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**FINAL REPORT**

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**SPACEFLIGHT AND IMMUNE RESPONSES OF RHESUS MONKEYS**

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## INTRODUCTION

Evidence from both human and rodent studies has indicated that alterations in immunological parameters occur after space flight [1]. The number of flight experiments has been small, and the full breadth of immunological alterations occurring after space flight remains to be established. Among the major effects on immune responses after space flight that have been reported are: alterations in lymphocyte blastogenesis and natural killer cell activity [1-3], alterations in production of cytokines [4,5], changes in leukocyte sub-population distribution [6], and decreases in the ability of bone marrow cells to respond to colony stimulating factors [6]. Changes have been reported in immunological parameters of both humans and rodents [1]. The significance of these alterations in relation to resistance to infection remains to be established.

The objective of the studies contained in this project is to determine the effects of space flight on immune responses of Rhesus monkeys. The hypothesis is that space flight and the attendant period of microgravity will result in alteration of immunological parameters. The parameters tested include: production of cytokines, composition of leukocyte subpopulations, functional activities of immunologically significant cells, and differences in effects on cells from primary and secondary lymphoid tissues. The expected significance of the work is a determination of the range of immunological functions of the Rhesus monkey, a primate similar in many ways to man, affected by space flight. Changes in immune responses that could yield alterations in resistance to infection may be determined. The duration of alterations in immune responses may also be determined. This could yield useful information for planning studies that could contribute to crew health. Additional information on the nature of cellular interactions for the generation of immune responses may also be obtained.

## METHODS

The methods used in the study have involved the determination of leukocyte subpopulations and the production of cytokines. For the leukocyte subpopulation analysis, whole blood was taken using EDTA as the anticoagulant or lymph node cells were harvested. Samples of whole blood or lymph node cells were stained with the following monoclonal antibodies obtained from the Becton-Dickinson Monoclonal Center, Mountain View, CA: Leu 2a, Leu 3a, Leu4, Leu 11a, Leu 12, Leu-M3, HLA-DR, IgG, and IL-2 receptor. Additional cells were stained with monkey anti-IgM and Monkey anti-IgM (Fab')<sub>2</sub>. Control cells were either stained with a monoclonal antibody directed against an irrelevant species or were unstained [7]. Cells were then analyzed for fluorescence using a Coulter profile II flow cytometer [7].

For the cytokine production, samples of whole blood were placed on ficoll-hypaque density gradients to purify mononuclear cells. The mononuclear cells or the lymph node cells were treated with either phytohemagglutinin-P or polyribosinic-polyribocytidylic acid. After the appropriate incubation period at 37°C, the culture supernatant fluids were harvested and stored at -70°C [8]. The supernatant fluids were assayed by biological assay using vesicular stomatitis virus and Hep-2 cells for interferon-alpha and -gamma production [8].

## RESULTS AND DISCUSSION

For the duration of this grant, we have been able to show the following:

- 1) We have been able to show that we can successfully collect blood, bone marrow and lymph node from adult rhesus monkeys in space flight conditions.
- 2) We have been able to show that we can carry out the following assays on blood - leukocyte subset analysis, interferon production and action, neutrophil activation.
- 3) We have been able to show that we can carry out the following assays on bone marrow cells - leukocyte subset analysis, interferon production, effects of exogenous colony stimulating factor GM on bone marrow cell colony formation
- 4) We have been able to show that we can carry out the following assays on lymph node cells - leukocyte subset analysis, interferon production and action
- 5) The results described in sections 3, 4, and 5 indicate that we can carry out all procedures on cells from adult rhesus monkeys as required for the space flight experiments originally laid out in our Rhesus Project funding proposal.
- 6) We have carried out initial studies on looking for message for cytokines and cytokine receptors on leukocytes, This is an additional refinement technique that will increase the power of our proposed flight studies and is currently under development.
- 7) We have carried out a powers analysis to show statistically the chances of our obtaining statistically significant data for our proposed flight studies
- 8) We have participated in the "SIT:" tests to determine the effects of the flight conditions (outside of flight itself) on the immunological parameters we were planning to observe on flight itself. We saw minimal effects on our immunological parameters.
- 9) We began participation in the "AART" tests to determine the effects of restraint on our

immunological parameters. The data are currently under analysis and results will be forwarded to the technical monitor as they are available.

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## PUBLICATIONS

1) Sonnenfeld, G., Schaffar, L., Schmitt, D.A., Koebel, D.A., and Smith, B.A. Interferon production by and leukocyte phenotyping of rhesus monkey lymph node and peripheral blood cells. *J. Interferon Research*, 13:259-265, 1993. **Appended to this report.**

2) Sonnenfeld, G., Schaffar, L., Schmitt, D.A., Peres, C., and Miller, E.S. The rhesus monkey as a model for testing the immunological effects of space flight. *Advances in Space Research*, In Press, 1994.

3) Sonnenfeld, G. Effects of space flight on immune responses of animals. *Proc. of Animals in Space Conference, Bordeaux*, In Press, 1994.

The following have been submitted for publication. They will be forwarded after acceptance of the manuscripts.

4) Schmitt, D.A., Sonnenfeld, G., Manié, S., and Schaffar, L. *In vitro* interleukin 1 and 2 production and interleukin-2 receptor expression in the rhesus monkey. Submitted for publication, 1994.

Additional manuscripts are expected to be prepared. They will be forwarded to the technical monitor as they are ready.

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## Interferon Production by and Leukocyte Phenotyping of Rhesus Monkey Lymph Node and Peripheral Blood Cells

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### ABSTRACT

The ability of peripheral blood leukocytes to produce interferon- $\gamma$  (IFN- $\gamma$ ) and be labeled with monoclonal antibodies against cell-surface markers was determined in this study. Both peripheral blood leukocytes and lymph node cells were able to produce IFN- $\gamma$  after challenge with mitogens. The rhesus monkey IFN- $\gamma$  was detectable by means of a biological assay but not by means of a radioimmunoassay for human IFN- $\gamma$ . Peripheral blood leukocytes and lymph node cells from rhesus monkeys (*Macaca mulatta*) were treated with fluoresceinated antibodies directed primarily against cell-surface antigens of humans. The degree of binding was determined by means of flow cytometry. Several of the anti-human antibodies did bind to the rhesus monkey peripheral blood leukocytes, as expected. In a novel study, the antibodies bound in a similar fashion to rhesus monkey lymph node cells. Binding of the antibodies was equivalent whether the cells came from inguinal or axillary lymph nodes. Rhesus monkey peripheral blood leukocytes incubated with recombinant human IFN- $\gamma$  showed enhanced expression of class II major histocompatibility complex antigens, as detected with anti-HLA-DR antibodies.

### INTRODUCTION

THE RHESUS MONKEY, *Macaca mulatta*, has been used as a nonhuman primate model for immunological studies.<sup>(1-3)</sup> Many human immunological reagents cross-react with rhesus monkey tissue, allowing studies that can be carried out longitudinally in situations in which human experimentation is not accomplished easily.<sup>(1-3)</sup> This includes studies involving infectious diseases such as the acquired immune deficiency syndrome (AIDS),<sup>(4)</sup> as well as studies involving space flight.<sup>(5)</sup>

In the present study, the ability of rhesus monkey peripheral blood and lymph node cells to produce interferon- $\gamma$  (IFN- $\gamma$ ) was determined, with optimum conditions for IFN- $\gamma$  production established. Although many studies have been carried out showing cross-reactivity of many human reagents, most of these studies have centered on analysis of peripheral blood cell markers.<sup>(2)</sup> In the present study, experiments were designed to determine if leukocyte phenotyping studies could also be carried out using lymph node cells from the monkeys. A comparison of fluorescein-conjugated antibody staining of peripheral

blood leukocytes with staining of lymph node cells was also carried out. A determination of the ability of human IFN- $\gamma$  to cause increases in class II major histocompatibility complex (MHC) antigen expression on both peripheral blood and lymph node cells was carried out. In this fashion, techniques allowing the use of the rhesus monkey as a surrogate for humans using readily available human reagents were validated.

### MATERIALS AND METHODS

*Animals:* Male, adult rhesus monkeys were bred at and obtained originally from Charles River Laboratories (Key Lois Island, FL). The monkeys were maintained at the NASA Ames Research Center Animal Care Center (Moffett Field, CA). All standard monkey health testing, including tuberculin and herpes B virus testing, were carried out routinely. The vivarium is AAALAC accredited, and all procedures were approved by the Ames Research Center and the University of Louisville

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animal care and use committees and carried out under the direct supervision of a veterinarian. At the time of use, the monkeys weighed from 8–15 kg.

**Tissue Sampling:** For blood sampling, the monkeys received a dose of 10 mg/kg of Ketamine and were under anesthesia for a time period of 45 min to 1 h. Blood was sampled from a vein and collected into tubes containing sodium citrate as the anticoagulant. For lymph node sampling, monkeys received a pre-anesthetic dose of Telezol (10 mg/kg), followed by up to 2% isoflurane with 1–2 liters/min of O<sub>2</sub> during surgery. The total time under anesthesia was approximately 2 h. One set of inguinal and/or axillary lymph nodes was removed from each animal.

**Induction and Assay of IFN- $\gamma$ :** IFN- $\gamma$  was induced by a modification of the method of Kirchner *et al.*<sup>(6)</sup> for human peripheral blood. Whole blood was diluted 1 : 10 with RPMI-1640 medium without serum. One milliliter of the diluted blood was added to a well on a 24-well tissue culture plate. Either 5  $\mu$ g of phytohemagglutinin-P or 5  $\mu$ g of concanavalin A (Sigma Chemical Co., St. Louis, MO) were added to each well and the cultures were incubated at 37°C in 5% CO<sub>2</sub> for 48 h. At that point, culture supernatant fluids were harvested and assayed for IFN- $\gamma$  activity. For some blood samples, mononuclear cells were separated from whole blood by centrifugation on Ficoll-Paque.<sup>(7)</sup> The mononuclear cells were resuspended in RPMI-1640 medium with 10% fetal bovine serum at a concentration of  $3 \times 10^6$ /ml and then plated in 24-well tissue culture dishes. The cultures were then induced to produce IFN- $\gamma$  as described above. For lymph node cells,  $3 \times 10^6$  cells were suspended in RPMI-1640 medium with 10% fetal bovine serum, plated in 24-well tissue culture dishes, and then IFN- $\gamma$  was induced as described above. Some of the samples were tested for INF- $\gamma$  in a commercial radioimmunoassay (RIA) for human IFN- $\gamma$  (Centecor Laboratories, Malvern, PA). Other samples were assayed by means of a cytotoxicity assay on HEP-2 cells using the Indiana strain of vesicular stomatitis virus as the test virus.<sup>(8–10)</sup> The titer of IFN- $\gamma$  was equal to the reciprocal of the last dilution of test that decreased viral cytotoxicity by 50%. In this assay, one unit of INF- $\gamma$  was equal to 0.8 international standard units.

**Leukocyte phenotype analysis:** For blood cell staining, 40  $\mu$ l of whole blood was placed in a microcentrifuge tube. For lymph node cell staining, the lymph nodes were teased into individual cells in RPMI-1640 medium with 10% fetal bovine serum (GIBCO-Life Technologies, Inc., Grand Island, NY). The cells were adjusted to a concentration of  $1 \times 10^7$ /ml and 100  $\mu$ l placed in a microcentrifuge tube. The cells were washed once in FTA buffer (BBL Microbiology Systems, Cockeysville, MD) with 0.1 % sodium azide and resuspended in residual buffer. The following fluorescein-conjugated antibodies against leukocyte cell-surface markers were purchased from Becton-Dickenson Immunocytometry Systems, San Jose, CA: anti-Leu 2a-CD8; anti-Leu 3a-CD4; anti-Leu 4-CD3; anti-Leu 7-CD57; anti-Leu 11a-CD16; anti-Leu 12-CD19; anti-Leu 14-CD22; anti-Leu M1-CD15; anti-Leu M3-CD14; anti-HLA-DR; anti-IL2R; anti-human IgM (purchased from Sigma Chemical Co., St. Louis, MO); anti-monkey IgG (purchased from Organon Teknika Corp., W. Chester, PA); F(ab')<sub>2</sub> fragment of anti-monkey IgG (purchased from Organon Teknika Corp., W. Chester, PA); fluorescein-conjugated goat anti-rabbit IgG (non-specific staining control; purchased from Accurate Chemical

and Scientific, Westbury, NY). A negative control was performed without antibodies needed.

Ten microliters of the appropriate antibody was added to each cell suspension, and the cultures incubated at 4°C for 25 min to allow optimum staining.<sup>(11)</sup> One milliliter of lysing solution (8.26 grams of ammonium chloride, 1.00 grams potassium bicarbonate, 37 mg of tetrasodium EDTA, brought to 1 liter with deionized water at pH 7.4) was added to each sample, and the cells were allowed to incubate at room temperature for 6 min to lyse erythrocytes. The cells were then centrifuged at  $1,000 \times g$  for 1.5 min and then resuspended in 1 ml of FTA buffer. Cells were again centrifuged and then fixed by resuspending in 0.5 ml of 1% paraformaldehyde. The cells were then analyzed for fluorescence, an indicator of presence of the antigen, with a FACSCAN flow cytometer (Becton-Dickinson Cytometry Systems, San Jose, CA) with Consort 30 software or with a Coulter Profile II flow cytometer (Coulter Electronics, Hialeah, FL). Lymphocytic and monocytic regions were gated on three-part differentials by use of forward *versus* side scatter plots. Negative gates were set by use of unstained samples. Fluorescein isothiocyanate fluorescence >530 nm was detected with the 488-nm line of an argon ion laser for excitation.

**Incubation of cells with IFN- $\gamma$ :** Human recombinant IFN- $\gamma$  (lot N9001AX, sp. act.,  $1.8 \times 10^7$  units/mg protein) was a gift of Dr. Christine Czarniecki (Genentech, Inc., S. San Francisco, CA). Whole blood samples were diluted 1 : 10 with RPMI-1640 medium and plated in duplicate in 24-well tissue culture plates (Falcon Plastics, Oxnard, CA). One-hundred units of IFN- $\gamma$  was added to each wells, and the cultures were incubated at 37°C in 5% CO<sub>2</sub> for 24 h.<sup>(12)</sup> The cultures were harvested by scraping with a plastic cell scraper, and 100  $\mu$ l of the cell suspension was placed in a microcentrifuge tubes. Twenty microliters of the anti-HLA-DR antibody was added, and staining carried out as described above.

## RESULTS

### Induction of IFN- $\gamma$

IFN- $\gamma$  was inducible by phytohemagglutinin or concanavalin A in both peripheral blood leukocytes and lymph node cells (Tables 1 and 2). Each protocol was repeated at least two times, and only the results from all monkeys of a representative experiment using concanavalin A are shown. Stimulation of whole blood with mitogens did not result in IFN- $\gamma$  production. Production was observed when ficoll-paque-purified mononuclear cells were challenged with mitogen (Table 1). The IFN- $\gamma$  was not detectable by use of an RIA specific for human IFN- $\gamma$ . The IFN- $\gamma$  biological antiviral activity was detectable, however, using an antiviral assay on human HEP-2 cells. Very low levels of innate antiviral activity were detected in the culture supernatants from purified peripheral blood mononuclear cells and lymph node cells not challenged with mitogen.

### Fluorescein staining of peripheral blood leukocytes

Cells from over 30 monkeys were analyzed, and only representative data are shown here. Staining of rhesus monkey pe-

TABLE 1. INDUCTION OF IFN- $\gamma$  IN RHESUS MONKEY PERIPHERAL BLOOD LEUKOCYTES BY MITOGENS

<i>Treatment</i>	<i>n</i>	<i>Assay</i>	<i>IFN-<math>\gamma</math> Titer <math>\pm</math> SD (units/ml)</i>
Whole blood	5	RIA	None detectable
Whole blood + mitogen	5	RIA	None detectable
Whole blood	5	Antiviral assay	None detectable
Whole blood + mitogen	5	Antiviral assay	None detectable
Mononuclear cells	4	RIA	None detectable
Mononuclear cells + mitogen	4	RIA	None detectable
Mononuclear cells	4	Antiviral assay	12 $\pm$ 13
Mononuclear cells + mitogen	4	Antiviral assay	1,205 $\pm$ 602

ipheral blood leukocytes was observed with the following antibodies: anti-Leu 2a, anti-Leu 3a, anti-Leu 11a, anti-HLA-DR, anti-human IgM, anti-monkey IgG, and anti-monkey IgG (Fab')<sub>2</sub> (Fig. 1). No appreciable levels of staining were observed using goat anti-rabbit IgG or any of the other antibodies directed against human leukocyte cell-surface antigens.

#### *Fluorescein staining of lymph node cells*

Cells from 5 monkeys were analyzed, and only representative data are shown here. Staining of the rhesus monkey lymph node cells followed the same pattern as that observed with peripheral blood leukocytes (Fig. 2), except all staining appeared to be more intense than that observed with peripheral blood leukocytes and the anti-Leu 4 also stained the lymph node cells. There were no apparent differences in staining patterns of cells from inguinal or axillary lymph nodes.

#### *Induction of class II MHC antigen expression by human IFN- $\gamma$*

Cells from 11 monkeys were analyzed, and only representative data are shown. Peripheral blood leukocytes from rhesus monkeys showed increased expression of class II MHC antigens [RhLA-D(DR)] after overnight incubation with human recombinant IFN- $\gamma$ , as detected by use of an anti-HLA-DR antibody (Fig. 3).

## DISCUSSION

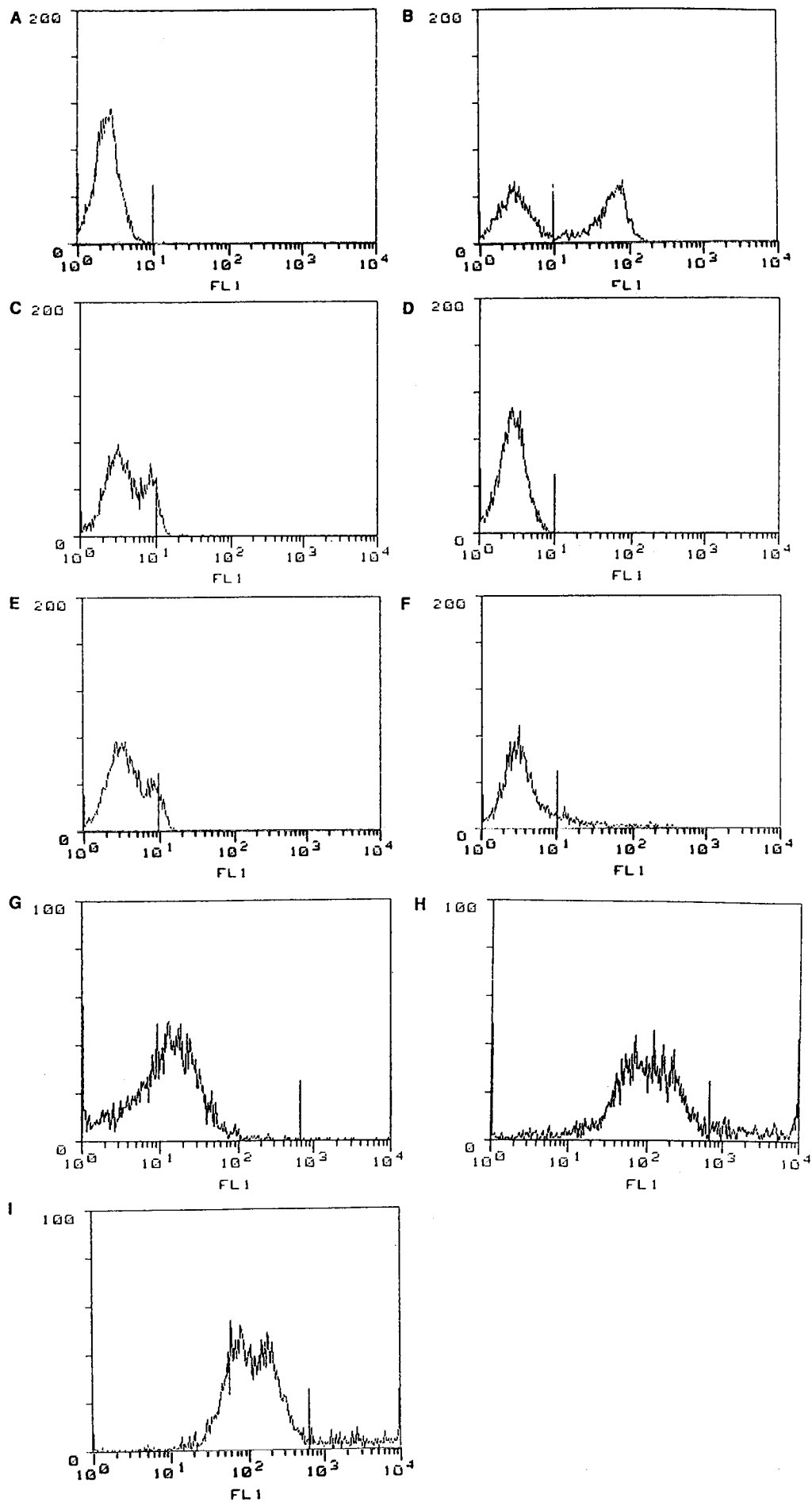
Studies involving immunological reagents developed for use in humans have been carried out using rhesus monkey cells.<sup>(1-3)</sup> This can be of great advantage in allowing the use of the rhesus monkey as a model for diseases or experimental situations in which human experimentation would be difficult.

In the present study, the production of IFN- $\gamma$  by both rhesus monkey peripheral blood leukocytes and lymph node cells after mitogenic challenge was also explored. Previous studies have indicated that human and rhesus monkey IFNs are cross-reactive.<sup>(13,14)</sup> However, in our hands, a commercial RIA kit for human IFN- $\gamma$  could not be used for the detection of IFN- $\gamma$  produced by rhesus monkey leukocytes. This appears to indicate that the monoclonal antibody used in the kit for detection of human IFN- $\gamma$  was not cross-reactive with rhesus monkey IFN- $\gamma$ . Therefore, the most reliable method for detection of rhesus monkey IFN- $\gamma$  appears to be a biological assay testing cell culture supernatants for antiviral activity. Apparently, this assay can be carried out conveniently using human HEP-2 or other human cells as the target cell for the virus infection.

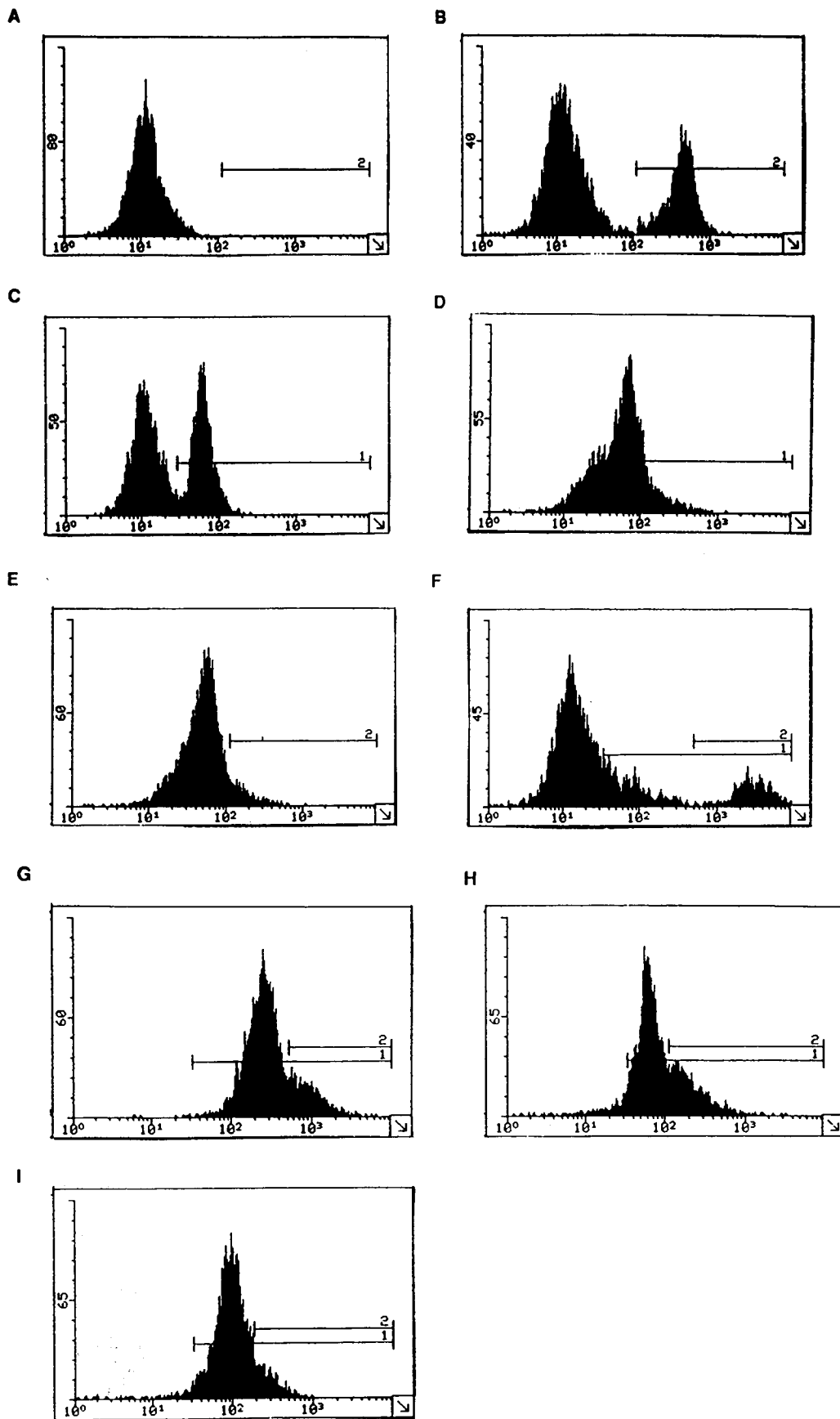
The whole blood culture technique used routinely for production of human IFN- $\gamma$ <sup>(6)</sup> does not appear to be effective for production of rhesus monkey IFN- $\gamma$ . The more cumbersome technique involving purification of mononuclear cells from peripheral blood does, however, appear to be very effective for the study of induction of IFN- $\gamma$  by the rhesus monkey. Induc-

TABLE 2. INDUCTION OF IFN- $\gamma$  IN RHESUS MONKEY LYMPH NODE CELLS BY MITOGENS

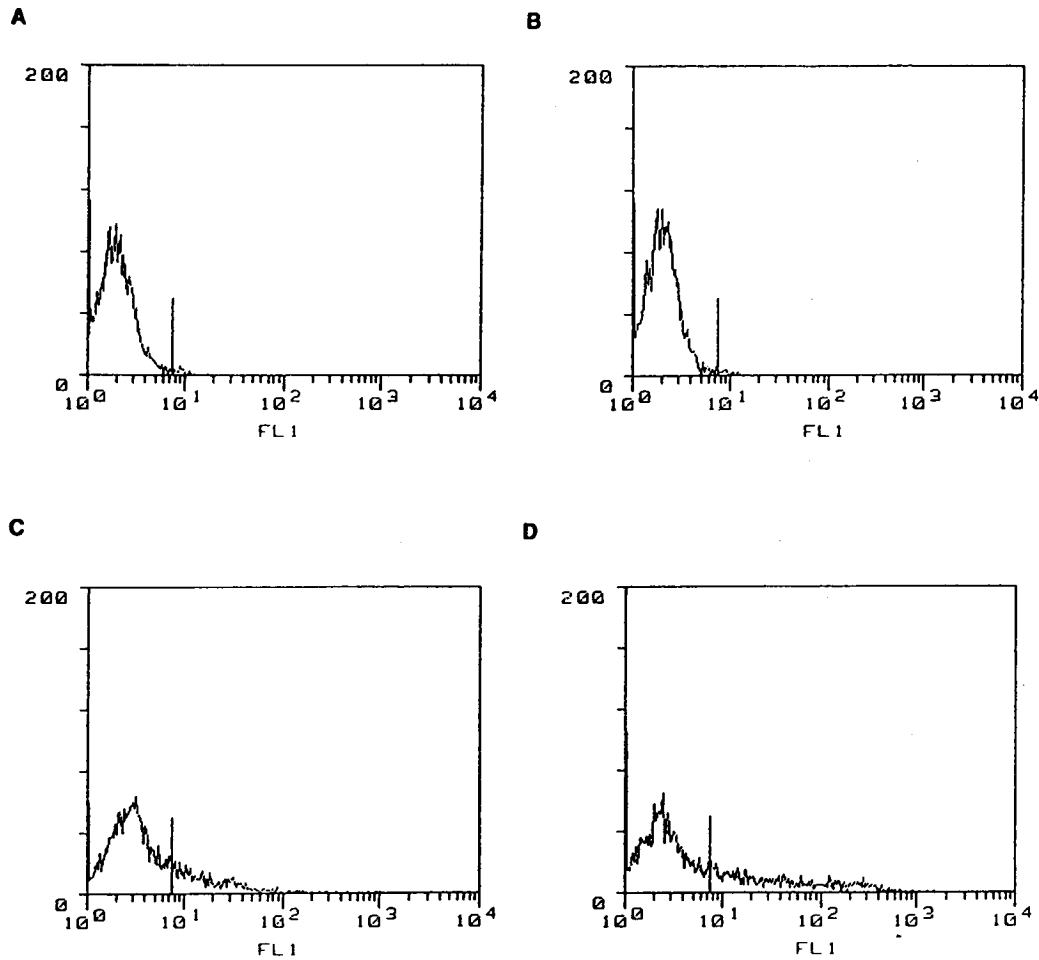
<i>Treatment</i>	<i>n</i>	<i>Assay</i>	<i>IFN-<math>\gamma</math> Titer <math>\pm</math> SD (units/ml)</i>
Lymph node cells	4	RIA	None detectable
Lymph node + mitogen	4	RIA	None detectable
Lymph node cells	4	Antiviral assay	21 $\pm$ 32
Lymph node cells + mitogen	4	Antiviral assay	285 $\pm$ 216



**Fig. 1.** Fluorescein staining of peripheral blood leukocytes. A. No stain. B. Anti-Leu 2a. C. Anti-Leu 3a. D. Anti-Leu 4. E. Anti-Leu 11a. F. Anti-HLA-DR. G. Anti-human IgM. H. Anti-monkey IgG. I. Anti-monkey IgG (Fab')<sub>2</sub>.



**Fig. 2.** Fluorescein staining of lymph node cells. A. No stain. B. Anti-Leu 2a. C. Anti-Leu 3a. D. Anti-Leu 4. E. Anti-Leu 11a. F. Anti-HLA-DR. G. Anti-human IgM. H. Anti-monkey IgG. I. Anti-monkey IgG (Fab')<sub>2</sub>.



**Fig. 3.** Induction of class II MHC antigen expression by human IFN- $\gamma$ . A. No treatment, no stain. B. Incubated with IFN- $\gamma$ , no stain. C. No treatment, anti-HLA-DR. D. Incubated with IFN- $\gamma$ , anti-HLA-DR.

tion of IFN- $\gamma$  by mitogens in rhesus monkey lymph node cell cultures was carried out without difficulty. It should be noted, that the IFN- $\gamma$  could have been contaminated with other interferons, as it was not purified or characterized further.<sup>(15)</sup>

The results of the current experiments also confirm that leukocyte surface antigen analysis can be successfully carried out using primarily antisera directed against human antigens.<sup>(2)</sup> A great number of cell-surface markers can be analyzed, but several markers on rhesus monkey cells, including macrophage markers, did not show a cross-reaction with antisera directed against human antigens.

A novel result of the present study is we have also shown that lymph node cells from rhesus monkeys can be analyzed for leukocyte surface antigen expression using primarily antibodies against human cell-surface markers. The range of antibodies that could be used was similar to that found effective on peripheral blood leukocytes, except the lymph node cells appeared to stain more intensely than the peripheral blood leukocytes.

In addition, we have demonstrated that human IFN- $\gamma$  can be used to enhance and study expression of class II [RhLA-D(DR)] MHC antigens on the surface of rhesus monkey peripheral blood leukocytes. The enhanced expression of the MHC anti-

gens after IFN- $\gamma$  treatment of the leukocytes was readily detectable using an antibody directed against HLA-DR antigens, and offer the potential of novel studies for antigen presentation.

The expansion of immunological assays that are achieved in the rhesus monkey using immunological reagents that are designed for use in humans can serve as a valuable research resource. Since new, potentially expensive, reagents specific for rhesus monkey antigens do not appear, on the whole, to require development, immunological experiments using a close-to-human surrogate such as the rhesus monkey can be carried out readily.

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