolabelled cells on uncoated and poly-HEMA coated plastic. The amount of cell spreading on surfaces with varying adhesiveness was determined by measuring the area of cells. Cells grown on medium and thick poly-HEMA films were rounded in shape. By contrast, on tissue culture plastic, they showed clear signs of spreading. The mitogenic response of lymphocytes grown on thick poly-HEMA films was reduced by up to 68% of the control (tissue culture plastic). Interferon-γ production was virtually nil when the cells were grown on the least adhesive substratum. These results show that activated lymphocytes need to anchor and spread prior to achieving an optimal proliferation response. We conclude that decreased lymphocyte adhesion could contribute to the depressed *in vitro* lymphocyte responsiveness found in the microgravity conditions of space flight.

lymphocyte / activation / adhesion / poly-HEMA / space flight / microgravity

Introduction

The *in vitro* responsiveness of human lymphocytes to mitogenic lectins is remarkably reduced in weightlessness (microgravity). On 2 Shuttle missions we have unequivocally demonstrated that the mitogenic response to con A was severely reduced by 90–97% under microgravity conditions compared with a 1 g control [2, 8, 9]. These observed *in vitro* effects in space have been explained as being due to reduced cell-cell contacts [20], and other, still unidentified factors [9]. However, an analysis of the cell aggregates using electron microscopy in flight and ground samples suggested that the cell-cell interactions were not affected by microgravity conditions [8, 9].

The proliferation of most cells is dependent upon attachment to a substratum. Folkman and Moscona [11] studied the role of cell shape in determining growth characteristics of attachment-dependent mammalian cells. They decreased the adhesiveness of tissue culture plastic by applying increasing concentrations of poly-HEMA to control the extent of cell spreading. Cells grown on the least adhesive substrata were rounded in shape whereas those grown on more adhesive substrata were flatter. Cell growth, measured by DNA synthesis, was highly correlated with cell shape. The flattened cells had significantly higher

growth rates than the rounded ones. Thus, cell adhesion and spreading are essential for optimal growth of mammalian attachment-dependent cells (bovine aortic endothelial cells, WI-38 cells, A-31 cells, and Swiss 3T3 cells).

Research in the field of lymphocyte contacts has focused upon lymphocyte-cell interactions. *In vitro* studies have shown the significance of cell-cell contacts for optimal antigenic or mitogenic stimulation of lymphocytes [16, 21]. At least two types of such interactions have been identified. Firstly, recirculating lymphocytes leave the blood by adhering to and migrating between endothelial cells in specific organs [14], in particular lymphoid sites, which have been termed lymphocyte homing [12]. The specific cell surface structures lymphocytes use for this process have been called homing receptors [12]. On lymphocytes, two groups of heterodimer receptors were found that are similar to extracellular matrix receptors from mammalian cells [4, 22]. One group includes the lymphocyte function-associated antigen LFA-1, Mac-1, and the p150,95 complex; the other group comprise the "very late antigens" (VLA-1 to VLA-5). Secondly, the CD3-T cell receptor complex of helper T cells recognize fragments of foreign antigens in association with class II MHC proteins on the surface of accessory cells [6, 25]. Thus, interactions between adhesion proteins and receptors involved in lymphocyte-cell contacts are assumed to play a vital role in the early events of an immune response.

Regarding lymphocyte-substratum interactions, the present body of knowledge is more limited. Sundqvist and Wanger [23], and Wanger and Sundqvist [26] reported that human lymphocytes attach and spread on glass and plastic surfaces. Moreover, the mitogenic response of lympho-

cytes was lower on bacterial plastic compared with tissue culture plastic [23]. This finding suggests that lymphocyte adhesion and spreading are related in order to achieve an optimal proliferation response. However, nothing is known as to how a complete blocking of lymphocyte adhesion, attachment and spreading affect the proliferation response. This is important to know in order to analyze earlier space flight experiments and to devise future *in vitro* lymphocyte studies in space. Recent *in vitro* experiments performed in weightlessness [2, 8, 9] indicate that lack of sedimentation and convection could markedly modify the mitogenic response of lymphocytes by reducing cell-substratum contacts. Thus, the depressed lymphocyte responsiveness observed *in vitro* under microgravity conditions could result from changes in the lymphocyte surface milieu and cell shape. This underscores the need to continue to clarify the effect of lymphocyte-substratum adhesion on responsiveness. We reduced the adhesiveness of tissue culture plastic by applying increasing concentrations of poly-HEMA. We have measured amounts of lymphocyte adhesion, and correlated these to changes in cell area as a means to estimate spreading, responsiveness, and interferon-γ production. This allows us to, in part, explain the results of lymphocyte *in vitro* experiments performed during space flight.

Materials and methods

Preparation of human peripheral lymphocytes

Lymphocytes were obtained from healthy donors by Ficoll-Hypaque gradient centrifugation [3, 19]. After determining the viable cell number (trypan blue exclusion) the cells were resuspended in 2–20 ml RPMI-1640 (Gibco, Paisley, Scotland, cat no 042/02511H) medium supplemented with 40 mM Hepes (Sigma, St Louis, USA, cat no H 3375), 5 mM $NaHCO_3$, 50 mg l^{-1} gentamicin (Biochrom KG, Berlin, Germany, cat no A 2712), 20% pooled type 0 human serum, and 4 mM glutamine (Biochrom KG, Berlin, Germany, cat no K 0282).

Lymphocyte proliferation assay

The procedure has been described earlier [2, 8]. Briefly, 10^6 lymphocytes in 1 ml (in triplicate) were stimulated in multidishes (Nunc, Roskilde, Denmark, cat no 1-43982) by the addition of 25 μg ml^{-1} con A (Pharmacia, Uppsala, Sweden, cat no 17-0450-01). After incubation for 72 h the rate of DNA synthesis was determined by the incorporation of ^{3}H-labelled thymidine (Amersham, Buckinghamshire, UK, TRK. 418). For this, the lymphocytes were resuspended and transferred from the wells into 5 ml tubes. 20 μl labelled thymidine (2 μCi) was added to 1 ml lymphocyte culture which was incubated for 2 h at 37°C. The labelling was stopped by the addition of 3 ml ice cold saline (9 g/l). Following rapid filtration over Whatman GF/C filters and 2 rinses of the tubes with 3 ml saline, the DNA was precipitated on the filters with 2 washes with 3 ml ice-cold trichloroacetic acid (5%) and 2 washes with 3 ml ice-cold ethanol. Radioactivity retained by the dried filters was counted in a scintillation spectrometer (Beckman Scientific Instruments, Irvine, USA, model LS 1800).

^{51}Cr labelling

Lymphocytes were labelled with ^{51}Cr by incubation of cells at 12.5 × 10^6 cells ml^{-1} with 250 μCi ml^{-1} $Na_2^{51}CrO_4$ (Amersham, Buckinghamshire, UK, cat no CJS.4) for 90 min at 37°C in medium without supplements [10, 27]. After washing the cells 2-times with medium the supplements were added and the cell density was adjusted.

Poly-HEMA coating of multidishes

Slideflasks (Nunc, Roskilde, Denmark, cat no 1709210) and multidishes were coated with dilutions of poly-HEMA (Aldrich Chemical Company, Milwaukee, USA, cat no 19206) according to Folkman and Moscona [11]. 6 g poly-HEMA was dissolved in 50 ml 95% ethanol and the mixture was slowly stirred overnight at 37°C. The viscous solution was then centrifuged at 2500 rpm for 30 min to remove possible particles. This stock solution was diluted 10-, 100-, and 10000-fold with ethanol (termed PH1, PH2, and PH4, respectively). 200 μl or 947 μl diluted poly-HEMA was filled in triplicates into the wells of the multidishes (1.9 cm^2) and slideflasks (9 cm^2), respectively. The culture vessels were dried (lids open) at room temperature for 48 h in a sterile workbench free of vibrations.

Lymphocyte adhesion assay

10^6 ^{51}Cr-labelled cells in 1 ml of medium were activated adding con A (25 μg/l) and placed into the wells of multidishes (in triplicate). The cultures were incubated for 4 h at 37°C. First, 50 μl of the supernatant without cells were sampled to check the viability of the lymphocytes [27]. This cell viability test relies on the fact that chromate is taken up by living cells and reduced by the metabolism. The reduced form remains trapped within the cells, unless the cell membrane becomes leaky or the cell lyses. Thus, radioactivity detected in a cell culture supernatant of ^{51}Cr-tagged cells is a means to check cell viability [27]. The rest of the supernatant and nonadhering cells were then aspirated (dishes tipped at an angle of 30°) using a pipette (samples 1), and replaced by pre-warmed HBSS. After shaking the dishes on a gyratory shaker for 15 s (220 rpm, 4 mm amplitude) to suspend loosely bound cells, the supernatant was removed again (sample 2). Lastly, adhering lymphocytes were lysed with NaOH (950 μl, 2 M) (sample 3). Radioactivity in the samples were determined in a γ-counter. The proportion of cells in the 3 samples was calculated by relating the radioactivity recovered in the 3 fraction to the original radioactivity in the total cell suspension (100%).

Lymphocyte spreading

Immediately after the addition of con A the cell suspension (4 ml containing 4 × 10^6 cells) was loaded into slideflasks and incubated for 18 h at 37°C. The cells were fixed by the addition of 800 μl glutaraldehyde (10%) for 20 min. After removing the liquid by aspiration, the bottom of the slideflasks, where the cells had bound, was separated and used as a microscopy slide. The fixed cells were allowed to dry at a room temperature, stained with Giemsa, mounted in a light microscope (1250-fold magnification), and photographed. The cell area of 80–100 lymphocytes from 4 different sites was determined on a graphic tablet (Summagraphics) connected to a Macintosh computer (Software: MacDraft and StatView) by following the lymphocyte contours.

Cytotoxicity and mitogenicity of poly-HEMA

Cytotoxic and mitogenic effects of poly-HEMA *per se* on human lymphocytes was tested as follows: ^{51}CrO₄-labelled lymphocytes were incubated in poly-HEMA coated multidishes (PH1, 2, and 4) and radioactivity in the supernatant to check for cell viability was assessed after 4 and 72 h [27]. To investigate whether poly-HEMA is mitogenic for human lymphocytes, the lymphocyte responsiveness was measured in poly-HEMA coated dishes (PH1, 2, and 4) and compared with the mitogenic responsiveness in uncoated dishes without the addition of con A.

Determination of interferon-γ concentrations

Interferon-γ is a T-lymphocyte specific lymphokine released into the culture medium following antigen or mitogen stimulation. Following an incubation of 72 h at 37°C, culture supernatants were frozen and stored at − 20°C until ready for analysis.

Interferon-γ was determined in the supernatants by a radioimmunoassay method (Centecor, Inc, Malvern, PA, USA).

Statistical analysis

Nonparametric test techniques for non-matched samples were applied. The Kolmogorov-Smirnov test (2-tailed) checks for differences in the median and homogeneity of all sort, whereas the Wilcoxon-Mann-Whitney U-test (2-tailed) is more powerful in detecting differences in the medians. P levels ≥ 0.05 were accepted as significant.

Results

We first tested whether poly-HEMA is cytotoxic or mitogenic for human lymphocytes. The ^{51}Cr-radioactivity recovered from lymphocyte culture supernatants of cells grown on poly-HEMA never exceeded the values of uncoated controls (5 experiments, results not shown). Since ^{51}Cr is retained by viable cells, but not by leaky or lysed cells, this finding means that poly-HEMA coated culture surfaces are not cytotoxic for human lymphocytes. To detect a possible mitogenic effect of poly-HEMA, the incorporation of acid-precipitable radiolabelled thymidine following a 72 h incubation of unstimulated cells placed on uncoated plastic and poly-HEMA coated surfaces was measured. No significant differences was found between the two sets of experiments (5 experiments, results not shown). Therefore, the dose of poly-HEMA used in our experiments is neither mitogenic nor toxic to human lymphocytes.

The effect of increasing the thickness of the poly-HEMA film on adhesiveness of con A activated lymphocytes is illustrated in figure 1. The proportion of non-adhering cells (represented by sample 1) increased with increasing thickness of the poly-HEMA films. In the control, 23% of the radioactivity was found in sample 1, compared to 74% of the highest poly-HEMA concentration used (PH1). Radioactivity in sample 2 (loosely bound cells) was highest in the wells with the intermediate poly-HEMA dilutions (PH2). The proportion of adhering cells (represented by sample 3) increased with increasing adhesiveness of the substratum. Almost no cells attached in the wells coated with the thickest polymer film (3%); 22% and 42% of the cells were recovered from the wells with the thinner coatings, PH2 and PH4, respectively. In the control, 59% of the original radioactivity was recovered in this fraction. Clearly, an inverse relationship exists between the thickness of the poly-HEMA film and lymphocyte adhesiveness.

The influence of substratum adhesiveness on the shape of activated lymphocytes was determined by assessing the cell area of adhering lymphocytes under a light microscope as an index of the extent of lymphocyte spreading (figure 2). We were unable to estimate the area of lymphocytes when using the thickest poly-HEMA layer as a substratum (PH1) since lymphocyte adherence was so poor that too few cells would be available for statistical analysis. By contrast, adhesion to the thinest film used (PH4) did not reveal any change in cell area compared with the control (data not shown). However, a statistically significant difference in the area of attached lymphocytes was detected on the medium dense polymer film (PH2). A significantly higher proportion of activated lymphocytes was spherical and smaller in area compared with a control after culturing for 18 h on poly-HEMA (PH2) in 5 independent experiments (table I).
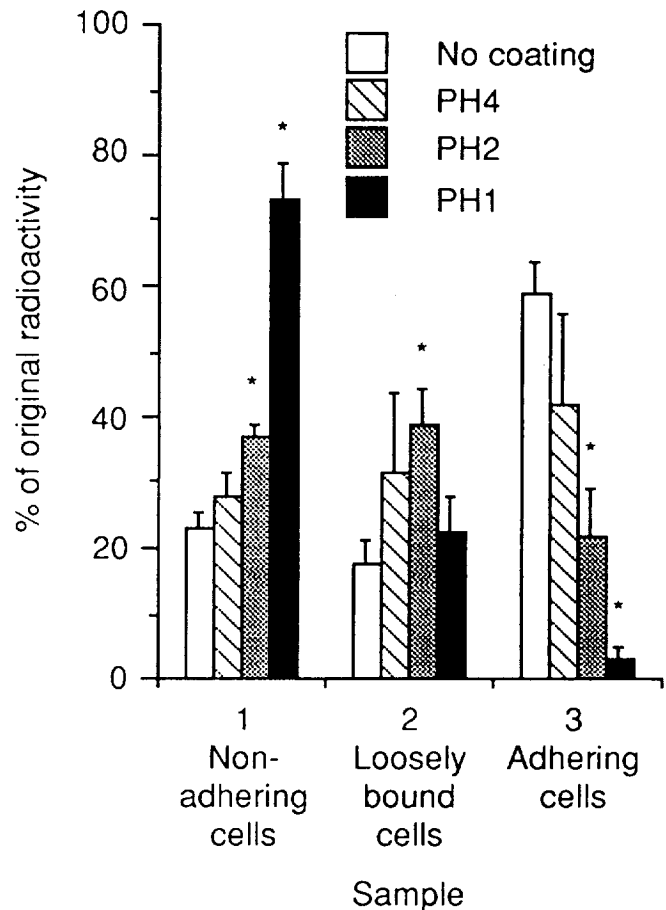
Fig 1. Effect of substratum adhesiveness on lymphocyte adhesion. ^{51}Cr-labelled lymphocytes were activated with con A and seeded on uncoated plastic (control) and on three different poly-HEMA films with decreasing adhesiveness (PH4, PH2, and PH1, which refer to a 10'000-, 100-, and 10-fold dilution of the poly-HEMA stock solution). Following an incubation of 4 h at 37°C, for each type of coating, the amount of radioactivity was measured in the three cell samples as indicated below the graph. The amount of radioactivity recovered in these 3 samples was expressed as a percentage of the total amount or radioactivity used in the amount of cells to be seeded on a specific type of coating. Each value is the mean ($\pm$ sd) from 5 experiments (ie 5 different donors). Statistical significance relative to the control was tested using the Mann-Whitney-U-test (2-tailed; $*P \leq 0.005$).

Table I. Effect of substratum adhesiveness on cell area. Immediately after con A addition, lymphocytes were plated on culture plastic or poly-HEMA coated plastic. The mean of 5 experiments (ie 5 different donors) is displayed. The median is given in brackets. Significant differences between control and assay were identified using nonparametric statistics (Kolmogorov-Smirnov-test).

	Cell area (μm^2)		
Exp (Donor)	No coating (control)	poly-HEMA (PH2) coating	Statistical analysis
1	44.1 (39.2)	36.0 (36.7)	*
2	48.1 (43.1)	40.5 (39.5)	**
3	43.1 (40.6)	35.8 (35.1)	**
4	49.5 (43.4)	39.3 (37.0)	**
5	47.8 (42.8)	39.6 (37.8)	**

$P \leq 0.005$; $**P \leq 0.001$

It is interesting that no large cells (> 83 μm^2) were observed in the poly-HEMA coated wells. By contrast, in all 5 experiments the occurrence of large lymphocytes was noted in the control wells. On average, 13% of the cells in the uncoated controls exhibited an area of between 83 and 165 μm^2. Furthermore in the uncoated wells the main peak of areas generally showed a shift in the distribution of cell size which included larger lymphocytes. This was never observed in the poly-HEMA coated wells. Likewise, only the lymphocytes grown in the control wells showed the typical signs of cell spreading (a broad rim of cytoplasm around the nucleus and filopodia). These findings suggest that the lymphocytes cultured on poly-HEMA could not anchor and spread. In attachment-dependent cells, proliferation is related to anchorage and spreading. We then tested the effect of reduced anchoring and spreading on activated lymphocytes and measured proliferation by the uptake of radiolabelled thymidine after 72 h (fig 3).

We determined that lymphocyte proliferation is inversely related to the thickness of the poly-HEMA film. Lymphocyte responsiveness was reduced by up to 68% of the control at PH1 (fig 3). At the lowest poly-HEMA dilution (PH4), lymphocyte responsiveness was unaffected.

When cell-substratum interactions are reduced after con A activation, lymphocytes cannot proliferate normally. Consequently, other lymphocyte activation events associated, such as interferon-γ production, should also be affected. In 2 separate experiments the production of interferon-γ was greatly reduced after plating con A activated lymphocytes on the thickest poly-HEMA coated plastic (table II).

Discussion

Poly-HEMA is a hydrophobic polymer that prevents cells from anchoring to and spreading on the coated surface. With increasing concentration of the polymer solution applied, there is a smaller left suitable area for establishing contact. Poly-HEMA is not cell-toxic as Folkman and Moscona [11] and as confirmed by us. In addition, Poly-HEMA has no mitogenic effect on human lymphocytes. Non-toxicity and non-mitogenic of poly-HEMA are important pre-requisites for the experiments presented here.

Our results obtained *in vitro* show that the mitogenic response of human peripheral blood lymphocytes is markedly reduced when cell adhesion to the substratum is excluded. Previous studies [23, 26] have shown that human lymphocytes attach to adequate substrata and display signs of cell spreading. However, the effect of completely preventing lymphocyte adhesion to a substratum on lymphocyte responsiveness has not been assessed before. This is important with respect to *in vitro* flight experiments because under microgravity conditions, lack of sedimenta-
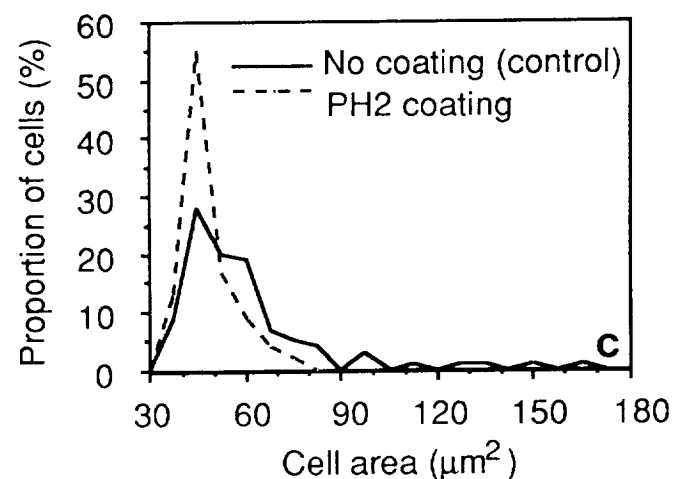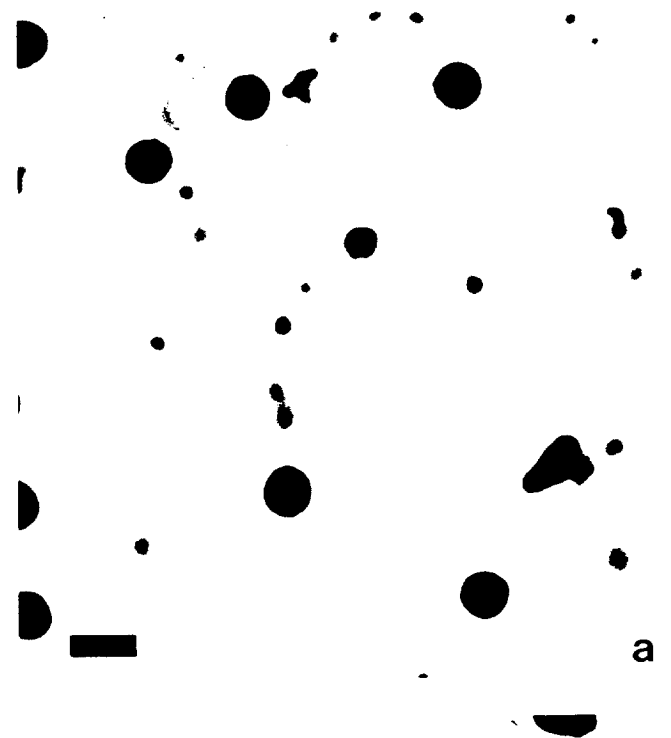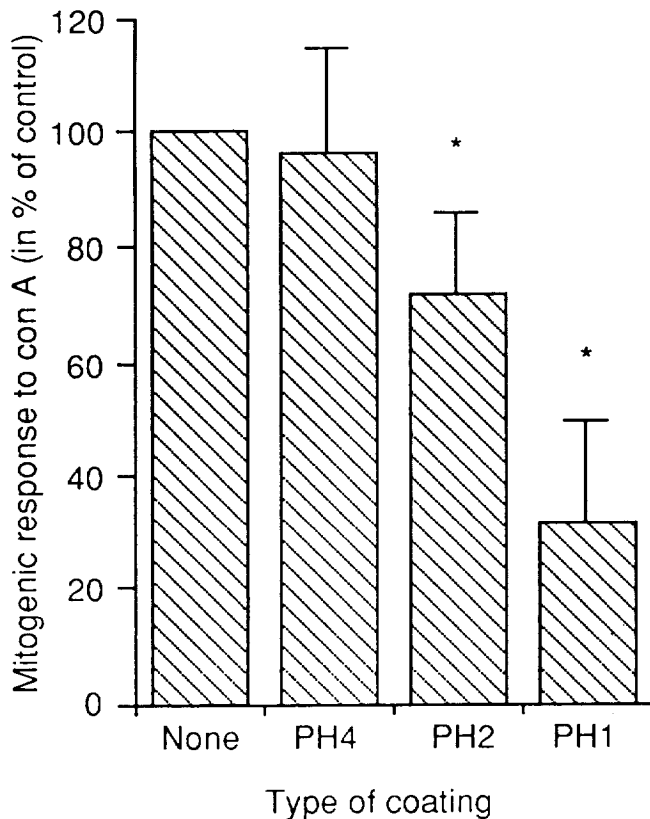

Fig 2. Effect of substratum adhesiveness on cell shape and frequency distribution of lymphocyte area. a. Lymphocytes placed on PH2. The cells remain rounded and small. b. Lymphocytes placed on tissue culture plastic. A considerable proportion of cells show the typical signs of cell spreading. c. Frequency distribution of lymphocyte area. A typical analysis of an experiment is depicted. In the uncoated control (*cf* fig 2b) a large portion of spread, large cells were found. In the coated sample (*cf* fig 2a) no large cells were found. These differences are significant (table I). Bar represents 10 μ.

Table II. Effect of substratum adhesiveness on interferon-γ production in con A activated lymphocytes. Stimulated and unstimulated lymphocytes were seeded on uncoated and poly-HEMA coated plastic as indicated. Interferon-γ produced by the lymphocytes was determined in the culture supernatants following an incubation of 72 h at 37°C.

		No coating (Units/ml)	PH4 (Units/ml)	PH2 (Units/ml)	PH1 (Units/ml)
With con A	Exp 1	74	69	44	9
	Exp 2	99	105	103	32
No con A	Exp 1	2	2	0	0
	Exp 2	5	2	2	1

Fig 3. Effect of poly-HEMA coated substratum on lymphocyte responsiveness to con A. Con A activated lymphocytes were seeded on uncoated plastic (control) and plastic with increasing thickness of the poly-HEMA film (PH4 to PH1). Substratum adhesiveness decreases with increasing thickness of the poly-HEMA layer. Following an incubation of 72 h at 37°C, lymphocyte proliferation was measured as the radioactivity incorporated into trichloroacetic acid-precipitable material. The mean (± sd) of 6 experiments (ie 6 different donors) is shown. In the control assays (no coatings) the cpm values of the incorporated radioactivity ranged between 130 000 and 313 000 for 10^6 cells. The incorporated radioactivity in the poly-HEMA coated dishes was expressed as a percentage of the corresponding control (100%). For statistical analysis the Mann-Whitney-U-test (2-tailed) was used ($*P \leqslant 0.01$).

tion and convection could prevent cell-surface interactions [2, 8, 9].

Our findings are similar to those reported by Folkman and Moscona [11] using non-transformed mammalian cells. Thus, in both non-transformed mammalian cells and human lymphocytes, cell attachment is indispensable prior to achieving optimal proliferation. Likewise, in both studies, the magnitude of the proliferation response is related to cell-substratum adhesiveness. The results suggest that lymphocytes do not differ greatly from non-transformed mammalian cells with regard to attachment and spreading. However, the present lack of data do not allow us to conclude that with lymphocytes there is a such close correlation between cell shape and proliferation rate as it was found in many other non-transformed mammalian cells [11]. Our data are consistent with an earlier finding that binding of lymphocytes to fibroblasts is improved following stimulation [1].

The results presented here can, in part, explain data of lymphocyte studies performed in space. In 3 independent in vitro experiments in space we have shown that lymphocyte activation is depressed by 90–97% compared with 1 g controls [2, 8, 9]. It is important to point out that normal cell-cell interactions were observed in space [8, 9] which implies that other factors than cell-cell contacts prevail in affecting lymphocyte responsiveness. However, the depression of lymphocyte responsiveness was more pronounced in space (90–97%) compared with even the thickest poly-HEMA film used (68%). This indicates that under weightlessness, cell adhesion and shape are even more affected, or that factors other than adhesion contribute to the reduced in vitro responsiveness. The effect of cell adhesion on lymphocyte responsiveness under microgravity conditions will be tested during the next Spacelab mission (SLS-1). Cytodex microcarriers will be added to the lymphocyte culture to allow for adhesion in microgravity (Lee and Cogoli, in preparation). Thus, we will determine to what extent mitogen-induced responsiveness is restored when lymphocytes bind to an adhesive substratum in space.

An interesting issue is the relation between the in vitro studies addressed in this paper and the depressed lymphocyte responsiveness to mitogenic lectins found in ex vivo lymphocyte cultures from astronauts during and after space flights. This finding was first reported by Soviet immunologists in 1973 [18], confirmed following US investigations on Skylab astronauts [17], and observed in the majority of Space Shuttle crew members [24]. Although such an impairment of lymphocyte function may possibly lead to lowered immunity, the astronauts and cosmonauts have maintained good health, both during and following their flights, with the exception of minor problems with respiratory tract infections and viral gastroenteritis on the Apollo flights [15] and a case of pneumonia on a Soviet Soyuz-Saljut mission [5]. Cogoli and Tschopp [7] have cautioned that one should not extrapolate from data obtained in in vitro experiments to the depressed ex vivo lymphocyte responsiveness in astronauts observed after space flight. It is more likely that the multiple stressors associated with space flight contribute to the depressed immunity observed in astronauts [13, 24]. It is conceivable that in in vitro studies lymphocytes obtained from relaxed donors are not primed by stress-associated hormones [13, 24, Gmünder et al, submitted]. Thus, the effect of space flight on lymphocyte function warrants more detailed investigations considering the ambitious activities planned for long-term flights on space stations and interplanetary travels.

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REDUCED LYMPHOCYTE ACTIVATION IN SPACE: ROLE OF CELL-SUBSTRATUM INTERACTIONS

F.K. Gmünder[1], M. Kiess[1], G. Sonnenfeld[2], J. Lee[1] and A. Cogoli[1]

[1] Institute of Biotechnology, Space Biology Group, ETH-Zürich, Switzerland;
[2] Dept. of Microbiology and Immunology, Univ. Louisville, Louisville, USA

Abstract

We investigated the effect of substratum adhesiveness on lymphocyte responsiveness by reducing and blocking cell adhesion with poly-HEMA (poly (2-hydroxyethyl methacrylate)) in a simple on-ground system. Cells grown on medium-thick and thick poly-HEMA films were rounded in shape and displayed no signs of spreading. By contrast, on tissue culture plastic and very thin poly-HEMA films, they showed clear signs of spreading. The mitogenic response of lymphocytes grown on thick poly-HEMA films was reduced by up to 68% of the control (tissue culture plastic). Interferon-γ production was virtually nil when the cells were grown on the least adhesive substratum. These results show that activated lymphocytes need to anchor and spread prior to achieving an optimal proliferation response. We conclude that decreased lymphocyte adhesion could contribute to the depressed *in vitro* lymphocyte responsiveness found in the microgravity conditions of space flight.

Key Words: lymphocyte, adhesion, con A, poly-HEMA, activation, spaceflight, immunity

Report

The *in vitro* responsiveness of human lymphocytes to mitogenic lectins is remarkably reduced after spaceflight. This finding was first reported by Soviet immunologists in 1973 (1), confirmed following U.S. investigations on Skylab astronauts (2), and observed on the majority of Space Shuttle crewmembers (3). Although such an impairment of lymphocyte function may possibly lead to lowered immunity, the astronauts and cosmonauts have maintained good health both during and following their flights, with the exception of minor respiratory and gastrointestinal problems on Apollo flights (4) and one reported pneumonia on a Soyuz-Saljut mission (5).

On 2 Shuttle missions we have unequivocally demonstrated that microgravity affects lymphocyte responsiveness *in vitro*. The mitogenic response to con A was severely reduced (6-8). These observed *in vitro* effects in space have been explained as being due to reduced cell-cell contacts, and other, still unidentified factors (7, 9).

The proliferation of most cells is dependent upon attachment to a substratum. Folkman and Moscona (10) studied the role of cell shape in determining growth characteristics of attachment dependent mammalian cells. They decreased the adhesiveness of tissue culture plastic

by applying increasing concentrations of poly-HEMA (poly(2-hydroxyethyl methacrylate)) to control the extent of cell spreading. Cells grown on the least adhesive substrata were rounded in shape whereas those grown on more adhesive substrata were more flattened (Fig. 1).

No poly-HEMA: Thin poly-HEMA   Thick poly-HEMA
full spreading   film: reduced   film: no spreading
                              spreading

Fig. 1. Effect of increasing thickness of a poly-HEMA films on cell shape of attachment-dependent mammalian cells. With increasing thickness, cells are more rounded in shape and growth rate decreases.

Cell growth, measured by DNA synthesis, was highly correlated with cell shape. The flattened cells had significantly higher growth rates than the rounded ones. Thus cell adhesion and spreading are essential for optimal growth of mammalian attachment dependent cells.

In vivo, a considerable proportion of lymphocytes remains suspended in the blood and lymph. *In vitro* studies have shown the significance of cell-cell contacts for optimal antigenic or mitogenic stimulation of lymphocytes (11, 12). Regarding lymphocyte-substratum interactions Sundqvist and Wanger (13) reported that the cultivation of lymphocytes on bacterial plastic petri-dishes resulted in a reduction of 10-50% of lymphocyte responsiveness compared with lymphocytes seeded on tissue culture plastic. Furthermore the authors (13) found that the lymphocytes placed on bacterial plastic did not show spreading. This finding suggests that lymphocyte adhesion and spreading is indispensable prior to achieving an optimal proliferation response. Therefore, in relation to the effects of spaceflight on the functioning of human lymphocytes in *in vitro* assays, it is interesting to know to what extent lymphocytes must depend upon attachment to a substratum and spreading before proliferation can proceed normally following antigenic stimulation and how this relates, if at all, to the *in vivo* situation.

Recent research in the field of lymphocyte contacts

has focused upon lymphocyte-cell interactions, and at least two types have been identified as a prerequisite for the early events of an immune response. Firstly, recirculating lymphocytes leave the blood by adhering to and migrating between epithelial cells in specific organs (14), in particular lymphoid sites which has been termed lymphocyte homing (15). The specific cell surface structures lymphocytes use for this process have been called homing receptors (15). On lymphocytes, two groups of heterodimer receptors were found that are similar to extracellular matrix receptors from mammalian cells (16, 17, 18). It is assumed that interactions between adhesion proteins and receptors involved in lymphocyte-cell contacts play a vital role in the early events of an immune response.

The *in vitro* experiments performed in weightlessness (6-8) indicate that lack of sedimentation and convection could markedly modify the mitogenic response of lymphocytes by reducing cell-cell and/or cell-substratum contacts (Fig. 2).
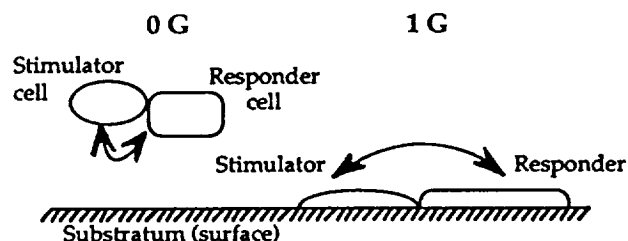
Fig. 2. *Effect of gravity on lymphocyte sedimentation and adhesion. Under microgravity conditions, stimulator cells (accessory cells like monocytes and macrophages) and lymphocytes (responders) do not sediment (diagram on the left). It is conceivable that in microgravity these cells cannot adhere, anchor and spread on the substratum. This might affect the mitogenic responsiveness. By contrast, under normal gravity cells may adhere, anchor and spread on the substratum (diagram) on the right provided that the adhesiveness of the surface is adequate.*

Thus, the depressed lymphocyte responsiveness observed *in vitro* under microgravity conditions could result from changes in the lymphocyte surface milieu. It is important to realize that we observed normal cell-cell interactions in lymphocyte cultures obtained after spaceflight (7, 8). This underscores the need to continue to clarify the effect of lymphocyte-substratum adhesion on responsiveness. We reduced the adhesiveness of tissue culture plastic by applying increasing concentrations of poly-HEMA. We have measured amounts of lymphocyte adhesion, and correlated these to changes in cell area as a means to estimate spreading, responsiveness, and interferon-gamma production. These experiments are important to help to understand earlier spaceflight results and to devise future experiments.

The effect of increasing the thickness of the poly-HEMA film on adhesiveness of con A activated lymphocytes is illustrated in Fig. 3.

The proportion of nonadhering cells (represented by sample 1) increased with increasing thickness of the poly-HEMA films. In the control 23% of the radioactivity was found in sample 1, compared to 74% of the highest poly-HEMA concentration used (PH 1). Radioactivity in sample 2 (slightly adhering cells) was highest in the wells with the intermediate poly-HEMA dilutions (PH 2). The proportion of adhering cells (represented by sample 3) increased
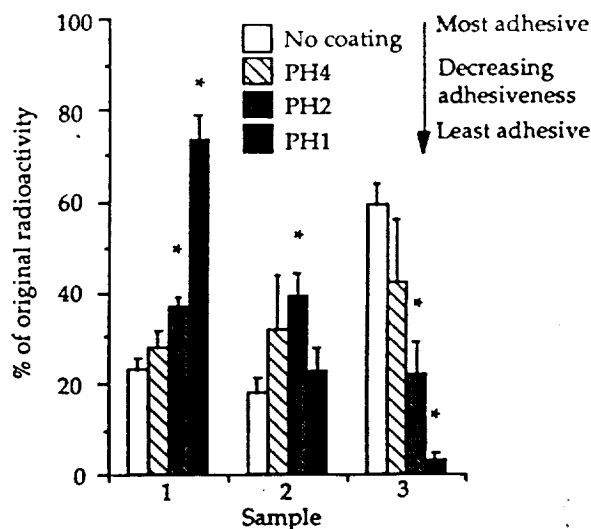
Fig. 3. *Control of lymphocyte adhesion by variation of substratum adhesiveness. Flat bottom multidishes (Nunc) were coated with increasing thickness of poly-HEMA films (10). Dilutions of the poly-HEMA stock solution (6 g/l in 95% ethylalcohol) were 10^{-4} (PH4), 10^{-2} (PH2), and 10^{-1} (PH1). Lymphocytes were prepared by Ficoll-Hypaque density centrifugation (19, 20). After labelling the lymphocytes with $^{51}Cr_2O_4$ (21, 22), 10^6 cells in 1 ml of medium were activated with concanavalin A (25 µg/l) and placed into the wells (in triplicates). The cultures were incubated for 4 h at 37 °C. First, 50 µl of the supernatant without cells were sampled to check the viability of the lymphocytes (21). Next, the rest of the supernatant and nonadhering cells were aspirated (dishes tipped at an angle of 30°) using a pipette (sample 1), and replaced by prewarmed HBSS. After shaking the dishes on a gyratory shaker for 15 s (220 rpm, 4 mm amplitude) to suspend loosely bound cells, the supernatant was removed again (sample 2). Lastly, adhering lymphocytes were lysed with NaOH (950 µl, 2 M) (sample 3). Radioactivity in the samples were determined in a g-counter. The proportion of cells in the 3 samples was calculated by relating the radioactivity recovered in the 3 fractions to the original radioactivity in the total cell suspension. Each value is the mean (± standard deviation) from 5 independent experiments using 5 different donors. For statistical analysis the Mann-Whitney-U-test (2-tailed) was applied (*, P≤0.005).*

with increasing adhesiveness of the substratum: Almost no cells attached in the wells coated with the thickest polymer film (3%); 22% and 42% were recovered from the wells with the thinner coatings, PH 2 and PH 4, respectively. In the control 59% of the original radioactivity was recovered in this fraction. Clearly, an inverse relationship exists between the thickness of the poly-HEMA film and lymphocyte adhesiveness.

The influence of substratum adhesiveness on the shape of activated lymphocytes was determined by assessing the cell area of adhering lymphocytes under a light microscope as an index of the extent of lymphocyte spreading (Fig. 4).

We were unable to estimate the area of lymphocytes when using the thickest poly-HEMA layer as a substratum (PH1) since lymphocyte adherence was so poor that too few cells would be available for statistical analysis. By contrast, adhesion to the thinnest film used (PH4) did not reveal any change in cell area compared with the control (data not shown). However, a statistically significant difference in the area of attached lymphocytes was detected on the medium dense polymer film (PH2). A significantly higher proportion of lymphocytes after con A addition
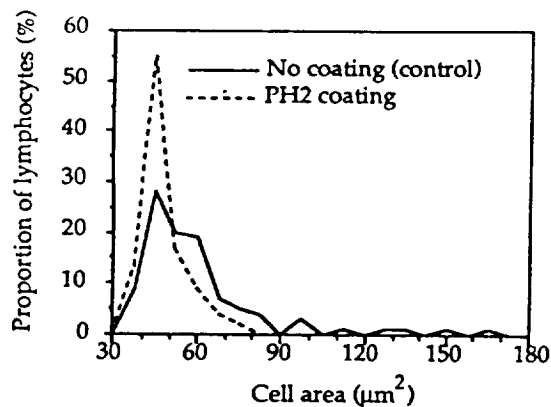
Fig. 4. Effect of substratum adhesiveness on cell shape. The frequency distribution of lymphocyte area of a typical experiment (one of of five) is depicted. Slideflasks were coated with poly-HEMA (PH2; s. Fig. 3). Immediately after con A activation the cell suspension (4 ml containing 4 10^6 cells) was loaded into slideflasks and incubated for 18 h at 37 °C. The cells were fixed by the addition of 800 µl glutaraldehyde (10%) for 20 min. The fixed cells were allowed to dry at room temperature, stained with Giemsa, mounted in a light microscope (1250-fold magnification), and photographed. The cell area of 80-100 lymphocytes from 4 different sites was determined on a graphic tablet (Summagraphics) connected to a Macintosh computer (Software: MacDraft and StatView) by following the lymphocyte contours.

was spherical and smaller in area compared with a control after culturing for 18 hours on poly-HEMA (PH2) in 5 independent experiments.

It is interesting that no large cells (> 83 µm²) were observed in the poly-HEMA coated wells (Fig. 4).

By contrast in all 5 experiments the occurrence of large lymphocytes (up to 165 µm²) was noted in the control wells. Furthermore in the uncoated wells the main peak of areas generally showed a shift in the distribution of cell size which included larger lymphocytes. This was never observed in the poly-HEMA coated wells. Likewise, only the lymphocytes grown in the control wells showed the typical signs of cell spreading (a broad rim of cytoplasm around the nucleus and filopodia). These findings suggest that the lymphocytes cultured on poly-HEMA could not anchor and spread. In attachment dependent cells proliferation is related to anchorage and spreading. We next tested the effect of reduced anchoring and spreading on activated lymphocytes and measured proliferation by the uptake of radiolabelled thymidine after 72 h (Fig. 5).
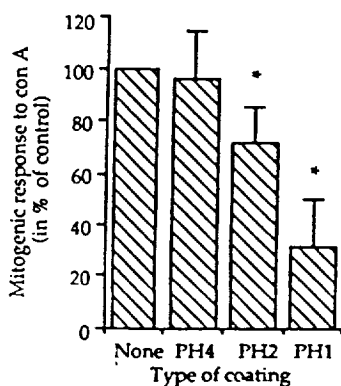
Fig. 5. Effect of poly-HEMA coated substratum on lymphocyte responsiveness. After con A stimulation, lymphocytes were seeded on untreated plastic of multidishes (control) and on increasing polymer film thickness (PH4, PH2, and PH1; s. Fig. 3) and incubated for 72 h. Then the rate of DNA synthesis was determined by the incorporation of ³H-labelled thymidine (20). The mean (± s.d.) of 6 independent experiments (6 donors) is shown. For statistical analysis the Mann-Whitney-U-test (2-tailed) was used (*, P≤0.01).

We determined that lymphocyte proliferation is inversely related to the thickness of the poly-HEMA film. Lymphocyte responsiveness was reduced by up to 68% of the control at PH1 (Fig. 5). At the lowest poly-HEMA dilution (PH4), lymphocyte responsiveness was unaffected. These results are similar to those reported by Folkman and Moscona (10) using nontransformed mammalian cells. Thus in both nontransformed mammalian cells and human lymphocytes the magnitude of the proliferation response is related to cell-substratum adhesiveness. Furthermore, in both studies, the growth rate determined by radiolabelled thymidine uptake appears to correlate with cell shape.

When cell-substratum interactions are reduced after con A activation, lymphocytes cannot proliferate normally. Consequently, other lymphocyte activation events associated, such as interferon-γ production, should also be affected.

In 2 separate experiments the production of interferon-γ was greatly reduced after plating con A activated lymphocytes on the thickest poly-HEMA coated plastic (results not shown).

Our results obtained in vitro suggest that human peripheral blood lymphocytes are attachment dependent cells which need adhesion to a substratum in order to facilitate anchoring and spreading for activation events and proliferation to proceed normally. Therefore lymphocytes do not differ greatly from nontransformed mammalian cells in respect to attachment and spreading. This is consistent with the complex functions of lymphocytes in vivo during the early events of the immune response. In particular, the interaction of lymphocytes with the endothelium lining of blood vessels is important for adhesion and diapedesing of lymphocytes into the lymphatic tissue (lymphocyte homing) and into sites of inflammation (23, 24). It is not surprising that resting, nonstimulated B and T lymphocytes both bind very poorly to fibroblasts (16, 23, 24). Only activated lymphocytes express the adhesion proteins that allow for attachment to other cells and to extracellular matrix proteins such as laminin (23).

Our findings that lymphocytes are adhesion dependent can in part explain data of lymphocyte studies performed in space. In 3 independent in vitro experiments in space we have shown that lymphocyte activation is depressed by 90-97% compared with 1 g controls (6-8). Note that in this paper we used exactly the same protocol as in these previous space experiments. However, the depression of lymphocyte responsiveness was more pronounced in space (90-97%) compared with even the thickest poly-HEMA film used (68%). This indicates that under weightlessness, factors other than adhesion may contribute to the reduced in vitro responsiveness. For instance, on ground cells still sediment and their shape may change although the cells cannot anchor on poly-HEMA. To check this hypothesis we subjected lymphocytes seeded on tissue culture plastic to increased gravity (20g) and normal gravity as a control. It is interesting that lymphocyte area was increased in all cells at 20g compared with 1g (Fig. 6).

In particular, the modal of the main peak showed a significant shift towards larger cells. In addition, a higher proportion of large cells (> 85 µm²) was found at 20g compared with 1g. This increase of cell size at 20g resulted in a significant increase of lymphocyte responsiveness to
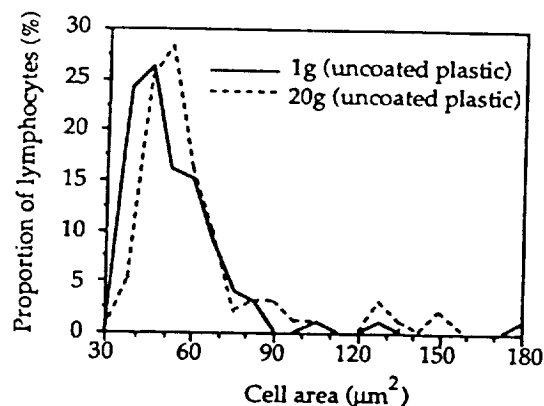
Fig. 6. Effect of increased gravity (20g) on cell shape. One typical experiment of 5 is shown. After the addition of con A, the lymphocytes were seeded on PH2 and cultured as described in the caption of Fig. 5 at 1g and 20g. The frequency distribution of a typical experiment is depicted.

con A of 40%, which is consistent with earlier results (25). Likewise, on poly-HEMA coated plastic (PH2) at 20g, the modal of the main peak was significantly shifted towards larger cell sizes in all five experiments (result not shown). No large cells (> 85 μm^2) were observed when the cells were seeded on poly-HEMA both at 1g and 20g. Thus it is not surprising that the mitogenic response in cells grown on poly-HEMA was not changed by increased gravity. This can be interpreted that increased gravity leads to an increase in lymphocyte proliferation when the cells are allowed to anchor and to spread.

The experiments presented here helped us to devise future experiments under microgravity conditions. During the next Spacelab mission (SLS-1) we will culture lymphocytes with Cytodex microcarriers to allow for adhesion under microgravity conditions (Lee and Cogoli, manuscript in preparation). We will determine to what extent mitogen-induced responsiveness is restored when lymphocytes bind to an adhesive substratum in space. With respect to the *in vivo* situation, Cogoli and Tschopp (26) have cautioned that one should not extrapolate from data obtained in *in vitro* experiments to the depressed lymphocyte responsiveness observed after spaceflight. It is more likely that the multiple stressors associated with spaceflight contributes to the depressed immunity observed in astronauts (3, 27, 28, 29). The effect of space flight on lymphocyte function warrants more detailed investigations considering the ambitious activities planned for long-term flights on space stations and interplanetary travels.

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Effect of Microgravity Modeling on Interferon and Interleukin Responses in the Rat

WALLACE D. BERRY,[1] JERRY D. MURPHY,[1] BETH A. SMITH,[1] GERALD R. TAYLOR,[2]
and GERALD SONNENFELD[1]

ABSTRACT

Rats were placed in whole-body harness suspension in three configurations: antiorthostatic hypokinetic/ hypodynamic suspension (AAH) to induce headward body fluid redistribution and unload the limbs, orthostatic hypokinetic/hypodynamic suspension (OHH) to unload the limbs without fluid redistribution, and harness restraint (HR) to produce the restraint stress of the model without fluid redistribution or musculoskeletal disuse. AHH and OHH suspension transiently increased interferon-γ (IFN-γ) production in response to the mitogen conconavalin A. Harness restraint alone did not affect IFN-γ response. However, both suspension modeling and harness restraint caused a transient reduction in interleukin-1 (IL-1) and IL-2 responses to mitogen. This suggests that factors associated with musculoskeletal unloading affected IFN-γ responses, while IL-1 and IL-2 responses were affected by the physiological stress of restraint.

INTRODUCTION

VARIOUS IMMUNOLOGIC CHANGES have been noted in humans, animals, and cell cultures during and after space flight.[1–12] Lymphoid organ involution,[4] changes in immune cell populations, and decreased response to colony-stimulating factor have been seen in rats following space flight.[8] Diminished lymphocyte blast transformation in response to mitogens has been observed consistently in space flight crews.[7,11,12] Lymphocytes taken from humans and rats immediately after space flight have reduced ability to produce interferons (IFNs) in response to mitogenic challenge.[5,9] In contrast, lymphocytes cultured during space flights show enhanced IFN production.[10] The underlying mechanisms of these effects, as well as their immunologic significance, are currently unknown.

Previous studies by investigators in our laboratory have examined IFN and interleukin (IL) responses of rats that had flown aboard the U.S. Space Shuttle. In these studies, lymphocytes harvested from rats flown in space had subnormal IFN-γ production but normal IL-3 production.[5] Ground-based suspension modeling has been used in attempts to reproduce or anticipate the immunologic effects of microgravity. Impaired IFN-α/β production was seen in mice and rats in suspension experiments.[13,14] Reduced IFN-α/β production in suspended

mice has been correlated to increased susceptibility to viral infection.[15] Rats and mice used in various suspension models have also shown reduced macrophage phagocytosis,[16] decreased superoxide production by peritoneal macrophages,[17] and decreased *in vivo* production of IFNs similar to that observed in rats flown on the U.S. Space Shuttle.[5,14,15]

Variations on the whole-body harness suspension model were used to test the hypothesis that redistribution of body fluids and musculoskeletal unloading contribute to the immunologic effects of suspension and therefore may be related to immunologic changes during space flight. Rats were placed in whole-body harness suspension[18,19] configured to produce musculoskeletal unloading and reduced limb movement (hypokinesia/ hypodynamia). Suspensions were conducted with rats in the head-down position (antiorthostasis) to induce fluid shifts, or in the level position (orthostasis). Rats were also placed in the suspension harnesses with full weight bearing on all limbs and without head-down tilt. This allowed examination of the contribution of restraint stress to the immunologic effects of suspension. Following suspension, the *in vitro* immunologic responses surveyed consisted of mitogen-stimulated production of the immunologically important cytokines IFN-γ, IFN-α/β, IL-1, and IL-2.

[1]Department of Microbiology and Immunology, School of Medicine, University of Louisville, Louisville, KY 40292; [2]NASA Johnson Space Center, Houston, TX.

MATERIALS AND METHODS

Suspension Modeling: Male, 350-400-gram CD rats (Charles River, Wilmington, MA) were placed in the whole-body harness suspension model.[18,19] Three different configurations of the model were used. In the first configuration, the rats were positioned in the harness system with 20° head-down tilt to induce antiorthostasis and with their hind limbs completely unloaded to produce hypokinesia and hypodynamia. This configuration is referred to as antiorthostatic hypokinetic/hypodynamic suspension (AHH). In the second configuration, referred to as orthostatic hypokinetic/hypodynamic suspension (OHH), rats of the same strain, sex, and size were placed in suspension with their hind limbs unloaded as before but without the head-down tilt. The third suspension configuration was used to examine the effects of restraint in the suspension harness. Rats were placed in suspension harnesses with no tilt and with full loading on their limbs (harness restraint, HR). Suspensions of 1, 3, 7, or 14 days duration were carried out for each suspension type. In each experiment, rats of the same size, sex, and strain were maintained as controls in normal caging. Food was provided to the control rats in amounts equal to the average daily food consumption of the suspended rats. The rats were maintained under National Institutes of Health (NIH) guidelines in an AAALAC accredited facility under the supervision of a veterinarian.

The rats were killed by a 100 mg/kg body-weight dose of sodium pentobarbital administered i.p. The spleen of each rat was excised under sterile conditions and washed four times in RPMI-1640 medium (GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, GIBCO) and antibiotics. Splenocytes were harvested by teasing apart the spleens in 5 ml of medium. The splenocytes were concentrated by centrifugation at 200 × g for 10 min at 4°C then suspended in 30 ml of medium. The cells were counted on a hemocytometer and viability was assessed by trypan blue exclusion. Cell viability was always found to be 90% or greater.

IFN-γ Production: Splenic lymphocytes from the suspended rats (1 ml at 3 × 10⁶ cells/ml) were incubated with a final concentration of 5 μg/ml concanavalin A (ConA, Sigma Chemical Co., St. Louis, MO) in 24-well culture plates (Falcon 3047, Becton Dickinson, Lincoln Park, NJ). The cells were cultured for 48 h at 37°C and 5% CO_2.[20] Following the incubation, the IFN-containing supernatants were collected, centrifuged at 200 × g for 10 min to remove cells, and stored at −70°C.

IFN-α/β Production: Splenic lymphocyte suspensions containing 3 × 10⁶ cells/ml in 1 ml of RPMI-1640 were incubated with 10 μg/ml polyriboinosinic/polyribocytidylic acid [poly(I:C), Sigma] and 20 μg/ml DEAE dextran (Pharmacia, Piscataway, NJ) in 24-well culture plates for 2 h at 37°C and 5% CO_2. Following the incubation, 1 ml of additional medium was added to each culture and the cultures were incubated for an additional 24 h.[20,21] Culture supernatants containing IFN-α/β were harvested by centrifugation at 200 × g to remove cells and were stored at −70°C.

Production of IL-1 and IL-2: Splenic lymphocytes from the suspended rats (1 ml at 3 × 10⁶ cells/ml) were incubated with 1

μg/ml indomethicin and 5 μg/ml ConA in 24-well culture plates.[22] The cells were cultured for 48 h at 37°C and 5% CO_2. Following the incubation, the supernatants were collected, centrifuged at 200 × g for 10 min to remove cells, and stored frozen at −70°C.

IFN Assays: IFN activity in supernatants was quantified by reduction of the cytopathic effect of Indiana strain vesicular stomatitis virus (VSV) on mouse L929 cells.[23] Serial tripling dilutions of the sample supernatants and standards were made in the assay medium which consisted of minimal essential medium (MEM, GIBCO) containing 2.5% HEPES (Sigma) and 5% FBS, in 96-well microtiter plates (Falcon 3072). A suspension of 1 × 10⁵ cells/ml was prepared from a confluent monolayer culture of L929 cells. Each assay well received 100 μl of the L929 suspension and was then incubated for 24 h at 37°C and 5% CO_2. Following the incubation, 200 plaque-forming units of VSV in 200 μl of assay medium was added to each well and incubation continued for 24 h or until cytopathic effects were observed microscopically. The plates were then drained and 50 μl of MTT (5 mg/ml in saline) and 50 μl of assay medium were added to each well.[24] Incubation was continued for 2 h and then 100 ml of 10% SDS was added. Following overnight incubation, the plates were scanned on a microtiter plate reader (Dynatech MR 600, Dynatech Laboratories, Alexandria, VA) at 570 nm. Titers were defined as the reciprocal of the supernatant dilutions giving a 50% increase in optical density compared to controls receiving virus without IFN.

IL-1 Assay: IL-1 activity in samples was quantified by a mouse thymocyte co-mitogenesis assay.[22] Thymocytes were harvested from 20-25-gram female Swiss Webster mice (Charles River) under sterile conditions. The mouse thymocytes were suspended at a concentration of 2 × 10⁶ cells/ml in RPMI-1640 supplemented with 5% FBS and antibiotics. Samples, standards, and controls were serially diluted two-fold in RPMI-1640 with 5% FBS and antibiotics in 96-well microtiter plates. ConA was added to each well at a final concentration of 2 μg/ml. Each well then received 100 μl of the thymocyte cell suspension. The cultures were incubated for 72 h at 37°C and 5% CO_2. IL-1 induced blast transformation of the thymocytes and quantified colorimetrically with MTT.[24] Following the 72-h incubation, 20 μl of a 5-mg/ml MTT solution was added to each well and allowed to incubate for 4 h as before. Each well then received 50 μl of 10% SDS with overnight incubation. The optical density of each well was read on a scanning microtiter plate reader at 570 nm. Titers were defined as the reciprocal of the dilution giving a 50% reduction in optical density compared to the highest standard.

IL-2 Assay: Stimulation of mouse splenoblast proliferation was used to assay IL-2 activity in samples from the suspended rats.[25] Splenocytes were sterilely harvested from 20 to 25-gram female Swiss Webster mice. The cells were suspended in 150-ml culture flasks (Falcon) at a concentration of 2 × 10⁵ cells/ml in RPMI-1640 containing 10% FBS, antibiotics, and 5 μg/ml ConA. The cells were incubated for 72 h at 37°C and 5% CO_2 to allow blast transformation. Following the incubation, the splenoblasts were washed three times, resuspended at 2 × 10⁵ viable cells/ml in medium, and incubated for 24 h. Serial

doubling dilutions of each sample, standards, and controls were made in medium in 96-well microtiter plates. Splenoblasts were added to each well (50 μl at a concentration of 2 × 10⁶ cells/ml) and incubated for 20 h. Following this incubation, 20 μl of 5 mg/ml MTT was added to each well and incubated for 4 h.[24] The plates were scanned at 570 nm on a microtiter plate reader following overnight solubilization of the reaction products with 100 μl of 10% SDS. Titers were defined as the reciprocal of the dilution giving a 50% reduction in optical density.

Statistical Analysis: Student's *t*-test was used to establish the statistical significance of differences between results for experimental and control samples. Alpha was set *a priori* at $p \leq 0.05$.

RESULTS

Body weight

Rats placed in suspension or harness restraint lost considerable body weight as did their pair-fed controls. The weight loss in suspended and restrained groups was nearly identical. Suspended rats initially lost weight at a faster rate than their pair-fed controls. However, by 14 days of suspension, total weight loss was the same in the controls and suspended animals.

IFN-α/β production

Lymphocytes from AHH suspended rats tended to produce less IFN-α/β activity than those from controls through day 7 of suspension; however, the numerical differences did not reach statistical significance. Cells from rats in the OHH configuration produced similar results with a significant depression of IFN-α/β production occurring at day 3 (Fig. 1). IFN-α/β production in HR suspended rats was similar to that of the AHH rats, with the exception that a significant depression of interferon production occurred at 7 days.
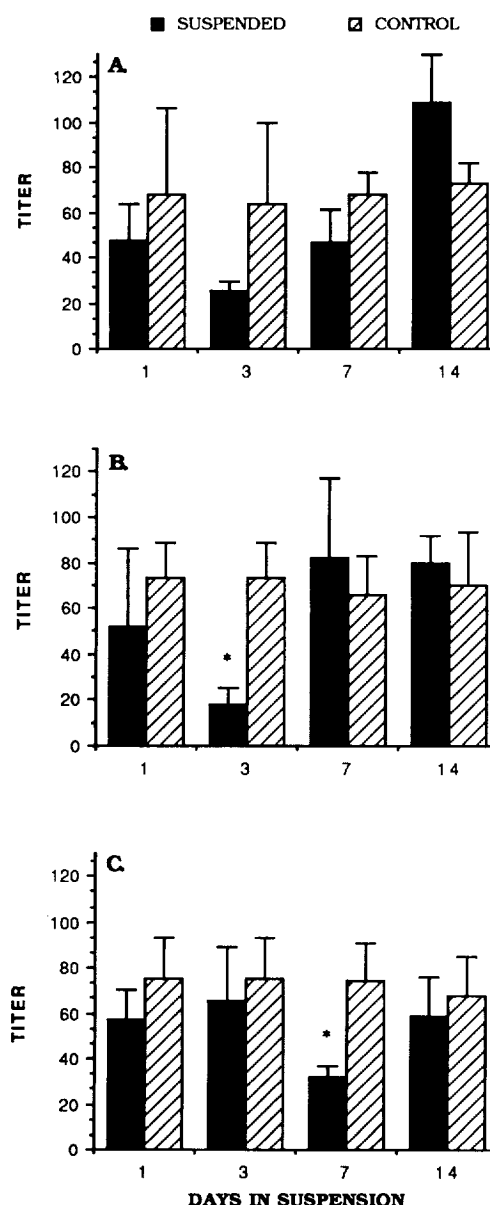
IFN-γ production

IFN-γ production by the lymphocytes of suspended rats was significantly increased at day 3 by both the AHH and OHH suspensions (Fig. 2). Afterwards, IFN titers were equivalent to controls in the AHH animals but were significantly enhanced at day 14 in the OHH rats.

IL-1 production

Lymphocytes from rats experiencing both head-down tilt and musculoskeletal unloading in the AHH suspension configuration produced significantly less IL-1 compared to controls following 1 or 3 days of suspension (Fig. 3). Production of IL-1 was normal at 7 or 14 days of suspension. Rats experiencing musculoskeletal unloading without head-down tilt (OHH) showed a similar trend in IL-1 production; however, the depression of IL-1 production was significant only on day 3. Harness restraint suppressed IL-1 production at days 1 and 7.
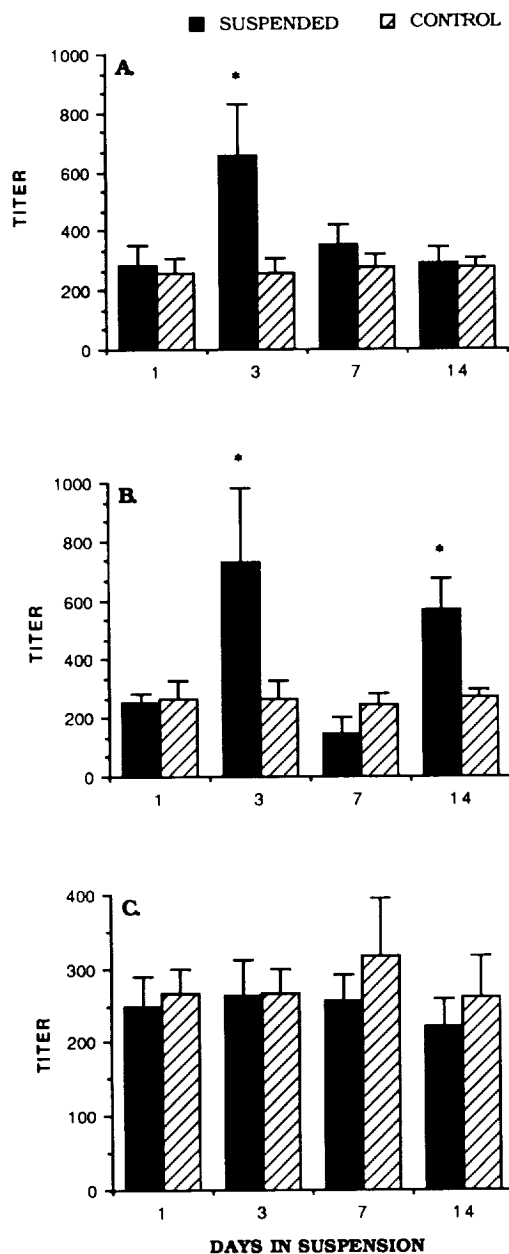
IL-2 production

All three of the suspension configurations transiently depressed IL-2 production by lymphocytes from suspended rats
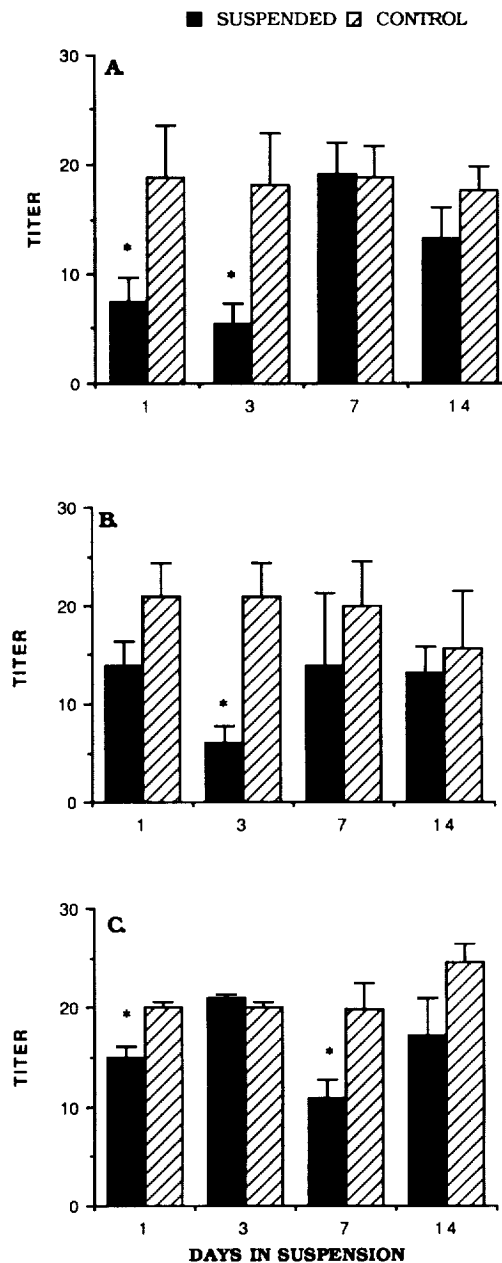

FIG. 1. Effects of suspension modeling on rat IFN-α/β production. Rats were placed in one of three harness suspension configurations to simulate the unloading and fluid redistribution of microgravity. Following suspension, splenocytes from suspended and control rats were stimulated with poly(I:C) and their IFN-α/β response was measured. A. Results for rats in AHH (hind limbs unloaded, 20° head down tilt). B. Results for rats in OHH (hind limbs unloaded, no tilt). C. Results for rats in HR (harness restraint). Each bar represents the mean titer ± SE of two separate experiments with 5 rats/experiment. *$p \leq 0.05$.

(Fig. 4). IL-2 production appeared to be depressed at 1 and 3 days of suspension regardless of the suspension type, with statistically significant depression occurring at 3 days of suspension in the AHH and OHH rats, and at 1 and 3 days in the HR rats.

FIG. 2. Effects of suspension modeling on rat IFN-γ production. Rats were placed in one of three harness suspension configurations to simulate the unloading and fluid redistribution of microgravity. Following suspension, splenocytes from suspended and control rats were stimulated with ConA and their IFN-γ response was measured. A. Results for rats in AHH (hind limbs unloaded, 20° head down tilt). B. Results for rats in OHH (hind limbs unloaded, no tilt). C. Results for rats in HR (harness restraint). Each bar represents the mean titer ± SE of two separate experiments with 5 rats/experiment. *$p \leq 0.05$.

FIG. 3. Effects of suspension modeling on rat IL-1 production. Rats were placed in one of three harness suspension configurations to simulate the unloading and fluid redistribution of microgravity. Following suspension, splenocytes from suspended and control rats were stimulated with ConA and their IL-1 response was measured. A. Results for rats in AHH (hind limbs unloaded, 20° head down tilt). B. Results for rats in OHH (hind limbs unloaded, no tilt). C. Results for rats in HR (harness restraint). Each bar represents the mean titer ± SE of two separate experiments with 5 rats/experiment. *$p \leq 0.05$.
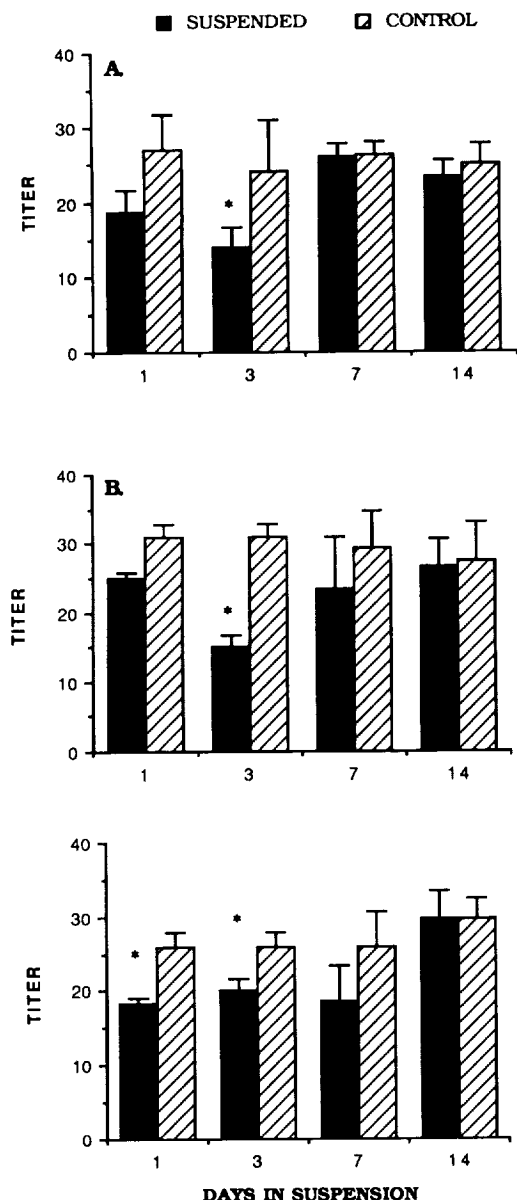
FIG. 4. Effects of suspension modeling on rat IL-2 production. Rats were placed in one of three harness suspension configurations to simulate the unloading and fluid redistribution of microgravity. Following suspension, splenocytes from suspended and control rats were stimulated with ConA and their IL-2 response was measured. A. Results for rats in AHH (hind limbs unloaded, 20° head down tilt). B. Results for rats in OHH (hind limbs unloaded, no tilt). C. Results for rats in HR (harness restraint). Each bar represents the mean titer ± SE of two separate experiments with 5 rats/experiment. $*p \leq 0.05$.

DISCUSSION

Microgravity or "weightlessness" in space flight is characterized by the absence or reduction of various biologically relevant forces. Mechanical loading, hydrostatic pressure, thermal buoyancy, and sedimentation are absent or greatly reduced in micro-

gravity. The absence of these forces may directly affect cellular activity or indirect effects may result as a consequence of physiologic responses to microgravity.

Reduced loading in microgravity results in loss of muscle mass[26] and reduced bone growth or loss of bone mass.[27-29] Body fluids, which tend to pool in the lower extremities under normal gravity, are redistributed to the upper body[30] during exposure to microgravity. The physiologic mechanisms controlling fluid balance, bone and mineral homeostasis, and muscle metabolism are known to integrate with the immune system through cytokines, hormones, neurotransmitters, and neuropeptides.[31,32] This raises the possibility that systemic endocrine changes due to fluid redistribution, musculoskeletal deconditioning, and stress contribute to the known or suspected immunologic effects of spaceflight.

Rodent suspension experiments model body fluid redistribution and musculoskeletal effects approximating those of space flight. Suspension modeling has been used as well to model and predict the immunologic effects of microgravity. In this study, suspension modeling was used to expand on previous studies of cytokine responses during suspension and to begin exploration of mechanisms of immunologic change during space flight.

The results of this study can be summarized as follows: (i) Suspension with or without head-down tilt transiently suppressed lymphocyte production of IL-1 and IL-2. IFN-α/β responses of lymphocytes from suspended rats tended to be suppressed during the first few days of suspension while IFN-γ responses were transiently enhanced; (ii) Simple restraint of rats in the suspension harness, with full load bearing on the forelimbs and hindlimbs, produced immunologic effects similar to those of musculoskeletal unloading regardless of head-down tilt.

The finding that restraint in the suspension harness produced immunologic effects essentially identical to those of suspension, regardless of head-down tilt, suggests that most of the immunologic effects are related to restraint rather than hypokinesia/hypodynamia or antiorthostasis. Restraint is a potent elicitor of the physiologic stress response, which is well known to suppress immunologic functions.[33] However, restraint of rats in the suspension harness, even with full loading on the limbs, entailed some reduction of limb movement. Therefore, it is possible that hypokinesia contributed to the effects of harness restraint as well as to those of suspension.

A previous study found that AHH suspension reduced the ability of mice to produce IFN-α/β but that mice suspended in the OHH position retained that ability.[15] In contrast to the previous study using mice, these results indicate that suspension modeling has little, if any effect on in vitro IFN-α/β production in rats regardless of body orientation or loading. This may indicate that the mouse model is more appropriate for studying the immunologic effects of body fluid redistribution in suspension modeling.

Reduced in vitro IFN-γ production has been seen in humans and rats following space flight.[5,9,10] In this suspension study, IFN-γ production was increased following suspension. However, the increase in IFN production was seen only in rats which experienced musculoskeletal unloading. Therefore, factors associated with musculoskeletal unloading appear to be responsible for the effects on IFN-γ. Considering the similarity between the musculoskeletal effects of suspension modeling and microgravity, then similar IFN-γ effects would be expected to occur in

space flight. However, increased IFN-γ production in AHH and OHH rats in this study contrasted with space flight results, which found that *in vitro* IFN-γ production was lower in rats following space flight.[5] Cells from the space flight rats were harvested after reentry and landing, and had to be transported at 1 gram for several hours, therefore, the effects seen in the flight study may have reflected readaptation to gravity following space flight rather than the effects of microgravity proper.

Comparisons of the effects of suspension on IL production with those of space flight are impossible at present since measurements of these responses have not been reported in humans or other animals during or following space flight. Suppression of the IL-1 and IL-2 response in the restrained rats as well as the suspended rats is consistent with stress-induced immunosuppression.[34]

It has been pointed out that IL-2 usually correlates with IFN-γ in cultures of ConA-stimulated lymphocytes. Reduced IL activity coinciding with increased IFN-γ activity in AHH and OHH suspended rats may represent an uncoupling of cytokine regulatory pathways.

The results of these studies show that rat suspension modeling of microgravity can induce functional changes in cellular immunity *in vitro*. Body fluid redistribution and musculoskeletal unloading do not appear to contribute to the observed immunologic changes with the exception of the increased *in vitro* IFN-γ response, which was apparently related to musculoskeletal unloading. The immunologic effects were otherwise consistent with immunosuppression related to the physiologic stress of restraint.

Assuming that activation of physiologic stress in the suspension model is comparable to that of actual space flight, then similar transient immunologic effects should occur in short-term flights. However, these findings do not rule out possible immunologic consequences of fluid redistribution and musculoskeletal deconditioning during long-term space flights. Experiments conducted *in vivo* during space flights will be necessary to answer these questions.

ACKNOWLEDGMENTS

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Effects of Microgravity on the Immune System

Gerald Sonnenfeld
Dept. of Microbiology and Immunology
University of Louisville
Louisville, KY

Gerald R. Taylor
NASA Johnson Space Center
Houston, TX

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Effects of Microgravity on the Immune System

Gerald Sonnenfeld
Dept. of Microbiology and Immunology
University of Louisville
Louisville, KY

Gerald R. Taylor
NASA Johnson Space Center
Houston, TX

ABSTRACT

Changes in resistance to bacterial and viral infections in Apollo crew members has stimulated interest in the study of immunity and space flight. Results of studies from several laboratories in both humans and rodents have indicated alterations after space flight that include the following immunological parameters: thymus size, lymphocyte blastogenesis, interferon and interleukin production, natural killer cell activity, cytotoxic T-cell activity, leukocyte subset population distribution, response of bone marrow cells to colony stimulating factors, and delayed hypersensitivity skin test reactivity. The interactions of the immune system with other physiological systems, including muscle, bone, and the nervous system, may play a major role in the development of these immunological parameters during and after flight. There may also be direct effects of space flight on immune responses.

INTRODUCTION

Until recently, reports on the effects of space flight on the immune system have been interesting, but often inconclusive (1-5).*
These studies have been limited by small sample size, the relatively small number of flights available for immunological studies, and, occasionally, by uncontrollable experimental conditions (1-5). Nevertheless, it is apparent from the results of these earlier studies that some alterations of immunological parameters do occur during, or immediately following, space flight. Several factors could contribute to these effects, including microgravity, stress, radiation, and interaction with other host biological systems. This

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*Numbers in parentheses designate references at end of paper.

report reviews our current understanding of the effects of space flight, and of systems used to simulate the effects of microgravity, on immune responses.

MICROGRAVITY MODELING SYSTEMS

As a result of the limited opportunities for space flight, ground models simulating some aspects of space flight have been used to study changes in immune responses. Immune responses of rodents have been the main subject of these studies, because modeling using these quadrupeds was more easily and economically carried out.
In one such study, mice were maintained in a "space cabin" environment in which barometric pressure was altered in a pressure chamber (6). These confined mice were more susceptible to mengovirus infection than were control mice. These data suggested that maintenance of mice in this restricted environment with alterations in pressure could have resulted in alterations in immune responses, such as interferon or antibody production, that decreased their resistance to viral infections.
By far the most widely used model to simulate some aspects of weightlessness that occur during space flight has been antiorthostatic, (15-20° head-down tilt), hypokinetic, hypodynamic suspension (no load on the hind-limbs) by tail (7) or by harness (8). The immunologic results have been obtained using either rats or mice suspended similarly (9). The suspension model promotes the development of physiological changes in muscle, bone, fluid-shifts, and certain other parameters that simulate changes observed following flight (7,8). For example, suspended rats showed involution of the thymus similar to that seen after space flight (10,11). Although rodent suspension is a less than perfect model for simulating the phsyiological effects of microgravity seen during space flight, it has proven useful in aiding the determination of which immunological parameters should be studied

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in rare flight experiments.

One set of experiments involved the study of cytokines. Cytokines are soluble, non-anti-body mediators that play a major role in cell-to-cell communication and regulation of immune responses (12). The major cytokines studied were the interferons. Interferons are a family of proteins that have antiviral activity and several other activities, including regulation of immune responses (12). Interferon-alpha is produced by leukocytes primarily after stimula-tion with viruses, double-stranded RNAs, or other non-specific inducers (12). Interferon-beta production, primarily by fibroblasts, is init-iated by the same stimuli as is interferon-alpha (12). In many cases, these two interferons are difficult to separate and are referred to common-ly as interferon-alpha/beta. The third type of interferon, interferon-gamma is a product of T-lymphocyte-mediated responses stimulated either with a specific antigen or with a mitogen such as concanavalin-A (12).

The first cytokine studies showed that suspension of rats in an antiorthostatic model resulted in severe inhibition of interferon-alpha/beta production (13). The rats were sus-pended antiorthostatically in this tail suspen-sion model for two weeks,and then challenged intavenously with polyriboinosinic-polyribocyt-idylic acid (poly-I·C, a double-stranded RNA inducer of interferon-alpha/beta). There was an 80% decrease in interferon-alpha/beta produc-tion compared to normally caged controls (13). In more recent studies, rats were suspended antiorthostatically for 1-2 weeks, and spleens were removed immediately after the rats were taken down from suspension. Spleen cells were placed in culture and then challenged with either poly-I·C or concanavalin-A, Both interferon-alpha/beta and interferon-gamma production was altered (14). These results suggest that anti-orthostatic suspension of rats resulted in altered interferon production, a finding similar to that observed when cosmonauts were tested for interferon production after space flight (15).

Similar results were observed when mice were suspended antiorthostatically (16). In an attempt to control for the stresses of confinement and suspension, mice were also sus-pended orthostatically in a harness with no head-down tilt. Mice suspended 1-2 weeks in the antiorthostatic orientation showed severely inhibited interferon-alpha/beta production com-pared to controls housed normally (16). Mice suspended in the orthostatic orientation showed no change in interferon-alpha/beta production compared to controls (16). This suggested that the antiorthostatic orientation of suspension was required for inhibited interferon-alpha/beta production, i.e. the stress of suspension alone could not account for inhibited interferon pro-duction. Mice suspended antiorthostatically and then allowed to recover in normal cages for one week regained their capacity to produce interferon-alpha/beta (16).

In addition, suspended mice were inoculated with the D variant of encephalomyocarditis virus (EMC-D virus). EMC-D virus is a convenient virus to work with, because alteration in a glucose tolerance test is all that is necessary to show successful infection with the virus. Females of the Swiss/Webster strain of mouse normally are totally resistant to infection with EMC-D virus (17). Resistance to EMC-D virus is mediated, at least in part, by inter-feron (17). Antiorthostatically suspended mice became susceptible to infection, while ortho-statically suspended mice remained resistant to infection (17). Alterations in resistance to EMC-D virus correlated with alterations in interferon production. These results indicated that changes in immunological parameters induced by antiorthostatic suspension had the potential to alter resistance to infection.

Other mouse suspension studies have shown that leukocytes from mice suspended antiortho-statically had impaired ability to produce superoxide, decreased ability to kill phagocy-tosed bacteria (Propionibacterium acnes) and had altered serum corticosterone levels (18). This indicated that antiorthostatic suspension could alter the inflammatory and phagocytic responses.

The suspension studies described above indicate that this model of some aspects of microgravity resulted in alterations of important immunological parameters. The resulting information has provided a basis for planning space flight studies.

IMMUNOLOGICAL STUDIES INVOLVING THE INTACT ORGANISM

Although cellular immune responses of U.S. and Soviet crew members have been studied by various methods for two decades, their remains a limited pool of reliable information from which to draw conclusions. Considerable immuno-logical testing was performed following 11 U.S. Apollo flights, 3 U.S. Skylab flights, and U.S./U.S.S.R. Apollo-Soyuz flights (19). In addition, postflight alterations in the in vitro responses of cosmonaut lymphocytes were reported following the flights of Soyuz 6, 7, 8, and 9 (20); for the two Soyuz visits to the Salyut 4 space station, Soyuz 24, 26 and 27; Salut 5; and the two Soyuz visits to Salyut 6 (21). However, because of small sample sizes, mission anomalies and constraints on analytical condit-ions, the resulting data were generally incon-clusive (19). Nevertheless, a pattern seemed to be developing. These studies appeared to suggest that lymphocyte activity may be depressed following spaceflight and that a postflight leukocytosis concurrent with a postflight lympho-cytopenia could be expected (19).

Extensive comparisons of preflight and post-flight immunological parameters were conducted with the first 41 U.S. Space Shuttle astronauts (4,5). This study demonstrated unequivocally that the absolute number of lymphocytes in the

peripheral circulation, the ability of these cells to respond to mitogenic stimulation (lymphocyte blastogenesis), and the number of eosinophils in the peripheral circulation were all decreased typically after flight. Conversely, there was an almost universal doubling of the absolute neutrophil number, and often a major change in the CD4/CD8 (helper T-cell/cytotoxic-suppressor T-cell) ratio. This latter event resulted from an increase in the helper (CD4) T-lymphocyte population as determined by flow cytometric analysis (5). Data from Space Shuttle flights 41B and 41D involving 11 crew members indicated a postflight decrease in circulating monocytes and B-lymphocytes. Further, the reduced T-lymphocyte blastogenesis was shown to correlate with monocyte count. Since monocytes serve a critical role during lymphocyte activation for blastogenesis as potent immunoregulatory cells through release of cytokines, these findings suggested a possible mechanism for blunted in vitro mitogen-induced blastogenesis (19).

Only recently has the effect of space flight on the ability of the human cell-mediated immune system to function normally in vivo been tested in flight. This was accomplished by using the delayed-type hypersensitivity (DTH) response to recall antigens as a simple, yet effective method for evaluating in-flight mediated abnormally diminished reactivity in U.S. Space Shuttle crewmembers. The cell-mediated immune mechanism was evaluated in 10 astronauts by measuring their in-flight DTH response to the common recall antigens of tetanus, diphtheria, tuberculin, Streptococcus, Proteus, Candida, and Trichophyton. Three of the subjects were evaluated after four days, three after five days, and four after ten days of space flight. The mean number of reactions within the population of 10 subjects was reduced from 4.5 preflight to 3.0 during flight, and the mean aggregate reaction size decreased from 21.4 mm before flight to 13.7 mm during flight. The authors reported that the data suggest that on day 4 of a Space Shuttle mission, the cell-mediated immune system is degraded measurably. Between day 5 and day 10, the depression reaches its maximum, and the cell-mediated immunity mechanism begins to adjust to the new conditions (Taylor, G.T., et al., presented at the 1990 American Society for Gravitational and Space Biology Meeting, Louisville, KY).

Soviet/Hungarian studies have also indicated that leukocytes from some cosmonauts had a severe decrease in production of interferon-alpha/beta immediately after return from flight (15). Additional Soviet studies have indicated dramatic decreases in natural killer and cytotoxic T-lymphocyte activity in the peripheral blood of cosmonauts obtained within 24 hours following space flight.

Studies with the human antibody-mediated humoral immune system have been more limited and inconclusive. These studies have shown that there did not appear to be alterations in the circulating levels of immunoglobulin classes in astronauts after flight in a Space Shuttle mission (22). There have been no studies to date to determine the effects of space flight on the ability to sensitize a host to specific antigen during flight, or the ability of a host to develop an immune response during flight after being sensitized on the ground. Therefore, the effects of space flight on antibody responses remain largely unexplored.

Additional flight studies have been carried out using rats. Several studies of rats flown in space have indicated that prolonged space flight resulted in hypoplasia of lymphoid organs and alterations in mitogen-induced blastogenesis (2,3). These effects were transient, and returned to normal after a postflight recovery period (2,3).

Several rats were flown in SpaceLab-3, and experiments were carried out to determine the effects of flight on cytokine production (23). The rats were flown on the Space Shuttle for 7 days, and after landing, a transcontinental airplane flight and a 12 hour delay occured prior to sacrifice (23). This delay & airplane flight could have affected the results of the study. After sacrifice, spleens were removed from the rats and placed in individual cell cultures. The cultures were then challenged with concanavalin-A to induce cytokines. After the appropriate time interval, culture supernatant fluids were harvested and assayed for interferon-gamma activity. Interferon-gamma production was reduced significantly in cells taken from rats flown in space, compared to cells from control rats (23). This flight experiment yielded results consistent to those observed after antiorthostatic suspension of rats, and with the results of impaired interferon production by cosmonauts after flight (15).

Production of another cytokine, interleukin-3, was also measured in the same SpaceLab-3 experiment (23). Interleukin-3 plays a major role as a growth factor for immunologically important cells. In this case, spleen cells from rats flown on SpaceLab-3 showed the same pattern of production of interleukin-3 as did cells from control rats (23). These data suggested that not all immunological parameters are affected by space flight.

Additional studies were carried out aboard the Cosmos # 1887 Soviet space flight. In this flight, the immunological studies were extended to additional areas. Experiments were carried out to determine the effects of space flight on the distribution of leukocyte subpopulations. The distribution of leukocyte subpopulations has been shown to be an important indicator of normal immunological function: for example, patients with acquired immune deficiency syndrome (AIDS) have an altered ratio of helper T-lymphocytes to suppressor T-lymphocytes (25). In the first set of experiments, spleens were removed from 5 rats flown for 12 and one-half days on biosputnik Cosmos # 1887. The biosputnik landed off-course, and a 48 hour delay and a trans-

continental airplane flight occured between landing of the space capsule and sacrifice of the rats, which may have affected the results observed. The spleens were dissociated into individual cells and separate samples were stained with different antibodies directed against cell surface markers. These antibodies were: anti-asialo GM-1 (natural killer cells), OX-39 (interleukin-2 receptor),OX-1 (pan-leukocyte marker), W3/25 (helper T-lymphocyte), OX-8 (suppressor/cytotoxic T-lymphocyte), W3/13 (pan T-lymphocyte), OX-4 (polymorphic Ia-class II histocompatibility antigen), anti-rat IgG Fab', anti-rabbit IgG (irrelevant antibody control), and no antibody (negative control). The stained cells were analyzed by flow cytometry. Although there may have been problems with nonspecific staining, the authors reported a trend suggesting a dramatic shift in some cell populations compared to synchronous controls (rats housed and treated in a similar fashion to flight animals with the exception of microgravity and radiation exposure of flight) and ground controls (vivarium controls in standard vivarium housing). These dramatic shifts were noted in populations of total T-lymphocytes, helper T-lymphocytes, suppressor/cytotoxic T-lymphocytes and interleukin-2 receptor bearing T-lymphocytes (24).

Additional studies carried out on rats flown in Cosmos # 1887 involved bone marrow cells. Due to the limited number of bone marrow cells from the femur available for the experiment, the distribution of only two cell populations was examined in the bone marrow, i.e. the total leukocyte population and the leukocytes carrying surface immunoglobulin. The analysis showed a larger number of myelogenous cells bearing surface immunoglobulin from flown rats as compared to synchronous and ground control rats (24). Myelogenous cells are monocyte/macrophage precursors and would not have been expected to have a surface immunoglobulin marker. In addition, the bone marrow cells were tested for their ability to respond to colony stimulating factor-monocyte (CSF-M). CSF-M stimulates the division of monocyte/macrophage precursors (26). The bone marrow cells were from flown rats were inhibited in their ability to form colonies in the presence of CSF-M, indicating a lack of division, and possibly maturation, on the part of the precursor cells (24). The bone marrow cell data suggested that an unusual response for myelogenous cells (i.e. possibly aberrent division and bearing of the inappropriate surface immunoglobulin marker) was induced by space flight, and those cells were refractory to additional exogenous stimulation by CSF-M upon return to earth. Monocytes/macrophages, therefore, are immunologically important cells that appear to be affected by space flight.

Additional Soviet studies carried out on Cosmos biosputnik flights have indicated that space flight resulted in decreased rat natural

killer cell and cytotoxic T-lymphocyte activity (27). Therefore, rodent studies confirm and extend the human studies that suggest that space flight results in severe alterations of immune responses.

IMMUNOLOGIC STUDIES INVOLVING CELL CULTURES

The studies described above indicate that certain immunological functions in animals and humans appear to be affected by space flight. Several studies have been carried out to determine the ability of isolated cells to sense and respond to changes in the gravity vector. Cogoli and his associates (28-30) developed an incubator that allowed them to carry out lymphocyte culture experiments during space flight. In this case, human lymphocyte cultures were exposed to mitogens during space flight. Lymphocyte blastogenesis was inhibited significantly compared to ground controls (28-30). Similar results were obtained when lymphocytes were maintained in a clinostat on the ground (31). A clinostat is a centrifuge-like device that randomly changes the direction of the gravity vector (31).

In a follow-up experiment, cells were also incubated during flight in a centrifuge that subjected the cells to 1 G. Much of the blastogenic capacity of the cells was regained (28-30). This could indicate a direct effect of gravity on the cellular processes, or perhaps some abnormal cell-to-cell contact in microgravity. An additional explanation could be that the· lack of blastogenesis was due to an impairment of the ability of receptors on the cells to interact with mitogen. Later studies using altered substrata for the cells on the ground indicated that decreased lymphocyte adhesion could contribute to the suppressed in vitro lymphocyte blastogenesis observed during space flight (32). In any case, the results of this series of experiments indicated that a cellular immune process was inhibited in isolated cells during space flight.

An additional study using cultures of human leukocytes was carried out by Talas and her associates (15). Various mitogens and inducers were added to the cultures to induce interferon-alpha/beta. The cells from the cultures produced strikingly higher levels of interferon-alpha/beta than did cells from control cultures maintained on the ground (15); however, it should be noted that this was a limited experiment with a small "n". This result was different from that observed from the cosmonauts after the same flight, and different than the results of the rodent studies in which interferon-alpha/beta production was inhibited.

It is not surprising that results differed between the cell culture studies and the studies of cells from intact organisms flown in space. In the latter case, the immunologically important cells may interact with other cells and other systems. In the host, the cell is in its natural

milieu, able to interact and cooperate with other cells and other systems. For example, the immune system interacts closely with the neuroendocrine system (33). The interactions of the immune system with other systems in space flight certainly require additional study.

CONCLUSIONS

It is apparent from the studies described above that space flight has profound effects on immune responses. The mechanisms of these effects remain to be established. Although cell culture studies and ground-based modeling have suggested that both microgravity and stress can play some role in the alterations of the immune response observed after space flight, the full nature of that role remains to be established. In addition, the role of radiation and other space flight factors has not been studied to a great extent.

In addition, it is clear that the full range and duration of the effects of space flight on the immune response have not been established. The effects of long-term flight as opposed to short-term flight remain to be established. The duration and reversibility of the effects of space flight on immune responses, particularly after long-term flights, has not yet been established.

Most importantly, the effects of space flight-induced immune alterations on resistance to infection has not been demonstrated. This could have profound effects on the ability to carry out long-term manned space flights. In addition, if resistance to infection is altered, the development of possible countermeasures should be undertaken.

In short, it is now clear that space flight affects immune responses. Much work remains to be done to establish the significance of these effects.

ACKNOWLEDGEMENTS

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