Gamma Interferon Production and Cytotoxicity of Spleen Cells from Mice Infected with Semliki Forest Virus

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(Accepted 3 February 1984)

SUMMARY

Interferon (IFN) production by spleen cells from normal mice, mice acutely infected with Semliki Forest virus (SFV) or mice immune to SFV was measured after stimulation in vitro with either infectious or inactivated SFV. All three classes of spleen cells made IFN-αβ in response to infectious SFV. Spleen cells taken from mice late, but not early, after infection, or from immune mice, made IFN-γ in response to inactivated SFV. Amounts of INF-γ and IFN-αβ were similar. Normal spleen cells made no IFN (of any type) in response to inactivated SFV. The cell type producing IFN-αβ appeared to be the macrophage, whilst both T-lymphocytes and macrophages were necessary for IFN-γ production. During the acute infection, the ability of spleen cells to lyse both virus-infected and uninfected target cells arose earlier than the ability to produce IFN-γ. However, cytotoxicity towards uninfected cells fell to near background levels by day 7, whilst cytotoxicity towards infected targets remained high at that time, when IFN-γ production was at its peak. IFN-γ production is therefore temporally associated with cytotoxicity specifically directed against virus-infected targets, and the ability to produce IFN-γ is a late response to SFV infection.

INTRODUCTION

Interferon (IFN) is produced when lymphoid cells of animals sensitized by a particular antigen are challenged with that antigen. Such immune-specific induction of IFN may occur either in vivo or in vitro, in response to bacterial or viral antigens. The first demonstration of immune-specific induction of IFN was reported by Glasgow (1966) who showed that spleen cells from mice immune to Chikungunya virus produced elevated amounts of IFN when challenged with the virus. Antigenically unrelated viruses induced no more IFN in Chikungunya virus-immune spleen cells than in normal spleen cells. Since that time, there have been several similar reports, describing immune-specific induction of IFN in lymphoid cells by a number of viruses, for example, influenza virus (Ennis & Meager, 1981); for review, see Wilkinson & Morris (1983). Generally, the viral ‘antigen’ preparations employed have been whole inactivated virus rather than purified antigenic components. Such preparations may also induce IFN in non-immune lymphoid cells; however, this IFN is IFN-αβ rather than the IFN-γ which is generally produced in immune-specific induction (Green et al., 1981; Langford et al., 1981; Osborne et al., 1980; Starr et al., 1980).

Broadly, it is agreed that the cell type producing IFN-γ under these circumstances is the T-lymphocyte, with cells of the monocyte series acting as accessory cells (see Wilkinson & Morris, 1983). T-lymphocytes have very clearly been implicated in recovery from a number of virus infections in animals (Lin & Askonas, 1981; Nash et al., 1980; Yap et al., 1978). It is not at all clear which of the multiple functions of T-lymphocytes are of major importance in the recovery from virus infection, but it would seem likely that local IFN release by T-lymphocytes could be involved. In this report we describe production of IFN by spleen cells challenged with Semliki Forest virus (SFV) from mice undergoing acute infections by SFV and mice immune to SFV. We found that the capacity of the spleen cells to produce IFN-γ in response to an SFV viral antigen preparation developed later than lymphocyte cytotoxicity against virus-infected targets. At the
peak of the response, as much IFN-γ was produced by spleen cells in response to viral antigens as IFN-αβ was produced in response to live virus. This implies that IFN-γ may well be quantitatively as important as IFN-αβ, late in the infection, and could play a role in recovery from infection. Quite plainly, IFN-γ plays no role early in infection.

**METHODS**

*Infection of mice.* Adult (6- to 8-week-old) C3H mice were infected intranasally with 4 x 10⁴ p.f.u. of SFV (avirulent strain 7, prepared by low multiplicity passage in BHK cells). Mice infected under these conditions show symptoms of encephalitis at about day 5, and about half the mice die on day 7 or 8 post-infection. Survivors are immune, being resistant to challenge with 10 LD₅₀ of SFV.

*Preparation and culture of spleen cells.* Mice were splenectomized aseptically and cell suspensions prepared from the spleens. These were cultured in RPMI 1640 medium, bicarbonate-buffered and supplemented with 10% foetal calf serum (Gibco) at 37 °C in 5% CO₂/air at a cell density of 3 x 10⁶ per ml.

*Virus challenge of spleen cells.* Spleen cell cultures were treated with SFV at about 10⁴ p.f.u./ml (m.o.i. approx. 6). For experiments with inactivated virus, the stock virus was treated with β-propiolactone (BPL). The virus was incubated with 0.1% BPL (Sigma, grade II) for 4 h at 4 °C and then dialysed to remove residual BPL and its breakdown products. The BPL-treated virus (BPL-SFV) was used at the same dilution as the infectious virus; its infectivity titre was more than 10⁵-fold less than the untreated virus (i.e. final virus titre in spleen cell cultures was < 100 p.f.u./ml) and it did not induce IFN in fibroblasts.

*IFN assay and characterization.* Supernatants from spleen cells treated with infectious SFV were dialysed prior to IFN assay for 5 days against pH 2 buffer to eliminate residual SFV. This procedure would destroy IFN-γ but not IFN-α or -β. Supernatants from spleen cells treated with BPL-SFV were not dialysed since the BPL-SFV did not interfere with the IFN assay and obviously the pH 2 dialysis would destroy the IFN-γ we wished to detect. IFN assay was by the INAS₅₀ method of Atkins et al. (1974) in mouse L-cells, using SFV to challenge. The IFN activity detected was characterized by neutralization assay using polyclonal rabbit antisera to IFN induced by Newcastle disease virus in L-cells (IFN-α + IFN-β) or to concanavalin A (Con A)-induced mouse spleen cell IFN (IFN-γ), prepared by C. Sutton in this laboratory. Briefly, IFN preparations were incubated with dilutions of the antisera and then assayed for residual antiviral activity. There was no significant cross-reaction when the two antisera were incubated with standard preparations of L-cell IFN or Con A-induced spleen cell IFN. Since our antisera to L-cell IFN does not distinguish between IFN-α and IFN-β, we refer to IFNs neutralized by this antisera as IFN-αβ.

IFN-αβ is expressed in international units; IFN-γ is expressed in laboratory units since no mouse IFN-γ standard is available. The IFN-γ standard used in the laboratory was prepared by induction of C3H mouse spleen cells (prepared as above) with Con A (5 μg/ml, Miles-Yeda, Rehovot, Israel). Supernatants were harvested at 2 days, and partially purified on phenylboronate-agarose (PBA-30: Pharmacia) to a specific activity of about 10⁵ units/mg. The preparation was diluted to a titre of 10⁻⁵ units/ml and stored at ~70 °C.

*Cytotoxicity assay.* Spleen cells were incubated at different effector :target (E:T) ratios with ⁵¹Cr-labelled target cells in microtitre tray wells. Target cells were syngeneic RDM4 tumour cells, either uninfected or infected with SFV, 4 h before addition of effector cells. ⁵¹Cr release was determined 5 h after addition of effector cells. Cytotoxicity was expressed as [(⁵¹Cr release in presence of effectors) − (spontaneous release)/total ⁵¹Cr] x 100. Release of ⁵¹Cr was essentially linear with E:T ratio over the range employed.

*Fractionation of spleen cells.* T-lymphocytes were depleted from spleen cells (3 x 10⁶ per ml) by treatment with an anti-Thy 1 monoclonal antisera (J11, the kind gift of Dr A. M. Schmitt-Verhulst, Centre d'Immunologie de Marseille-Luminy, France) together with complement (C': a 1:40 dilution of normal rabbit serum). Spleen cells were enriched for T-lymphocytes by passage over a nylon wool column (Julius et al., 1973). The enriched populations, greater than 90% Thy 1-positive as judged by indirect immunofluorescence using anti-Thy 1, were resuspended at 3 x 10⁶ cells/ml. Macrophages were depleted from spleen cells by repeated cycles of adherence to plastic and by treatment with silica. Such cultures contained no detectable adherent cells. Macrophage cultures were prepared by allowing cells to adhere to plastic Petri dishes followed by repeated washing to remove lymphocytes. These cultures consisted of greater than 90% phagocytic cells as judged by uptake of Indian ink. Cell numbers in macrophage cultures were equivalent to those in whole spleen cell cultures.

**RESULTS**

*Production of IFN by spleen cells from SFV-immune and control mice.*

Spleen cell suspensions from SFV-immune or control mice were treated with live SFV or BPL-SFV. Supernatant fluids were harvested 1, 2 or 4 days later and assayed for IFN. Table 1 shows that live SFV induced similar amounts of IFN from either immune or control spleen cells. On
Table 1. Production of IFN by spleen cells from immune and non-immune C3H mice challenged in vitro with live SFV or BPL-SFV

<table>
<thead>
<tr>
<th>Spleen cell source</th>
<th>Treatment in vitro</th>
<th>IFN yield*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td>Non-immune mouse</td>
<td>None</td>
<td>&lt;10</td>
</tr>
<tr>
<td></td>
<td>Live SFV</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>BPL-SFV</td>
<td>15</td>
</tr>
<tr>
<td>Immune mouse</td>
<td>None</td>
<td>&lt;10</td>
</tr>
<tr>
<td></td>
<td>Live SFV</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>BPL-SFV</td>
<td>100</td>
</tr>
</tbody>
</table>

* Units/ml IFN in supernatant fluid.

Table 2. IFN production by spleen cell subpopulations treated with infectious SFV

<table>
<thead>
<tr>
<th>Cell population</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
<th>Expt. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total spleen cells</td>
<td>300</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>Total spleen cells depleted of adherent cells†</td>
<td>40</td>
<td>ND†</td>
<td>16</td>
</tr>
<tr>
<td>Macrophages</td>
<td>200</td>
<td>60</td>
<td>ND</td>
</tr>
<tr>
<td>Nylon wool non-adherent cells</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

* Units/ml in supernatant fluid.
† In Expt. 1, by adherence to plastic; in expt. 3, by adherence to plastic and silica treatment.
‡ ND, Not done.

The other hand, BPL-SFV induced little or no IFN in non-immune spleen cells but quite large amounts in immune spleen cells. Non-induced spleen cells made no IFN. Antibody neutralization tests showed the IFN induced by live SFV to be IFN-αβ whilst that induced by BPL-SFV in the immune spleen cells to be IFN-γ.

Cell source of IFNs

Treatment of immune spleen cells with anti-Thy 1 + C′ prior to induction with either live SFV or BPL-inactivated SFV showed that non-T-cells and T-cells, respectively, are chiefly responsible for IFN production in response to these two inducers (data not shown). More detailed experiments with non-immune spleen cells also indicated that non-T-lymphocytes were chiefly responsible for IFN production in response to live SFV. Depletion of adherent cells from the spleen cells by plastic adherence substantially reduced IFN production, as did treatment of spleen cells with silica. Spleen cell cultures depleted of adherent cells and enriched for T-lymphocytes by nylon wool adherence of non-T-lymphocytes made essentially no IFN in response to live SFV. On the other hand, macrophage cultures derived from spleen cells made IFN in quantities similar to those produced by whole spleen cells (Table 2).

Similar experiments with immune spleen cells showed that both plastic-adherent cells and nylon wool non-adherent cells together were necessary for IFN production in response to BPL-SFV; neither cell type alone made IFN. Reconstitution experiments employing cells from either immune or non-immune mice confirmed the role of adherent cells and indicated that immune memory depended on the lymphocyte rather than adherent cell (Table 3).

IFN production by spleen cells from mice acutely infected with SFV

Spleen cells prepared from mice at intervals after infection with SFV were treated with live SFV or BPL-SFV. Fig. 1 shows the titres of IFN obtained. The amounts of IFN produced by spleen cells in response to live virus during the acute infection were similar to the amounts produced by non-immune spleen cells. In repeated experiments no correlation between the
amount of IFN produced and the time post-infection was found. The IFN produced was pH 2-stable and was shown to be IFN-αβ by neutralization tests. Under the conditions of the experiment, we could not show whether IFN-γ was also produced since to eliminate residual SFV (which would interfere with the IFN assay) pH 2 treatment was used. On the other hand, no (or very little) IFN was produced in response to BPL-inactivated SFV early in the infection, significant amounts first being produced on day 5 and reaching a maximum on about day 7 (Fig. 1). The production of IFN in response to BPL-SFV by 7-day spleen cells was abolished by pretreatment with anti-Thy 1 + C’. The IFN produced was shown to be IFN-γ by neutralization tests.

Spleen cells from acutely infected mice which were not treated with SFV in vitro (live or BPL-treated) made no IFN. We were unable to detect IFN in the serum of infected mice.

Cytotoxicity of spleen cells from mice acutely infected with SFV

The cytotoxicity of fresh spleen cells taken from mice at intervals after infection was assessed using infected or uninfected targets. Early in infection, both targets were killed with almost equal efficiency; later (after 7 days) cytotoxicity towards uninfected targets fell markedly whilst cytotoxicity towards infected targets remained high (Fig. 2). The same cells were treated with BPL-SFV in vitro and tested 5 days later. As expected, levels of cytotoxicity were markedly

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Table 3. Requirement for both immune lymphocytes and macrophages for production of IFN in response to BPL-SFV

<table>
<thead>
<tr>
<th>Source of &quot;T&quot;-lymphocytes*</th>
<th>Source of macrophages†</th>
<th>IFN yield upon challenge with BPL-SFV‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune spleen</td>
<td>None</td>
<td>&lt;10</td>
</tr>
<tr>
<td></td>
<td>Non-immune spleen</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>Immune spleen</td>
<td>80</td>
</tr>
<tr>
<td>Non-immune spleen</td>
<td>None</td>
<td>&lt;10</td>
</tr>
<tr>
<td></td>
<td>Non-immune spleen</td>
<td>&lt;10</td>
</tr>
<tr>
<td></td>
<td>Immune spleen</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

* Nylon wool-enriched cells.
† Plastic-adherent cells.
‡ Units/ml IFN in supernatant fluid.
IFN-γ production in SFV-infected mice

Fig. 2. Cytotoxicity (% at 30:1 effector:target ratio) of spleen cells taken from mice at intervals after infection. ○, Uninfected target; ●, infected target.

Fig. 3. Cytotoxicity (% at 30:1 effector:target ratio) of spleen cells taken from mice at intervals after infection and stimulated in vitro. ○, Uninfected target; ●, infected target.

higher towards the infected target, with little or no cytotoxicity detected against the uninfected target. Normal spleen cells treated with BPL-SFV showed some cytotoxicity towards virus-infected targets, but with spleen cells from infected mice cytotoxicity was clearly elevated above this level by day 5 post-infection and reached a maximum by day 7 (Fig. 3).

DISCUSSION

We have demonstrated that IFN-γ is produced by spleen cells from SFV-immune animals upon stimulation with SFV antigens. In agreement with other investigations (Epstein et al., 1971; Sonnenfeld et al., 1979; Valle et al., 1975) we found that IFN-γ production is dependent upon both T-lymphocytes and adherent, phagocytic cells, and that (as would be expected) the 'memory' for the response lies with the lymphocyte. The adherent, phagocytic cell, probably a macrophage, presumably acts in some accessory role. On the other hand, non-T-cells, probably macrophages, make IFN-αβ in response to live virus. Although the exact nature of the cell
populations being studied is not clear (the 'macrophage' cultures may have been contaminated with non-macrophage cells) it does seem that quite different cell populations are involved in production of IFN-αβ and IFN-γ. The ability of spleen cells to make detectable IFN-γ in response to BPL-SFV develops about 5 days post-infection, and is maximal by about day 7. In contrast, the ability of spleen cells to make IFN-αβ throughout the acute period of infection is about the same as that of non-immune (or immune) spleen cells.

Cytotoxicity of spleen cells towards infected target cells develops early in the infection, before the spleen cells are capable of making detectable IFN-γ. However, the character of the cytotoxicity observed early (3 days) and late (7 days) after infection is different. In particular, the cytotoxicity of fresh spleen cells towards infected or uninfected target cells is about the same at 3 days, but markedly different at 7 days, and the cytotoxicity of spleen cells towards infected targets is markedly amplified by a 5 day stimulation in vitro with BPL-SFV whilst cytotoxicity towards uninfected targets is not. In view of other investigations of spleen cell cytotoxicity in SFV-infected mice, it is reasonable to ascribe the later (7 day) cytotoxicity to virus-specific cytotoxic T-lymphocytes, whilst the earlier (3 day) cytotoxicity may be due to non-T-cells (macrophages or natural killer cells). Thus, Rodda & White (1976) have shown this to be the case in SFV infection of mice, as have Welsh & Zinkernagel (1977) and Welsh (1978) for lymphocytic choriomeningitis virus infection of mice. It therefore appears that the ability of spleen cells from SFV-infected mice to produce IFN-γ in response to SFV antigens develops concomitantly with T-lymphocyte cytotoxicity towards SFV-infected cells. The early cytotoxicity may well be IFN-stimulated, since spleen cells can make IFN-αβ early in infection in response to live virus; it is well established that virus-induced IFN stimulates natural cytotoxicity (Gidlund et al., 1978; Welsh, 1981) and IFN-αβ will clearly stimulate cytotoxicity of spleen cells towards uninfected or SFV-infected target cells (M. J. Blackman & A. G. Morris, unpublished data).

The role of IFN-γ has not been delineated under any circumstances, in any pathological conditions. IFN-αβ, on the other hand, is clearly important in host defence against virus infections. Thus, administration of exogenous IFN-αβ can modify the course of a number of infections, including SFV in mice (Bradish & Titmuss, 1981; Finter, 1966), and administration of anti-IFN globulins to mice exacerbates a number of virus infections (Gresser et al., 1976a, b) including SFV (Fauconnier, 1970). No analogous experiments employing purified IFN-γ or specific antibodies to IFN-γ have, as yet, been carried out, since neither of these reagents is yet available. However, our present experiments indicate that although quite plainly IFN-γ can be of no relevance in the early stages of SFV infection, it could play a role in late stages of infection leading to recovery, and could be an important facet of T-lymphocyte function in virus infection.

We wish to thank the Cancer Research Campaign for financial support.

REFERENCES

IFN-γ production in SFV-infected mice


(Received 13 December 1983)