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**SPACEFLIGHT AND IMMUNE RESPONSES OF RHESUS MONKEYS**

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# **Movement Limitation and Immune Responses of Rhesus Monkeys**

Science Report

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## SUMMARY

The effects of restraint on immunological parameters was determined in an 18 day ARRT (adult rhesus restraint test). The monkeys were restrained for 18 days in the experimental station for the orbiting primate (ESOP), the chair of choice for Space Shuttle experiments. Several immunological parameters were determined using peripheral blood, bone marrow, and lymph node specimens from the monkeys. The parameters included: response of bone marrow cells to GM-CSF (granulocyte-macrophage colony stimulating factor), leukocyte subset distribution, and production of IFN- $\alpha$  (interferon-alpha) and IFN- $\gamma$  (interferon-gamma). The only parameter changed after 18 days of restraint was the percentage of CD8+ T cells. No other immunological parameters showed changes due to restraint. Handling and changes in housing prior to the restraint period did apparently result in some restraint-independent immunological changes. Handling must be kept to a minimum and the animals allowed time to recover prior to flight. All experiments must be carefully controlled. Restraint does not appear to be a major issue regarding the effects of space flight on immune responses.

## INTRODUCTION

Space flight has been shown to affect immune responses (Taylor, 1993). Due to the limits of human experimentation, a search is currently in progress to develop surrogate models to study the effects of space flight on immune responses. One model with great advantages is the rhesus monkey. The rhesus monkey has many similar immunological parameters to man; in fact, many reagents used for human research and testing cross-react with rhesus monkey tissue (Sonnenfeld, *et al.*, 1993).

In order for rhesus monkeys to be flown in space craft, they must be restrained in some fashion. They can not be allowed free access to the space craft they occupy for both safety and experimental reasons.

One major question that can be raised in any space flight experiment carried out with restrained monkeys is: What is the contribution of the restraint procedure to the results obtained? Effects of restraint procedures could confound the effects of space flight on physiological parameters (Minton and Blecha, 1990).

The adult rhesus restraint test (ARRT) and the systems integration test (SIT) were carried out to determine whether or not restraint of adult monkeys in apparatuses similar to those proposed for use in the Space Shuttle could affect physiological parameters of rhesus monkeys. The focus of this report is an experimental protocol to determine the effects of restraint on immunological responses of rhesus monkeys. The ARRT and SIT tests were similar in design. Only the ARRT data will be presented in this report, since the results of both tests were similar regarding immune responses.

In the ARRT, monkeys were restrained for 18 days. Bone marrow, blood, and lymph node samples were obtained prior to and after the restraint period and analyzed for a variety

of immunological parameters. The results of this study will be used in an attempt to determine the *role of restraint* in immunological changes induced by space flight in rhesus monkeys.

## MATERIALS AND METHODS

Six adult male rhesus monkeys (*Macacca mulatta*) from the NASA Ames Research Center colony were selected for the study. The monkeys were divided into two groups: four restraint subjects and four control subjects. One of the restraint monkeys had to be removed from the study for technical reasons, leaving a group of 3 restraint monkeys. Unless otherwise noted, all samples are from 4 restraint and 2 control monkeys prior to restraint, and 3 restraint and 2 control monkeys after restraint. The monkeys were restrained for 18 days in the ESOP hardware. Prior to restraint, they were instrumented for other experiments and were for restraint. Details concerning instrumentation, restraint training, and the restraint procedures and hardware are described in the *Mission Description* section of this technical memorandum.

Tissue sampling from the monkeys occurred as follows (time related to the restraint period): blood : -76 days, -41 days, -10 days, -4 days, + 17 days, + 24 days; bone marrow and lymph node: -41 days; + 17 days. By definition, restraint commenced on day 0.

Bone marrow samples were obtained through needle biopsy of the posterior of the head of the humerus (left or right) of monkeys under Ketamine/Xylazine anesthesia. Each bone marrow sample was transferred to a 15 ml polypropylene centrifuge tube containing McCoy's medium (Gibco BRL, Grand Island, NY) supplemented with antibiotics, sodium bicarbonate, hepes buffer, L-glutamine, and fungizone. Bone marrow cells were washed, centrifuged and resuspended in supplemented McCoy's medium with 10% fetal bovine serum (FBS). Cell counts were obtained using a Coulter Counter (Coulter Laboratories, Hialeah, FL). Lymph node cells were obtained by excision of axillary lymph nodes and dissociation of

the lymph nodes into single cell suspensions in supplemented RPMI-1640 medium with 10% FBS.

For the colony stimulating factor (CSF) assay,  $2 \times 10^5$  bone marrow cells/mL were resuspended in a 2% methylcellulose solution prepared in supplemented McCoy's media containing 30% FBS (Shadduck and Nagabhushanam, 1971). Medium for experimental group cultures contained a concentration of 40 ng/ml recombinant human GM-CSF (a gift of Dr. Steven Gillis, Immunex Research and Development Corp, Seattle, WA). The GM-CSF was from lot 620-028-5, and had a specific activity of at least  $5 \times 10^7$  units/mg protein. For each animal tested, six 35 mm tissue culture dishes, each containing 1 ml of the bone marrow cell suspension, were set up for control ( - CSF) and for experimental ( + CSF) groups. Dishes containing suspended cells were incubated in a 37°C incubator with 5% CO<sub>2</sub> (Shadduck and Nagabhushanam, 1971). After 10 days of incubation, 18 microscope grid squares from each petri dish were evaluated for the number of colonies formed, a colony represented by aggregates of 50 or more cells (Sonnenfeld, *et al.*, 1990). The GM-CSF data was analyzed using Student's t test and paired Student's t test.

The following procedure was used to determine cell surface antigenic markers. For bone marrow and lymph node (Jackson and Warner, 1986),  $1 \times 10^6$  cells suspended in supplemented RPMI-1640 medium with 10% FBS, were allocated to 10 separate microcentrifuge tubes, washed with FTA buffer (BBL Microbiology Systems, Cockeysville, MD) at pH 7.4 and containing 0.1% sodium azide and 0.5% normal mouse serum (Sigma Chemical Co., St. Louis, MO), and centrifuged for 1.5 min at 1,000 x g. The supernatant fluid was removed, and 5 µl of the appropriate antibody was added to each microcentrifuge tube. For peripheral blood, 20 µl of heparinized blood and 10 µl of appropriate antibody were added to each microcentrifuge tube. Background values were determined by including a microcentrifuge tube containing cells, but no antibody, for each animal tested. Cells and

antibody (or no antibody) were allowed to incubate at 4°C for 25 min. Antibodies used in this study were obtained from Becton-Dickinson Immunocytometry Systems, San Jose, CA, except as noted below:

- A. No antibody added
- B. Leu 4 (CD-3 signal transducer for T lymphocyte)
- C. Leu 3a (CD-4, helper T lymphocyte)
- D. Leu 2a (CD-8, cytotoxic T lymphocyte)
- E. Leu 11 a (CD-16, Natural killer cell/monocyte)
- F. Anti-HLA-DR (human class II Major histocompatibility antigen)
- G. Anti-human IgM (B cell - purchased from Sigma Chemical Co., St. Louis, MO)
- H. Anti-monkey IgG (B cell - purchased from Organon-Teknika Corp. W. Chester, PA)
- I. Anti-monkey IgG F(ab')<sub>2</sub> (B-cell - purchased from Organon-Teknika Corp.)
- J. Goat anti-Mouse (IgG) (Fab specific - Sigma Chemical Co., St. Louis, MO)

After the 25 minute incubation, red blood cells were lysed for 6 minutes at room temperature with one ml of lysing solution/microcentrifuge tube (0.15 M ammonium chloride, 10 mM potassium bicarbonate, 0.097 mM tetrasodium EDTA, pH 7.4). Cell suspensions were then centrifuged for 1.5 min at 1,000 x g, and resuspended in FTA buffer, pH 7.4, containing 0.1% sodium azide. Cell suspensions were again centrifuged for 1.5 min at 1,000 x g, supernatant removed, and cells fixed by resuspension in 0.5 ml of 1% paraformaldehyde prepared in FTA buffer.

Fixed cells from blood and bone marrow and lymph node were maintained at 4°C until assayed to determine the percentage of various surface markers using a Facstar flow cytometer (Becton-Dickinson, Corp., San Jose, CA). White blood cells were gated on three-part differentials using forward vs. side scatter plots.



The following procedures were used for induction and assay of cytokines. Peripheral blood leukocytes were placed on Ficoll-Paque and centrifuged to allow purification of mononuclear cells (Sonnenfeld, *et al.*, 1990). The mononuclear cells were resuspended in supplemented RPMI-1640 medium with 10% FBS at a concentration of  $3 \times 10^6$ /ml in tissue culture plates. Five  $\mu$ g of lipopolysaccharide (Sigma Chemical Co., St. Louis, MO) was added per well to induce interferon-alpha and 5  $\mu$ g of concanavalin-A (Sigma Chemical Co., St. Louis, MO) was added per well (different wells for each inducer) to induce interferon-gamma (Sonnenfeld, *et al.*, 1990). Assay of the interferons was by cytotoxicity inhibition assay of the Indiana strain of vesicular stomatitis virus on Hep-2 cells for interferon-alpha (Sonnenfeld, *et al.*, 1990) and by commercial Elisa assay for interferon-gamma (Biosource International, Camarillo, CA).

## RESULTS

### **Effect of restraint on the response of bone marrow cells to GM-CSF**

Bone marrow samples were taken on 41 days prior to restraint (day - 41) and on the day of release (day + 17) (2 restrained and 2 control animal samples). No differences between the restraint and vivarium control animals were observed, except one vivarium control animal did not appear to respond to GM-CSF, possibly because of experimental error (Figure 1).

### **Effect of restraint on the percentage of cells expressing cell surface antigenic markers**

There were no significant differences observed between control and restraint groups during the restraint period (day 0 to day 17) in any blood leukocyte cell surface antigenic markers observed, except for a small, not statistically significant ( $p = 0.52$ ) difference in CD8 (Figure 2). There was, a decrease in cells expressing CD8 and other antigens in the time period just prior to restraint (-10 to -4 days). This was true in both the restrained and control animals. There were no significant differences observed in bone marrow cells. Although lymph node cells were viable and stained with antibodies, no determination could be made on the effects of restraint because of a very small number of samples available due to technical difficulties.

### **Effect of restraint on the production of cytokines**

There were no significant differences observed in the production of interferon-alpha and interferon-gamma by peripheral blood leukocytes at any time point between control groups and restraint groups (Figures 3 and 4).

## DISCUSSION

Restraint could pose a problem in studies designed to determine the effects of space flight on immune responses of rhesus monkeys. In order for rhesus monkeys to be flown in space, they must have restricted movement in the space craft cabin because of space and operational limitations. Restraint could serve as a stress that could result in alteration of immune responses (Minton and Blecha, 1990).

The results of the current study suggest that restraint would not be expected to play a major confounding role on the effects of space flight on immune responses of rhesus monkeys. Effects of restraint on the immunological parameters studied were minimal.

The lack of effect of restraint on immunological parameters could have been due to the training of the rhesus monkeys for the restraint apparatus. The ESOP is the chair apparatus that would have been used for Space Shuttle experiments, and the animals were trained for restraint in the ESOP. Because of the training, the animals may not have perceived the restraint period as stressful, and their immune responses may not have been affected.

It is of interest that there were several alterations in immunological parameters of both restrained and control monkeys in the week prior to the restraint period. At that period of time, the monkeys had several experimental manipulations performed and had changes in housing. These types of changes have previously been shown to result in alterations in immune responses (Rabin and Salvin, 1987, Salvin, *et al.*, 1990; Cunnick, *et al.*, 1991). Therefore, handling of monkeys independent of restraint could affect immune responses of rhesus monkeys.

## SUMMARY AND CONCLUSIONS

It appears that restraint will not play a major effect in the effects of space flight on immune responses of rhesus monkeys. Most immunological parameters were unaffected by an 18 day restraint period in the ARRT.

Manipulation and housing changes of the monkeys can affect immunological parameters independent of restraint. Care must be taken to minimize such effects and to allow the monkeys sufficient recovery time prior to flight. In any case, all experimental procedures must be carefully controlled.

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### FIGURE LEGENDS

Figure 1. Colonies formed after exposure of bone marrow cells of restrained and control animals to GM-CSF.

Figure 2. CD8+ T cell subpopulation in whole blood of restrained and control animals. Results are shown +/- standard error of the mean.

Figure 3. Levels of IFN- $\alpha$  after lipopolysaccharide stimulation of blood lymphocytes of restrained and control animals. Results are shown +/- standard error of the mean.

Figure 4. Levels of IFN- $\gamma$  after concanavalin-A stimulation of blood lymphocytes of restrained and control animals. Results are shown +/- standard error of the mean.

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