Human Interferon Alpha and Gamma Production by Lymphocytes During the Generation of Influenza Virus-specific Cytotoxic T Lymphocytes

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SUMMARY

We analysed the production of interferons (IFN)-alpha and -gamma during the generation of human influenza-virus specific cytotoxic T lymphocyte (CTL) responses using monoclonal antibodies in a specific radioimmunoassay. The results showed that the peripheral blood mononuclear cells (PBM) of all donors tested produced IFN-gamma and had influenza A virus-specific CTL activity after stimulation. The amount of IFN-gamma produced and the level of CTL activity were significantly correlated. The PBM of some donors also produced IFN-alpha. The level of IFN-gamma produced was low during the first few days and increased subsequently, but IFN-alpha, when it was detected, was produced on day 1. The kinetics of the increase in IFN-gamma correlated with the increase in CTL activity. We also observed an increased percentage of cells bearing interleukin-2 receptors, which may have been a response to the production of IFN-gamma. The T cells active in lysing influenza A virus-infected target cells and in producing IFN-gamma were determined after separating effector cells with monoclonal antibodies. The CTL effector cells were mainly in the T8 + subset, but IFN-gamma-producing cells were found in both T4 + and T8 + subsets. These results suggest that influenza virus-specific T8 + CTL produce IFN-gamma in response to virus, and that T4 + cells which are not CTL effectors also produce IFN-gamma after restimulation with influenza A virus-infected cells.

INTRODUCTION

We reported the production of high titres of interferon (IFN) from human lymphocytes following stimulation with influenza virus-infected cells in an earlier publication (Ennis & Meager, 1981). IFNs-alpha and -gamma were detected in the supernatant fluids, and most of the IFN appeared to be gamma. Subsequently, others have reported that primarily human IFN-alpha was produced by peripheral blood mononuclear cells (PBM) (Chonmaitree et al., 1981), macrophages (Roberts et al., 1979) and large granular lymphocytes (LGL) (Djeu et al., 1982; Burlington et al., 1984) after exposure to influenza virus. It was also reported that PBM exposed to influenza virus produced an acid-labile IFN-alpha and not IFN-gamma (Balkwill et al., 1983).

It is important to define more precisely whether influenza virus antigens induce human IFN-alpha and IFN-gamma, in order to evaluate the role of these lymphokines in the generation of influenza virus-specific cytotoxic T lymphocytes (CTL) which have been shown to be important in protection (e.g. Yap et al., 1978; McMichael et al., 1983; Wells et al., 1981a, b). Farrar et al. (1981) suggested that the production of IFN-gamma was important in induction of allogeneic CTL, since addition of specific antiserum to IFN-gamma to mixed lymphocyte reaction (MLR) cultures inhibited the CTL response. On the other hand, a murine influenza virus-specific CTL clone (Morris et al., 1982) and an allogeneic CTL clone (Klein et al., 1982) released IFN-gamma...
when they were co-cultured with antigen-bearing cells, suggesting that IFN-gamma produced by CTL might prevent spread of virus from infected cells and could also activate other immune cells. Therefore, IFN-gamma may have two roles in relation to CTL: a regulatory effect on the generation of CTL and a protective role against virus infection.

The development of a radioimmunoassay (RIA) using monoclonal antibodies to human IFNs-alpha and -gamma has enabled us to measure specifically the quantity of IFNs produced by human PBM. We performed experiments to determine the relationship between the generation of the influenza A virus-specific CTL response and the production of human IFNs-alpha and -gamma following stimulation with influenza A virus-infected autologous cells. The results show that the stimulated PBM of all donors produced IFN-gamma and developed influenza A virus-specific CTL activity and that these two responses were significantly correlated. Experiments were performed to determine the subset of IFN-gamma-producing cells, and demonstrated that T cells with either T4 or T8 surface markers produced IFN-gamma after antigenic stimulation with influenza A virus-infected cells.

METHODS

Virus. Influenza A virus, A/Port Chalmers/1/73 (A/PC; H3N2) and influenza B virus, B/Hong Kong (B/HK) were propagated in 9-day-old embryonated chicken eggs. Infected allantoic fluids were harvested 3 days after infection and clarified by low-speed centrifugation. The supernatant fluids were divided into vials and stored at -80 °C until use. The titres of both virus strains were 10^8.2 plaque-forming units/ml, determined in MDCK cells.

Stimulation of lymphocytes. PBM of 12 healthy adult donors were separated by Ficoll-Hypaque density centrifugation, and cryopreserved using a programmable liquid nitrogen freezer (CRYO-MED, Mt. Clemens, Mich., U.S.A.) and stored in liquid nitrogen until use. The PBM were stimulated following the methods of McMichael & Askonas (1978) with minor modifications. Cells were thawed and divided into three portions. One portion was suspended at a concentration of 1 x 10^6 cells/ml in RPMI 1640 medium containing 10% human AB serum (Flow Laboratories) for use as responder cells. A second portion of cells was exposed to 0.1 ml of A/PC virus-containing undiluted allantoic fluid/1 x 10^6 cells for 90 min at 37 °C, washed twice and resuspended in medium containing 10% human AB serum at a concentration of 1 x 10^6 cells/ml and used as stimulator cells. Responder cells were cultured with or without autologous stimulator cells at a responder: stimulator ratio of 9:1 at 37 °C in 5% CO_2 for 6 days. A third sample of cells was resuspended at a concentration of 5 x 10^6 cells/ml in medium containing 10% human AB serum, incubated at 37 °C in 5% CO_2 and phytohaemagglutinin M form (PHA) (Gibco) was added to these cells 3 days before the CTL assay at a final dilution of 1:200. These were used as target cells.

Phenotype determination and depletion. The phenotypes of effector cells were determined using OKT4 and OKT8 monoclonal antibodies (Ortho Diagnostic System, Raritan, N.J., U.S.A.) by indirect immunofluorescence staining. Interleukin-2 (IL-2) receptor-bearing cells were determined by indirect immunofluorescence staining with anti-TAC monoclonal antibody (Depper et al., 1983; kindly provided by Dr T. Waldmann).

To deplete OKT4- or OKT8-positive cells, effector cells were reacted with a 1:10 dilution of OKT4 or OKT8 monoclonal antibodies and a 1:6 dilution of rabbit complement (C'; Cedarlane Laboratories, Hornby, Ontario, Canada).

Panning of effector cells. The method described by Wysocki & Sato (1978) was used with minor modifications. Briefly, goat anti-mouse IgG (Cappel Laboratories) was diluted in 0.05 M-Tris buffer pH 9.5, at a concentration of 20 μg/ml and 10 μl was poured into a 100 x 15 mm non-tissue culture-treated Petri dish. The dishes were incubated at room temperature for 1 h, rinsed repeatedly with phosphate-buffered saline, and then incubated with medium containing 1% foetal calf serum (FCS).

Effector cells obtained on day 6 after stimulation were reacted with OKT4 or OKT8 monoclonal antibody for 1 h at 4 °C, washed twice and resuspended with medium containing 5% FCS at a concentration of 2 x 10^6 cells/ml. Three ml of the cell suspensions was placed into each dish and incubated for 2 h at 4 °C. Non-adherent cells (negative fraction) were collected from dishes. Dishes were then washed three times. Adherent cells (positive fraction) were removed from dishes by scraping with a rubber policeman. The purity of positive fractions was generally more than 90%; however, negative fractions sometimes contained more than 10% positive cells.

Maintenance of CTL lines. Unseparated or separated effector cells were maintained in medium containing 10% FCS and 2% human T cell growth factor (TCGF) (Meloy Laboratories, Springfield, Va., U.S.A.) at a concentration of 5 x 10^4 cells/ml. Cells were washed and resuspended in new medium every 3 days. Autologous X-irradiated (2500 rads) A/PC- or B/HK-infected stimulator cells were added to the cultures 3 days after separation. Supernatant fluids from these lines were harvested 3 days before and after re-stimulation to assay IFN titres. The CTL activity of these cells was determined 6 days after re-stimulation.
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CTL assay. A standard 51Cr release assay was used. Briefly, 1 × 10⁶ target cells were washed and infected with 0-5 ml of undiluted allantoic fluid containing A/PC or B/HK virus for 90 min at 37°C. After washing, target cells were re-incubated in medium containing 10% FCS for another 60 min. After centrifugation, 0-1 ml of medium containing 125 μCi of Na⁵¹CrO₄ (New England Nuclear) was added and they were re-incubated at 37°C for 90 min. Target cells were washed twice and resuspended in medium containing 10% FCS at a concentration of 5 × 10⁶ cells/ml. Effector cell cultures were centrifuged, supernatant fluids were harvested for IFN assays, and cells were washed twice and resuspended in medium containing 10% FCS. One-hundred μl of target cell suspensions was incubated with 100 μl of effector cell suspensions at various effector : target (E : T) ratios in 96-well round bottom microplates (Linbro) at 37°C in 5% CO₂ for 6 h. The supernatant fluids were harvested using frames (Skatron, Sterling, Va., U.S.A.) and the radioactivity was counted in a gamma counter (Packard). Per cent specific lysis was calculated as [(experimental release — minimum release)/(maximum release — minimum release)] × 100. Maximum and minimum releases were obtained by incubating target cells with 10% Renex 30 solution (Ruger Chemical Co., Irvington, N.J., U.S.A.) and medium, respectively.

IFN assay. Total IFN titres were assayed by a cytopathic effect reduction (CPER) assay as previously described (Ennis & Meager, 1981). The IFN level of culture fluids were also measured by RIAs specific for human IFN-alpha and IFN-gamma (Secher, 1981). Briefly, 6-5 mm diameter etched polystyrene beads coated with sheep polyclonal antiserum to IFN-alpha or IFN-gamma were incubated with culture fluids for 2 to 3 h at 4°C, washed and then incubated with either ¹²⁵I-MT4/E4 monoclonal antibody to human IFN-alpha₂ (Exley et al., 1984) or ¹²⁵I-5J monoclonal antibody to human IFN-gamma (Meager et al., 1984) for 16 h at 4°C. The beads were re-washed and counted in a gamma counter. The values obtained were calibrated in IU/ml by use of the relevant international standards for human IFN-alpha (69/19) and IFN-gamma (Gg 23-901-530). Neutralization of antiviral activity was carried out by using antibodies to human IFN-alpha and IFN-gamma, i.e. rabbit polyclonal antibody to human IFN-alpha (Interferon Sciences, New Brunswick, N.J., U.S.A.) and mouse monoclonal antibody to human IFN-gamma (Interferon Sciences). Samples were incubated with the same volume of antibody containing 2000 neutralizing units/ml for 2 h at room temperature and the residual IFNs were assayed by the CPER test.

In experiments to determine the effect of the addition of anti-IFN-gamma on CTL generation, an additional mouse monoclonal antibody to human IFN-gamma (Meloy Laboratories, Springfield, Va., U.S.A.) and rabbit polyclonal antibody to human IFN-gamma (Interferon Sciences) were also used.

RESULTS

IFN production and CTL activity

We stimulated the PBM of 12 donors to analyse their ability to generate influenza A virus-specific CTL responses, and to measure the amount of IFNs-alpha and -gamma produced using RIAs as well as a CPER assay. The results shown in Table 1 were obtained from cultures 6 days after stimulation with influenza A virus-infected autologous stimulator cells. Each donor had a detectable influenza A virus-specific CTL response (effector cells did not lyse target cells infected with influenza B virus; data not presented) and all donors' PBM produced IFN-gamma, and some produced IFN-alpha. Interestingly, several donors' PBM did not produce any IFN-alpha following 6 days of stimulation despite high titres of IFN-gamma and generating CTL activity. Unstimulated PBM did not kill target cells. Some donors' PBM produced a low level of IFN-gamma without stimulation. As shown in Fig. 1, the levels of CTL activity were significantly correlated with the levels of IFN-gamma production (P <0.01).

Treatment of culture fluids with antisera to IFNs-alpha or -gamma showed that anti-IFN-alpha generally neutralized some antiviral activity and anti-IFN-gamma neutralized almost all antiviral activity (Table 2). Simultaneous treatment with anti-IFNs-alpha and -gamma completely neutralized antiviral activity.

Kinetics of IFN production and CTL activity

A kinetic study was then performed to analyse the development of the CTL response and the production of IFNs in more detail. Two donors whose PBM had been demonstrated to have high levels of CTL activity, and to produce high levels of IFN-gamma, with (donor J.R., Fig. 2b), or without (donor K.K., Fig. 2a) IFN-alpha production were chosen for this experiment. The PBM of donor J. R. produced IFN-alpha during the first 24 h after stimulation, but there was no increase thereafter. IFN-gamma production began later and rose sharply after day 3, along with the increase in influenza virus-specific CTL activity. The PBM of donor K.K. showed a similar
Fig. 1. Correlation between CTL activity and IFN-gamma production of human PBM stimulated with influenza virus-infected cells. Data are based on results contained in Table 1 ($r = 0.752$, $P < 0.01$).

Table 1. *IFN production and CTL activity of human PBM following stimulation with influenza virus-infected cells*

<table>
<thead>
<tr>
<th>Effector</th>
<th>Donor</th>
<th>Virus stimulation*</th>
<th>CTL† (% lysis)</th>
<th>RIA-γ (IU/ml)</th>
<th>RIA-α (IU/ml)</th>
<th>Bioassay (IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/PC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J.R.</td>
<td></td>
<td>100</td>
<td>500</td>
<td>&lt; 20</td>
<td>&lt; 20</td>
<td>875</td>
</tr>
<tr>
<td>S.C.</td>
<td></td>
<td>44.5</td>
<td>275</td>
<td>&lt; 20</td>
<td>268</td>
<td></td>
</tr>
<tr>
<td>K.K.</td>
<td></td>
<td>41.0</td>
<td>295</td>
<td>&lt; 20</td>
<td>378</td>
<td></td>
</tr>
<tr>
<td>J.D.</td>
<td></td>
<td>40.6</td>
<td>225</td>
<td>28</td>
<td>308</td>
<td></td>
</tr>
<tr>
<td>P.P.</td>
<td></td>
<td>40.2</td>
<td>145</td>
<td>91</td>
<td>500</td>
<td></td>
</tr>
<tr>
<td>E.K.</td>
<td></td>
<td>39.5</td>
<td>150</td>
<td>&lt; 20</td>
<td>168</td>
<td></td>
</tr>
<tr>
<td>R.B.</td>
<td></td>
<td>37.4</td>
<td>400</td>
<td>54</td>
<td>1580</td>
<td></td>
</tr>
<tr>
<td>F.E.</td>
<td></td>
<td>35.5</td>
<td>202</td>
<td>&lt; 20</td>
<td>252</td>
<td></td>
</tr>
<tr>
<td>M.P.</td>
<td></td>
<td>31.4</td>
<td>291</td>
<td>&lt; 20</td>
<td>325</td>
<td></td>
</tr>
<tr>
<td>J.G.</td>
<td></td>
<td>26.6</td>
<td>158</td>
<td>40</td>
<td>325</td>
<td></td>
</tr>
<tr>
<td>E.M.</td>
<td></td>
<td>17.7</td>
<td>101</td>
<td>&lt; 20</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>J.S.</td>
<td></td>
<td>10.5</td>
<td>42</td>
<td>38</td>
<td>181</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J.R.</td>
<td></td>
<td>0.7</td>
<td>&lt; 10</td>
<td>&lt; 20</td>
<td>&lt; 10</td>
<td></td>
</tr>
<tr>
<td>S.C.</td>
<td></td>
<td>4.3</td>
<td>&lt; 10</td>
<td>&lt; 20</td>
<td>&lt; 10</td>
<td></td>
</tr>
<tr>
<td>K.K.</td>
<td></td>
<td>7.6</td>
<td>22</td>
<td>&lt; 20</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>J.D.</td>
<td></td>
<td>2.6</td>
<td>72</td>
<td>&lt; 20</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>P.P.</td>
<td></td>
<td>3.0</td>
<td>10</td>
<td>&lt; 20</td>
<td>&lt; 10</td>
<td></td>
</tr>
<tr>
<td>E.K.</td>
<td></td>
<td>1.0</td>
<td>&lt; 10</td>
<td>&lt; 20</td>
<td>&lt; 10</td>
<td></td>
</tr>
<tr>
<td>R.B.</td>
<td></td>
<td>2.8</td>
<td>107</td>
<td>&lt; 20</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>F.E.</td>
<td></td>
<td>2.0</td>
<td>90</td>
<td>&lt; 20</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>M.P.</td>
<td></td>
<td>2.5</td>
<td>115</td>
<td>&lt; 20</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>J.G.</td>
<td></td>
<td>5.5</td>
<td>30</td>
<td>&lt; 20</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>E.M.</td>
<td></td>
<td>1.0</td>
<td>&lt; 10</td>
<td>&lt; 20</td>
<td>&lt; 10</td>
<td></td>
</tr>
<tr>
<td>J.S.</td>
<td></td>
<td>0.5</td>
<td>&lt; 10</td>
<td>&lt; 20</td>
<td>&lt; 10</td>
<td></td>
</tr>
</tbody>
</table>

* In vitro stimulation by exposure to autologous A/PC-infected PBM for 6 days.
† Per cent specific lysis of PHA-stimulated A/PC-infected autologous cells at an E : T ratio of 50 : 1.
‡ Culture fluids were harvested on day 6 from cultures with cell concentrations of $1 \times 10^6$/ml.
Fig. 2. Kinetics of IFN production measured by bioassay (△), RIA-γ (●) and RIA-α (○) and CTL generation (□). (a) Donor K.K.; (b) donor J.R. The CTL activities of A/PC-stimulated PBM are expressed as per cent specific lysis of PHA-stimulated A/PC-infected autologous target cells at an E: T ratio of 50:1.

Table 2. Characterization of IFN produced by human PBM stimulated with influenza virus-infected cells by neutralization with antisera to human IFNs

<table>
<thead>
<tr>
<th>Donor</th>
<th>Anti-alpha</th>
<th>Anti-gamma</th>
<th>Anti-alpha + Anti-gamma</th>
</tr>
</thead>
<tbody>
<tr>
<td>J.R</td>
<td>&gt;94</td>
<td>88</td>
<td>&gt;94</td>
</tr>
<tr>
<td>P.P.</td>
<td>62</td>
<td>&gt;97</td>
<td>&gt;97</td>
</tr>
<tr>
<td>R.B.</td>
<td>50</td>
<td>97</td>
<td>&gt;99</td>
</tr>
<tr>
<td>K.K.</td>
<td>25</td>
<td>&gt;94</td>
<td>&gt;94</td>
</tr>
<tr>
<td>S.C.</td>
<td>50</td>
<td>&gt;84</td>
<td>&gt;84</td>
</tr>
<tr>
<td>M.P.</td>
<td>53</td>
<td>&gt;87</td>
<td>&gt;87</td>
</tr>
</tbody>
</table>

* These supernatant fluids were obtained from cultures of lymphocytes contained in Table 1.

time course in the development of the CTL responses and the production of IFN-gamma, and they did not produce IFN-alpha. There was a reasonable correlation between the level of IFN-gamma detected by RIA and the total amount of IFN detected in the bioassay when no IFN-alpha was produced as in the case of donor K.K.; however, the PBM of donor J.R. produced both IFNs-alpha and -gamma detected by RIAs, and it appeared that this resulted in a synergistic increase in antiviral activity measured by bioassay.

**Determination of T cell subsets that have CTL activity or produce IFN-gamma**

Experiments were then performed to help define the nature of the PBM which were active in CTL activity and in producing IFN-gamma. Lymphocytes of donor S.C. were reacted with OKT4 or OKT8 monoclonal antibodies and complement following 6 days of stimulation in an effort to deplete T4⁺ or T8⁺ cells from effector cells. The CTL activity of the remaining cells was then determined. The results shown in Fig. 3 indicate that effectors were highly active in lysing autologous lymphoblasts infected with influenza A virus, even if they had been treated with OKT4 + C' or with C' alone. Their CTL activity was also detected on A/PC-infected HLA-A and -DR-matched target cells. These effector cells did not lyse any of the following target cells: A/PC-infected HLA-A,B- and -DR-mismatched cells, A/PC-infected HLA-A,B-mismatched,
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Fig. 3. CTL activity of A/PC-stimulated PBM of donor S.C. (HLA-A1, A9, B5, B8, DR3, DR5) after depletion of T4+ or T8+ cells following stimulation for 6 days. ■, Untreated; ○, OKT4 + C'; △, C'; ●, OKT8 + C'.

DR-matched cells, or B/HK-infected autologous cells. The treatment with OKT8 + C' reduced the level of CTL activity, suggesting that killer lymphocytes were mainly contained in the T8+ subset; however, T8- cells may also have had some killing activity, since a lower level of lysis did remain after treatment with OKT8 + C'.

We further analysed the nature of PBM active in lysing target cells, and in producing IFN, following fractionation of effector cells based on their surface marker, T4 or T8. The PBM of donor S.C. were separated by panning after stimulation for 6 days with A/PC-infected autologous cells. Separated and unseparated cells were maintained in medium containing TCGF and were re-stimulated with X-irradiated A/PC-infected autologous cells 3 days later. They were cultured for 6 more days and then tested for the CTL activity. As shown in Fig. 4, cell fractions depleted of T8+ cells had low levels of CTL activity on autologous A/PC-infected target cells, but the level of lysis was very high in fractions depleted of T4+ cells, probably because the T8+ effector cell population was enriched in these fractions. Lysis of target cells matched at the HLA-A and -DR loci was detected using the fractions that showed a high level of lysis on autologous target cells. Lysis of target cells matched at only a HLA-DR locus was markedly reduced, and no lysis was observed on HLA-mismatched target cells or on B/HK-infected autologous target cells. In the same experiment, supernatant fluids were tested for their IFN content prior to re-stimulation and 3 days after re-stimulation with A/PC- or B/HK-infected X-irradiated autologous cells. The results shown in Table 3 demonstrate that there was a rise in IFN-gamma production following re-stimulation with A/PC-infected autologous cells. Fractions enriched for T4+ and T8+ cells both produced high amounts of IFN-gamma regardless of the difference in CTL activity, and there was no such rise in IFN-gamma production following re-stimulation with B/HK-infected stimulators.

**Effect of addition of anti-IFN-gamma antibody to stimulated culture**

In order to determine whether IFN-gamma was required to induce CTL activity, antisera to IFN-gamma were added to the stimulated cultures (final dilutions of 1:5 to 1:540) when the culture was initiated. This experiment was performed several times using three different
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Fig. 4. CTL activity of A/PC-stimulated PBM of donor S.C. (HLA-A1, A9, B5, B8, DR3, DR5) after separation by panning. Effector cells were separated following stimulation for 6 days, and they were maintained in the presence of TCGF. On day 9 they were re-stimulated with X-irradiated A/PC-infected autologous cells and the CTL activity was determined on day 15. O, T4⁺; ●, T4⁺; △, T8⁻; ▲, T8⁺; ■, unseparated.

Table 3. **IFN-gamma production by separated lymphocytes before and after re-stimulation with influenza virus-infected cells**

<table>
<thead>
<tr>
<th>Fraction*</th>
<th>Before re-stimulation ‡</th>
<th>After A/PC-re-stimulation</th>
<th>After B/HK-re-stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfractionated</td>
<td>37</td>
<td>570</td>
<td>62</td>
</tr>
<tr>
<td>T4⁺ (89.9)§</td>
<td>14</td>
<td>340</td>
<td>ND</td>
</tr>
<tr>
<td>T8⁺ (84.3)¶</td>
<td>13</td>
<td>435</td>
<td>ND</td>
</tr>
<tr>
<td>Stimulator cells alone**</td>
<td>ND</td>
<td>20</td>
<td>50</td>
</tr>
</tbody>
</table>

* Effector cells of donor S.C. were separated following stimulation for 6 days with influenza A virus-infected autologous cells, and were cultured in the presence of TCGF at a concentration of 5 × 10⁵ cells/ml.
‡ Measured by RIA-gamma.
§ Re-stimulation was performed using X-irradiated A/PC- or B/HK-infected autologous cells on day 9 at an effector : stimulator ratio of 2 : 1. Culture fluids were harvested for IFN analysis before and 3 days after re-stimulation.
¶ Percentage of positive cells reacting with the monoclonal antibody OKT8 1 day after panning.
** Supernatant fluids from cultures which contained X-irradiated influenza A or B virus-infected stimulator cells, but no effector cells, were tested as control.

antisera, and the results varied in these experiments (data not shown). In some experiments, CTL activity decreased to about 50% of the level of lysis by cells that had not received antiserum, but in other experiments there was no effect of antiserum on the level of CTL activity despite a decrease of antiviral activity in the culture fluids.

**DISCUSSION**

The results reported in this manuscript indicate that all donors tested have influenza A-specific CTL responses, although CTL activity varied among donors. The lymphocytes of all donors produce IFN-gamma during the induction of the CTL response. These results confirm and extend our previous observations (Ennis & Meager, 1981) concerning the production of IFN-gamma during the induction of human CTL responses to influenza virus.

We directly measured the amounts of IFN-gamma produced in these experiments using a RIA for human IFN-gamma. This is a more specific technique than those we used earlier (Ennis & Meager, 1981) and those which have been used by others (Chonmaitree et al., 1981; Roberts et al., 1979; Djeu et al., 1982; Burlington et al., 1984; Balkwill et al., 1983), which have
characterized IFNs produced by cells exposed to influenza virus by pH treatment and neutralization with polyclonal antisera. There was a significant correlation between the levels of influenza A virus-specific CTL activity and the amount of IFN-gamma in culture fluids measured by RIA following stimulation of human lymphocytes by influenza A virus-infected autologous cells.

These observations led us to investigate the phenotypes of the effector cells that have CTL activity and of the cells that produce IFN-gamma by enrichment for T4⁺ and T8⁺ lymphocytes after initial stimulation of lymphocytes in bulk cultures. Cell depletion studies with monoclonal antibodies and complement suggested that most of the lysis observed was due to T8⁺ cells, since the depletion of T8⁺ cells reduced CTL activity. Panning of lymphocytes provided results similar to those obtained in the experiment using monoclonal antibody and complement, i.e. virus-specific killing activity was mainly associated with T8⁺ enriched cell fractions, and less killing was seen with T4⁺ cells. The killing activity of these lytic cells was influenza A virus-specific and restricted by class I antigens, and was not restricted by class II antigens. These observations confirm in general earlier reports as to the surface markers and HLA-restriction of CTL (Biddison et al., 1981; Fleischer, 1982; McMichael et al., 1982; Shaw & Biddison, 1979). However, Kaplan et al. (1984) developed human CTL clones to influenza virus and their clones were T4⁺ and restricted by class II antigens. We observed a low level of killing by T4⁺ cells using PHA-stimulated target cells. This difference in phenotype may therefore be due to the preferential growth of CTL clones with class II antigens, but the precise reasons are not known. Our results suggest that influenza virus-specific T4⁺ CTL are present but may be a minor population in bulk cultures stimulated with influenza virus-infected cells. On the other hand, increased production of IFN-gamma by highly enriched populations of T4⁺ and T8⁺ lymphocytes was observed after re-stimulation with influenza A virus. Since IFN-gamma was only produced after re-stimulation with influenza A virus which was used for initial stimulation in the culture and not after re-stimulation with influenza B virus, IFN-gamma production was specific for influenza A virus. These results suggest that there are two types of IFN-gamma-producing cells: T8⁺ influenza A virus-specific CTL and T4⁺ cells which do not have CTL activity and may have helper functions. In mice, the former type of cell has been demonstrated, i.e. an influenza A virus-specific CTL clone was Lyt-2⁺ and produced IFN-gamma on contact with the H-2-matched influenza A virus-infected cells (Morris et al., 1982). Although Djeu et al. (1982) suggested that large granular lymphocytes (LGL) of a seropositive donor produced IFN-gamma in response to influenza virus, the effector cells in our experiments which produced IFN-gamma were not natural killer cells, since they did not kill influenza A virus-infected HLA-mismatched target cells or influenza B virus-infected autologous cells.

IFN production was generally not detected in unstimulated cultures, but the PBM of several donors did produce some IFN-gamma. Martinez-Maza et al. (1984) reported that they detected IFN-gamma-secreting cells in the blood from normal adults without in vitro stimulation. Therefore, it appears that the PBM of some humans may produce IFN-gamma without defined antigenic stimulation.

IFN-gamma is a lymphokine with many biological roles (Trinchieri & Perussia, 1985) including macrophage activation (Le et al., 1983; Mannel & Falk, 1983), increasing expression of major histocompatibility complex class II molecules (Steeg et al., 1982; Kelley et al., 1984; Becker, 1985), and activating the expression of receptors for IL-2 (Johnson & Farrar, 1983). Farrar et al. (1981) reported that IFN-gamma may be required in generating a murine allogeneic CTL response, since addition of an antiserum to murine IFN-gamma blocked the CTL response. It is possible that the production of IFN-gamma helps to generate the influenza virus-specific CTL response. We, however, have not been able consistently to suppress the CTL responses with antibodies to IFN-gamma, although these antibodies decreased the level of extracellular IFN. It remains possible, however, that small amounts of cell-bound IFN-gamma might remain after addition of antibody and is sufficient to generate the CTL response. Since IFN-gamma seems to activate the expression of IL-2 receptor (Johnson & Farrar, 1983) and CTL clones to influenza virus are reported to express high levels of IL-2 receptor after antigenic stimulation (Andrew et al., 1985), we examined the number of IL-2 receptor-bearing cells and observed that the number increased following stimulation for 6 days (data not presented) when
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IFN-gamma production and CTL activity increased. Additional experiments are needed to define the role of IFN-gamma in the CTL response.

Earlier reports indicated that IFN-alpha was produced following addition of influenza virus to human PBM (Chonmaitree et al., 1981), human macrophages (Roberts et al., 1979) and human LGL (Djeu et al., 1982; Burlington et al., 1984). We find on the other hand that IFN-alpha was produced in lower titre than IFN-gamma, and by the cells of fewer donors. We presume that these differences are due to the methods we employed which were developed for generating CTL responses. In our methods, virus-infected stimulator cells were added to uninfected responder cells after washing to remove unadsorbed virus, and the culture medium contains 10% pooled human sera which contain neutralizing antibodies to influenza viruses. These methods limit the number of infected cells to the initially infected stimulator cells, and decrease the effect of live virus spread to other cells in the culture in comparison with the methods described by other reports (Chonmaitree et al., 1981; Roberts et al., 1979; Djeu et al., 1982; Balkwill et al., 1983) and this probably results in less production of IFN-alpha.

Balkwill et al. (1983) concluded that a new type of IFN-alpha which was acid-labile was produced after addition of influenza virus to cultures of human PBM, since antiviral activity of the culture fluid was reduced after either pH 2 treatment or neutralizing with anti-IFN-alpha serum. However, their results could be explained by the possibility that both IFN-alpha and IFN-gamma may be present in the culture fluids. IFN-alpha and IFN-gamma may act synergistically in antiviral activity, if these IFNs are present together (Fleischmann et al., 1979; Zerial et al., 1982; Czarniecki et al., 1984). Therefore, residual antiviral activity after depletion of one of the IFNs appears to be lower than might be expected from the original titre if the culture fluid contains both IFNs. We observed that the antiviral activity of culture fluids was reduced by treatment with either anti-IFN-alpha or anti-IFN-gamma antibody, and that these culture fluids contained both IFN-alpha and IFN-gamma when they were tested in specific radioimmunoassays. These results suggest the possibility that their culture fluids may have contained both IFN-alpha and IFN-gamma, not an acid-labile IFN-alpha.

The results of this study confirm that human T cells are activated following stimulation with influenza virus-infected cells, and that IFN-gamma is produced during the generation of the influenza virus-specific CTL response. In addition, the levels of IFN-gamma produced are significantly correlated with the levels of influenza virus-specific CTL activity, and T8+ influenza A virus-specific CTL and T4+ non-lytic cells produce IFN-gamma after re-stimulation with influenza A virus-infected cells. These two host responses may have important roles in protection against influenza virus infection.

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REFERENCES


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