# Title

Potential for Therapy with Immune Interferon

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## Distribution Statement

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## Abstract

It is known from in vitro studies that immune interferon can influence the host response to virus infections in several ways, e.g., aids in the induction of cytotoxic T lymphocyte responses, by protecting host cells including macrophages against viral infection, by activating macrophages, and by enhancing the expression of HLA and B antigens. The goal of this study is to determine the role of immune interferon in vivo in recovery from challenge with virus infections.
Our findings during the first year of this study indicate:

1) Cytoxic T lymphocytes (Tc) are very effective immunotherapeutic agents in treating viral pneumonia.

2) The effective Tc are MHC and virus specific.

3) The Tc produce immune interferon when they recognize specific viral and MHC antigens on the infected target cell.

4) Gamma interferon can be detected in the cytoplasm of these antigen stimulated lymphocytes shortly after exposure to infected target cells.

5) Clones of virus specific cytotoxic or helper Tc which produce gamma interferon, have been developed and will be used in immunotherapy studies.
Abstract

We have analyzed the immunotherapeutic effect of cytotoxic T lymphocytes transferred to mice with influenza and demonstrated that highly cytotoxic influenza A specific H-2 restricted killer T cells are able to significantly reduce the pulmonary virus titer of mice with influenza pneumonia. The effect is dose dependent with significant decreases in lung virus titers observed at immunotherapeutic doses of $5 \times 10^6$ through $30 \times 10^6$ stimulated spleen cells. The reduction in virus titers is impressive, an 90% decrease at a dose of $5 \times 10^6$ cells and 99% decrease at a dose of $30 \times 10^6$ cells, when lungs were tested 4 days after challenge. The effector cells must be cytotoxic to influenza A infected syngeneic cells in vitro in an H-2 restricted virus specific fashion, if they are to protect. Interferon titers were performed on the same lungs which were tested for influenza virus, and the recipients of the cytotoxic cells which had the greatest reduction in pulmonary virus had no detectable antiviral activity in their lungs. A low level of interferon was detected in the recipients of lower doses of cells but the highest levels of interferon were detected in the mice who were infected but did not receive cytotoxic cells.

Experiments were performed to analyze the effect of major histocompatability complex (MHC) control over the production of interferon when the cytotoxic T cell comes in contact with the viral infected target cell. Influenza virus, of course, induces alpha interferon but higher levels of interferon are observed when previous immunized animals were the source of spleen cells which were added to virus infected H-2 matched target cells. The higher levels of interferon are induced when virus infected target cells of the same H-2 type were exposed to H-2 matched lymphocytes, which had been previously stimulated with same or a related influenza A virus that was used to infect the target cells, but not when exposed to influenza B virus infected target cells. There is a decrease in the level of antiviral activity noted following pH 2 treatment of fluids which were obtained from H-2 matched target cells which had been exposed to influenza A virus stimulated cells, and this interferon is neutralized by antiserum to gamma interferon. In the presence of unprimed lymphocytes or of infected target cells expressing the relevant influenza A virus but with different H-2 antigens there is little or no interferon detected, and the interferon detected is pH2 stable., and is neutralized by antiserum to a interferon.

Production of interferon during stimulation of memory lymphocytes by syngeneic virus infected stimulator cells was also analyzed. Peak interferon titers are reached by 18 hours after exposure of responder lymphocytes to such stimulating cells. The highest titers of antiviral activity in supernatant fluids were obtained when memory lymphocytes from previously immunized mice were stimulated with a related influenza A virus, although the antigenic relatedness is crossreactive among influenza A subtypes. The interferon produced by memory cells that had previously been infected with the same influenza A virus or one of another A subtype is gamma interferon as characterized with specific antigamma interferon antibodies. Immune cells cultured without
restimulation, and non-immune lymphocytes stimulated by virus infected cells, produce alpha interferon which is neutralized by specific antiserum to alpha interferon.

In preliminary experiments we have begun to characterize the lymphocytes producing alpha and gamma interferon in these cultures. A large percentage of cells in these responding lymphocyte cultures contain antigens which react with anti-murine interferon alpha serum, or anti-murine interferon gamma serum. Using normal rabbit serum the level of background staining was about 1%. The percentage of cells which contain murine interferon alpha following antigenic stimulation with virus infected syngeneic cells in culture for 18 hrs is approximately 6%, and 18% contained interferon gamma interferon. When these cells were added to H-2 matched target cells infected with influenza A virus there is a marked increase in the percent of cells reacting with the antiserum to murine gamma interferon to 50%, without an increase in the number of cells reacting with antiserum to alpha interferon. Thus, we have evidence that gamma interferon producing cells are present in the population of cells which are cytotoxic in vitro and which are highly effective as immunotherapeutic agents when transferred into mice with influenza pneumonia. The striking increase in the number of cells containing gamma interferon when they are exposed to the virus infected target cells suggests an important role for gamma interferon in recovery from influenza pneumonia.

We have developed clones of helper or cytotoxic virus specific MHC restricted T lymphocytes which produce gamma interferon to various levels following stimulation by infected antigen presenting cells. These clones will be used in immunotherapeutic protocols to determine the contribution of the Tc and/or gamma interferon in recovery from viral pneumonia.
Narrative Summary

We have focused our attention during the first few months of this project to the respiratory viral challenge model. We had published some earlier experiments using these techniques, and we were anxious to establish more quantitative information concerning this model of immunotherapy of acute respiratory virus pneumonia prior to performing extensive therapeutic intervention experiments using antiserum to murine alpha and gamma interferon. We have available specific antisera against murine alpha and gamma interferon. The antisera which we have used in these experiments was kindly provided by our collaborators: Dr. Kawade of Kyoto gave us a high titered specific anti-interferon alpha antisera prepared in rabbits, and we have two antisera to murine gamma interferon provided by Dr. E. Havell of New York and Dr. H. Johnson of Galveston.

The general methods employed in the immunotherapeutic protocol are summarized in Figure 1. Very briefly, we immunized the donor animals with sub-lethal doses of virus given by the natural intra-nasal route. Three weeks or more later we remove spleen cells from the donor animals and stimulate them in vitro with syngeneic stimulator cells infected with influenza A virus, the unrelated influenza B virus, or with uninfected stimulator cells. Following 5 days of culture the responding cells are tested for their ability to kill influenza infected target cells in a $^{51}$Cr cytotoxicity assay. The spleen cells from immunized donor animals will routinely become highly cytotoxic in vitro if stimulated by a related influenza A virus, but immune cells stimulated with an unrelated virus, or by uninfected stimulator cells will not become cytotoxic. We measure the production of interferon by these stimulated cytotoxic T cells, and transfer them to recipient animals who have influenza pneumonia as a result of previous administration of influenza virus. Three days after cell transfer we assess the therapeutic effects by measuring pulmonary virus titers, and interferon activity in the lungs of the recipient mice. The interferon which is measured in the supernatant fluids of the stimulated cells when they are exposed to infected target cells or in the lungs of infected mice is titered by pretreatment of L929 cells which is subsequently challenged with VSV virus. Initial characterization is done by the pH treatment and detailed interferon analysis is performed using specific antisera.

The effect of Tc transfer on influenza virus titers in the lungs of recipient mice is shown below as Experiment 1 in Table 1.
IMMUNOTHERAPY OF INFLUENZA PNEUMONIA

DONOR

ALB/c

i.n. approx. 100 PFU

3 weeks or more

spleen

Stimulated with virusinfected
syngeneic spleen cells.

37°C

5 days

Virus

IFN

IFN activity

(L\textsubscript{ac29}, VSV)

IFN production

3 days

In vivo transfer

1 day

RECIPIENT

BALB/c

i.n. 1000 PFU

i.v. (3-30 x 10\textsuperscript{6}
cells/mouse)

\textsuperscript{51}Cr - release assay (P815)

In vitro

SNF
### Table 1
Effect of Transfer on Influenza Virus Titers in the Lungs of Recipient Mice

**A. Expt. 1**

<table>
<thead>
<tr>
<th>Effector Cells Transferred</th>
<th>% Specific Lysis of Target Cells&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Virus Titer in the Lung&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uninfected</td>
<td>Infected</td>
</tr>
<tr>
<td>Stimulated Immune</td>
<td>56.7</td>
<td>2.1</td>
</tr>
<tr>
<td>Non Stimulated Immune</td>
<td>13.5</td>
<td>0.9</td>
</tr>
</tbody>
</table>

a) E:T ratio of 3:1  

b) Pulmonary Virus Titer - Egg Infections Dose 50 (EID<sub>50</sub>/0.1 ml) on day 4 after challenge.  
c) Cells transferred: 12 x 10<sup>6</sup>/recipient on day 1 after viral challenge.

**B. Expt. 2**

<table>
<thead>
<tr>
<th>Effector Cells Transferred</th>
<th>% Specific Lysis of Targeted Cells&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Virus Titer in the Lung&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uninfected</td>
<td>Infected</td>
</tr>
<tr>
<td>Stimulated-30 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>18.6</td>
<td>1.1</td>
</tr>
<tr>
<td>Stimulated-10 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>None</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

a) E:T ratio - 3:1  
b) Pulmonary Virus Titer - EID<sub>50</sub>/0.1 ml.

The stimulated immune cells were highly cytotoxic even at the low effector target ratio of 3:1 when they are added to syngeneic target cells infected with the influenza A virus that was used to infect the donor mice. This is a standard 51Cr release assay and the percent specific lysis is calculated by the formula:  

\[
\text{% Specific Immune Lysis} = \frac{\text{Exp CPM} - \text{Media CPM}}{\text{Max CPM} - \text{Media CPM}} \times 100
\]

The effector cells that have been cultured for 5 days were obtained from immune animals as described above; some were cultured in the presence of syngeneic influenza A infected cells and others in flasks which did not contain such stimulator cells. These were assessed in the 51Cr release assay, and even at the low effector target ratio of 3:1 they caused very high specific killing of infected target cells. When these cells were transferred to recipient mice there was an approximately 90% reduction in lung virus titus. The lower portion of Table 1, reveals data from Experiment 2 in which we studied the effects of 2 doses of stimulator cells, 30 x 10<sup>6</sup> and 10 x 10<sup>6</sup>, after transfer to virus infected recipients. Recipients of 10 x 10<sup>6</sup> cells had a
90% reduction in pulmonary virus titer, and when the dose is increased to 30 x 10^6 the reduction in virus titer was approximately 99%.

A more detailed dose response study was performed and results of this experiment are represented in Table 2. There was a 90% reduction in pulmonary virus titer at doses of 5 to 10 million cells but when 20 to 30 million cells were given greater than 99% of reduction in virus titer was observed.

**TABLE 2**

Dose Response Effect of Transferred T\(_c\) on Influenza Virus Titers in Lungs of Recipient Mice

<table>
<thead>
<tr>
<th>Effector Cell Transferred (Immune-Stimulated)</th>
<th>Virus Titer in the Lung</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 x 10^6</td>
<td>3.63 ± 1.2(^a)</td>
</tr>
<tr>
<td>20 x 10^6</td>
<td>3.97 ± 1.3</td>
</tr>
<tr>
<td>10 x 10^6</td>
<td>4.53 ± 1.2</td>
</tr>
<tr>
<td>5 x 10^6</td>
<td>4.83 ± 0.9</td>
</tr>
<tr>
<td>0</td>
<td>5.97 ± 0.07</td>
</tr>
</tbody>
</table>

\(^a\)Logjo EID\(_{50}\) expressed as Mean ± S.D., 3 lung/group.

Table 3 contains the results of pulmonary virus and lung interferon assays measured in the recipients of the cytotoxic T lymphocytes in this dose response experiment. Following administration of the cytotoxic T lymphocytes there was a marked decrease in the pulmonary virus titers. The lung homogenates were measured for their content to interferon after being treated with a high titered specific antisera to influenza hemagglutinin to neutralize all residual viral infectivity, which was confirmed (data not present), and the lung interferon titers are shown in Table 3.
## TABLE 3

### Interferon Titer and Pulmonary Virus Titer in Recipients of Tc

<table>
<thead>
<tr>
<th>Cells Transferred</th>
<th>Pulmonarya Virus Titer</th>
<th>Percentb Reduction</th>
<th>IFN in lungc</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 x 10^6</td>
<td>3.63</td>
<td>98</td>
<td>&lt;4</td>
</tr>
<tr>
<td>20 x 10^6</td>
<td>3.97</td>
<td>95</td>
<td>&lt;4</td>
</tr>
<tr>
<td>10 x 10^6</td>
<td>4.52</td>
<td>87</td>
<td>&lt;4</td>
</tr>
<tr>
<td>5 x 10^6</td>
<td>4.83</td>
<td>82</td>
<td>9.8d</td>
</tr>
<tr>
<td>None</td>
<td>5.97</td>
<td></td>
<td>78.8d</td>
</tr>
</tbody>
</table>

a) Pulmonary Virus Titers determined as EID_{50} 3 days after cell transfer, 4 days after viral challenge.
b) Percent reduction in the mean influenza virus titer in the lungs of recipient mice compared to group which received no Tc (three mice/group).
c) International Units of Interferon detected in lungs 4 days after infection, 3 days after cell transfer.
d) pH2 treatment did not change IFN titer of these tested samples.

The results indicate that at the higher dose of transferred cells there was no detectable antiviral activity in the pulmonary homogenates. With a lower number of transferred cells, and replication of over 4 logs of virus, low levels of antiviral activity were detected in the lungs. Animals that received no cells had high pulmonary virus titers, and had a high titer of interferon in the lung homogenate. The interferon detected was demonstrated to be pH 2 resistant, and is alpha interferon in neutralization assays.

We then performed an experiment in which we analyzed the content of interferon in the recipient lungs shortly after cell transfer. The results presented above were obtained 72 hours after cell transfer and 4 days after virus infection.

## TABLE 4

### Interferon Activity in the Lungs of Mice with Influenza Infections

<table>
<thead>
<tr>
<th>Cells Transferred</th>
<th>Hours after Cell Transfer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Stimulated Immune (30x10^6)</td>
<td>&lt;4</td>
</tr>
<tr>
<td>None</td>
<td>&lt;4</td>
</tr>
</tbody>
</table>
Table 4 contains results which indicate that no interferon activity was detected in the recipients of the secondarily stimulated cytotoxic T lymphocytes at any time point from 3 to 72 hours after infection, and that the virus infected mice which had not received such cells had antiviral activity detected at 72 hours when pulmonary virus titers were high. These results do not demonstrate a high production of gamma interferon in the lung of the immunotherapeutically treated animals. Although there is no measurable antiviral activity in the lungs of recipient mice that received the potent immunotherapy with cytotoxic T lymphocytes, the experiment clearly does not rule out the possibility that gamma interferon was produced at the site of target cell interaction with the stimulated effector cells which could be highly active.

In order to examine in a more sensitive way the production of interferon during interaction between killer cells and infected target cells we assessed the antiviral activity of supernatant fluids following exposure of cytotoxic T lymphocytes to virus infected target cells in vitro. The results are summarized in Table 5.

### Table 5

**Interferon Production During Tc Assay of Killer and Infected Target Cells**

<table>
<thead>
<tr>
<th>Effector:Target Cell Ratio</th>
<th>30</th>
<th>10</th>
<th>3</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target Cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infected</td>
<td>25a</td>
<td>23.3</td>
<td>23.3</td>
<td>21.8</td>
</tr>
<tr>
<td>Uninfected</td>
<td>23.3&lt;2</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td></td>
</tr>
<tr>
<td>No Target Cells</td>
<td>2</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td></td>
</tr>
</tbody>
</table>

a) Dilution of supernatant fluid which had antiviral activity.

There is little or no interferon produced when influenza A stimulated effector cells are added to wells containing no target cells. At a 30:1 E:T ratio there was a low level of antiviral activity detected when stimulator cells were added to uninfected target cells and none was detected at lower effector:target ratios. When the stimulated cells were added to infected target cells, interferon activity was detected in the supernatant fluids at all effector target ratios studied. Thus, in a period of just five hours following exposure of immune highly cytotoxic effector cells there was production of antiviral activity when the effector cells were in contact with the target cell. Although there is some interferon alpha in this supernatant fluid since the target cell is infected with influenza virus, the material also contains gamma interferon as determined by neutralization with specific antisera which was produced by the lymphocytes when they contacted the infected target cell. In addition to being able to kill influenza A infected cells these stimulated lymphocytes are able to produce gamma interferon when they recognize the appropriate viral infected target cell.

In the next experiments we analyzed the effect of major histocompatibility of antigen; as well as viral antigentic specificity on interferon production by cytotoxic T lymphocytes when they come into contact with the virus.
infected target cells. The results indicate that the addition of immune lymphocytes which have been secondarily stimulated in vitro by influenza A viruses produce much higher levels of interferon when they are placed on target cells infected with related influenza A viruses than on target cells that are infected with unrelated influenza B virus or are not infected. The addition of immune cells to target cells which were have mismatched MHC antigens resulted in no increase in interferon activity over that produced by the virus itself in the absence of added lymphocytes.

TABLE 6

Effect of MHC Antigen and Virus Antigens on IFN Production by Tc

A. Previous Stimulation of H-2D Effectors

<table>
<thead>
<tr>
<th>Primary</th>
<th>Secondary</th>
<th>P815 (H-2d) Target Cells</th>
<th>L929 (H-2k) Target Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A/PC</td>
<td>A/BK B</td>
<td>A/PC</td>
</tr>
<tr>
<td>A/PC</td>
<td>A/PC</td>
<td>16 8 2 2</td>
<td>8</td>
</tr>
<tr>
<td>A/PC</td>
<td>A/BK</td>
<td>64 16 2 &lt;2</td>
<td>8 &lt;2</td>
</tr>
<tr>
<td>A/PC</td>
<td>B</td>
<td>8 4 2 2</td>
<td>8</td>
</tr>
<tr>
<td>A/PC</td>
<td>0</td>
<td>16 &lt;2 2 &lt;2</td>
<td>8 &lt;2</td>
</tr>
<tr>
<td>0</td>
<td>A/PC</td>
<td>32 -a 2 &lt;2</td>
<td>-</td>
</tr>
<tr>
<td>0</td>
<td>A/BK</td>
<td>- 4 2 &lt;2</td>
<td>-</td>
</tr>
<tr>
<td>0</td>
<td>8</td>
<td>8 4 2 2</td>
<td>8</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>16 &lt;2 2 &lt;2</td>
<td>8 &lt;2</td>
</tr>
</tbody>
</table>

*a*Indicates not tested

B. Effect of pH2 treatment on IFN Activity

<table>
<thead>
<tr>
<th>Previous Stimulation of Effectors (H-2d)</th>
<th>P815 (H-2d)</th>
<th>L929 (H-2k)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td>Secondary</td>
<td>A/PC</td>
</tr>
<tr>
<td>A/PC</td>
<td>A/PC</td>
<td>4</td>
</tr>
<tr>
<td>A/PC</td>
<td>A/BK</td>
<td>16</td>
</tr>
<tr>
<td>A/PC</td>
<td>B</td>
<td>4</td>
</tr>
<tr>
<td>A/PC</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>0</td>
<td>A/PC</td>
<td>&lt;2</td>
</tr>
</tbody>
</table>

C. Tc Lysis of $^{51}$Cr labelled Target Cells in this Experiment

<table>
<thead>
<tr>
<th>Effector:Target</th>
<th>%Specific release of $^{51}$Cr from target cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Infected</td>
</tr>
<tr>
<td>30:1</td>
<td>91.9</td>
</tr>
<tr>
<td>10:1</td>
<td>82.0</td>
</tr>
<tr>
<td>3:1</td>
<td>68.1</td>
</tr>
</tbody>
</table>
The results presented in Table 6 indicate that increased levels of interferon are detected after adding immune lymphocytes to virus infected target cells. Increased interferon is detected if the lymphocytes have been stimulated by the related influenza virus previously, and when the stimulated lymphocytes are placed on H-2 matched target cells but not on target cells infected with an irrelevant virus, influenza B, on uninfected targets or on target cells infected with the relevant virus but which do not share H-2 antigens. The wells from which supernatant fluids were obtained in this experiment contained target cells which were not labelled with $^{51}$Cr, but we confirmed that active cytotoxic T cell activity was present in a parallel experiment performed on $^{51}$Cr labelled cells in another plate. The results shown on the bottom of Table 5 indicate a high percentage of the infected cells were killed in a dose responsive fashion by the cytotoxic T lymphocytes. Some of the supernatant fluids from this experiment were treated at pH2 and assessed for the effective antiviral activity. There was little or no change seen in antiviral activity with supernatant fluids from the H-2 mismatched effector and target cells after pH2 treatment, so that interferon is acid resistant. The supernatant fluids from wells that contained H-2 matched effector and target cells contains some interferon which decreases in titer following treatment at pH 2.

This indicated that there is MHC restriction and influenza virus antigenic specificity in the production of interferon when the cytotoxic T lymphocyte recognizes infected target cells. To further confirm the presence of gamma interferon in such wells we attempted to neutralize the activity of the interferon activity with the specific antialpha IFN antisera provided by Dr. Kawade and antigamma IFN by Dr. Johnson. Immune cells that were stimulated by a related virus were added to target cells infected by a related virus produced interferon that was blocked by antimirine gamma antibody. Non-immune lymphocytes exposed to infected target cells had interferon activity that was neutralized by antisera to alpha interferon, but not by the anti-sera to murine gamma interferon as shown in the table below.

Table 7

<table>
<thead>
<tr>
<th>Lymphocyte Donor</th>
<th>Stimulation</th>
<th>Titer of Interferon After Antisera$^a$ Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>- NRS</td>
</tr>
<tr>
<td>Immune</td>
<td>+</td>
<td>32</td>
</tr>
<tr>
<td>Immune</td>
<td>-</td>
<td>16</td>
</tr>
<tr>
<td>Non-Immune</td>
<td>+</td>
<td>4</td>
</tr>
<tr>
<td>Non-Immune</td>
<td>-</td>
<td>8</td>
</tr>
</tbody>
</table>

$^a$Antisera and NRS used at 1:30 dilution, as supplied by Dr Kawade (R anti α) and Dr. Johnson (R anti ).
To follow up on these observations that lymphocytes produce gamma interferon when they come into contact with the virus infected target cell, we have begun experiments to quantitate and further characterize the nature of the lymphocytes producing a gamma interferon in this system. In the first of these experiments, the results of which are briefly summarized in the table below, we examined cells by indirect fluorescence antibody techniques for the presence of interferon in the population of immune cells which were highly cytotoxic in vitro and therapeutic in vivo. These lymphocytes had been stimulated in vitro with influenza A infected syngeneic cells for 18 hrs and then were placed on target cells, matched at the H-2 locus, which were either uninfected or infected with the same influenza A that had been used to stimulate the donor lymphocytes. These lymphocytes were removed from wells containing no target cells, uninfected H-2 matched target cells, or virus-infected H-2 matched target cells, and reacted with normal rabbit serum, hyperimmune specific antimurine alpha interferon serum (Dr. Kawade) or antimurine gamma interferon (Dr. Johnson). The results indicated that approximately 25 to 35% of the lymphocytes in the culture following 5 days of stimulation by influenza A infected stimulator cells contained cytoplasmic antigen which reacted with the antimurine interferon alpha antibody or the antimurine gamma interferon antibody. The percentage of cells containing gamma interferon increased to over 50% when the stimulator cells were placed on H-2d matched target cells infected with the homologous virus. Thus, the data in the table suggest that following antigenic stimulation, bulk cultures of lymphocytes contain a high percentage of lymphocytes which contain interferon gamma in their cytoplasm as a result of interaction with influenza A infected stimulator cells. Following further stimulation by contact with target cells infected with influenza viruses there is an increase in the percentage of cells which contain interferon, especially those which contain murine gamma interferon.

**TABLE 8**

Percentage of In Vitro Stimulated Cells Containing Interferon Antigen

<table>
<thead>
<tr>
<th>Addition of Stimulators to Target Cells</th>
<th>NRS</th>
<th>Anti mu IFN α</th>
<th>Anti mu IFN</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Target</td>
<td>0</td>
<td>0</td>
<td>4.6</td>
</tr>
<tr>
<td>Uninfected P815</td>
<td>0</td>
<td>0</td>
<td>17.5</td>
</tr>
<tr>
<td>A/PC Infected P815</td>
<td>1.7</td>
<td>6.1</td>
<td>50.2</td>
</tr>
</tbody>
</table>

*Used at 1:30 dilution in an indirect FA assay.

The antiserum to IFN (supplied by Dr. H. Johnson of Galveston, Texas) which was used in the indirect fluorescent antibody in this experiment was used at a 1:30 dilution. In a subsequent experiment the antisera was diluted and detected cytoplasmic antigen in a high percentage of the stimulated lymphocytes at dilutions tested up to 1:7680. Thus, it is very likely that this antiserum to gamma interferon was reacting with gamma interferon in the
cytoplasm of the stimulated lymphocytes, and a lower percentage of cells reacted with a specific antiserum to murine \( \alpha/\beta \) interferon (supplied by Dr. Kawade of Kyoto).

Table 9

Percentage of cells FA positive at serum dilution

<table>
<thead>
<tr>
<th>Antisera</th>
<th>1:30</th>
<th>1:120</th>
<th>1:480</th>
<th>1:1920</th>
<th>1:7680</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti murine ( \gamma )IFN</td>
<td>50.9</td>
<td>49.4</td>
<td>52.3</td>
<td>40.0</td>
<td>30.3</td>
</tr>
<tr>
<td>Anti murine ( \alpha/\beta )IFN</td>
<td>28.4</td>
<td>22.9</td>
<td>22.6</td>
<td>20.7</td>
<td>15.5</td>
</tr>
</tbody>
</table>

Clones of virus specific \( T \) lymphocytes and of \( T \) cells producing gamma interferon

We have recently begun to establish clones of virus specific \( T \) cells which are cytototic in an \( H-2 \) restricted virus-specific fashion and/or produce gamma interferon when stimulated by MHC matched virus-infected stimulator cells, as described above. These clones have been produced following repeated stimulation in vitro of immune spleen cells by \( x \)-irradiated virus-infected stimulator cells, and are maintained with 20% human \( T \) cell growth factor. Several clones have been developed and preliminary characterization of these clones is contained in Table 10.

Table 10

Summary of Preliminary Data on Influenza Specific \( T_C \) Clones*

<table>
<thead>
<tr>
<th>Clones</th>
<th>CTL activity( xx )</th>
<th>Surface phenotype</th>
<th>MuIFN production</th>
<th>Protection*</th>
</tr>
</thead>
<tbody>
<tr>
<td>B8</td>
<td>+ (56%)</td>
<td>Thy1(^+), Lyt1(^{-2+})</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>C3</td>
<td>+ (53%)</td>
<td>Thy1(^+), Lyt1(^{-2+})</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>C9</td>
<td>+ (36%)</td>
<td>Thy1(^+), Lyt1(^{-2+})</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>C12</td>
<td>+ (55%)</td>
<td>Thy1(^+), Lyt1(^{-2+})</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>D1</td>
<td>+ (32%)</td>
<td>Thy1(^+), Lyt1(^{-2+})</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>D9</td>
<td>+ (34%)</td>
<td>Thy1(^+), Lyt1(^{-2-})</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>E11</td>
<td>- (0%)</td>
<td>Thy1(^+), Lyt1(^{-2+})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F5</td>
<td></td>
<td>Thy1(^+), Lyt1(^{-2+})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G5</td>
<td></td>
<td>Thy1(^+), Lyt1(^{-2+})</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( * \) Blank spaces indicate not tested; protection data based on one immunotherapy experiment and reduction in lung virus titer of approximately 3-4 fold for +clones and none by -clones (\( p<0.04 \)).

\( xx \) ( ) values indicate level of specific immune lysis using clones of \( T \) cells at 5 or 3:1 E:T ratios.
In summary, these experiments indicate that gamma interferon is produced following interaction between stimulated T lymphocytes and target cells infected with the same virus. In addition, we are developing clones of T lymphocytes which are virus specific and produce gamma interferon. The production of this interferon is controlled by MHC restriction and is viral antigen specific. Lymphocytes exposed to the appropriate antigen presenting cells, infected with influenza A virus and sharing the same major histocompatibility antigens, contain gamma in their cytoplasm. Upon exposure to target cells infected with the same virus there is a further increase in the number of lymphocytes which have gamma interferon detected in their cytoplasm.

In the coming year, we will characterize the cells which produce gamma interferon when they recognize influenza infected cells in vitro and in vivo, using techniques such as those recently reported from DeLey’s Laboratory. In addition we will use two conjugates, flourescein and rhodamine to determine whether given cells contain just alpha interferon, or gamma interferon interferon antigens. Other work in progress includes developing clones of lymphocytes that have been stimulated with influenza A infected stimulator cells, which are highly cytotoxic and/or very active producers of gamma interferon. These clones should provide valuable information on the relative contribution of gamma interferon and/or cytotoxic destruction of the virus infected organ in recovery following immunotherapy.

With the rapid development of recombinant DNA methods for preparing purified interferons, we have decided to await the very likely availability in the near future of purified murine gamma interferon before performing direct immunotherapeutic experiments with gamma interferon. We could prepare murine gamma interferon following the methods of Havel, but the recent publication of the complete sequence of murine gamma interferon indicates there is very likely to be a commercial source of recombinant DNA prepared murine gamma interferon in the near future, as well as a monoclonal antibody to the same. Thus we have focused our efforts in these first months on tightening our immunotherapeutic protocol and on dissecting in some detail the possible contribution of gamma interferon in this immunotherapeutic approach. We expect in the coming year to extend experiments on the gamma interferon production when virus infected target cells are exposed to sensitized lymphocytes. With the availability of higher titer monoclonal antibodies to alpha and gamma interferon we will perform in vivo experiments to determine whether administration of antagonamma interferon will block this protected immunotherapeutic effect. Although we have been kindly provided antisera to gamma interferon by Drs. Havell and Johnson, they are polyclonal and are not of high enough titer to use in the complex in vivo challenge and transfer studies. We have used them extensively for characterization of the interferons produced in vivo and in vitro.
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