Studies on the Expression of Spontaneous and Induced Interferons in Mouse Peritoneal Macrophages by Means of Monoclonal Antibodies to Mouse Interferons

By FILIPPO BELARDELLI, 1 * SANDRA GESSANI, 1 ENRICO PROIETTI, 1 CHIARA LOCARDI, 1 PAOLA BORGHI, 1 YOSHIHIKO WATANABE, 2 YOSHIMI KAWADE 2 and ION GRESSER 3

1 Laboratory of Virology, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy, 2 Institute for Virus Research, Kyoto University, Kyoto 606, Japan and 3 Laboratory of Viral Oncology, Institut de Recherches Scientifiques sur le Cancer, 94802 Villejuif Cedex, France

(Accepted 5 May 1987)

SUMMARY

Monoclonal antibodies (MAbs) to mouse interferons (MuIFN) have been used to characterize the interferon-like activities spontaneously expressed in mouse peritoneal macrophages freshly explanted from normal pathogen-free mice. Injection of mice with MAbs to MuIFN-α or -β resulted in a significant increase of vesicular stomatitis virus (VSV) multiplication in peritoneal macrophages. Addition of these MAbs to freshly explanted mouse macrophages accelerated the decay of the antiviral state to VSV during the 'ageing' in vitro of these macrophage cultures. Furthermore, these MAbs to MuIFN-α or -β markedly inhibited the transfer of the antiviral state from freshly explanted peritoneal cells or macrophages to syngeneic macrophages 'aged' in vitro permissive for virus replication. These effects were not observed using a non-neutralizing antibody to MuIFN-β, nor with a MAb to MuIFN-γ. In all experiments sheep polyclonal antibodies to MuIFN-α/β were more effective than the corresponding amount of MAbs to MuIFN-α or -β. A mixture of both these MAbs was more effective than either alone. Interferons produced after stimulation of peritoneal macrophages with Newcastle disease virus (NDV) and of total peritoneal cells with lipopolysaccharides (LPS) have also been characterized by means of MAbs to IFNs. The results of neutralization studies with these antibodies indicated that MuIFN-β was the major component of peritoneal cell IFN (induced by both NDV and LPS) and MuIFN-α was a minor component (13 to 17%). These data indicate that both MuIFN-α and -β, but not MuIFN-γ, are spontaneously present in/on mouse peritoneal macrophages and are produced after stimulation with NDV or LPS.

INTRODUCTION

The important role of macrophages in non-specific natural resistance to virus infections has been widely documented (Mogensen, 1979; Morahan et al., 1985). The resistance of resting mouse peritoneal macrophages to vesicular stomatitis virus (VSV) and encephalomyocarditis virus (EMCV) can be abolished by injection of mice with sheep antibody to mouse interferon α/β (MuIFN-α/β) (Belardelli et al., 1984). An inverse correlation was observed between the intracellular levels of (2'-5')oligoadenylate (2-5A) synthetase activity in peritoneal macrophages and the sensitivity of these cells to VSV (Gresser et al., 1985). Thus, the levels of 2-5A synthetase activity in peritoneal macrophages from mice injected with antibody to MuIFN-α/β were markedly lower than in macrophages freshly harvested from control mice (Gresser et al., 1985). Moreover, although IFN could not generally be recovered in the peritoneal washings or tissue extracts of the majority of normal mice (Sen, 1982; Gresser et al., 1983; Galabru et al., 1985), we have recently demonstrated that peritoneal cells or macrophages freshly harvested from young
mice were capable of transferring an antiviral state (to VSV and to EMCV) to syngeneic mouse macrophages 'aged' in vitro as well as to different mouse cell monolayers permissive for virus replication (Proietti et al., 1986). This transfer of the antiviral state was prevented by the addition of polyclonal antibodies to MuIFN-α/β (Proietti et al., 1986). These data suggested that IFN was produced constitutively in normal young mice.

In the experiments reported in this article we further studied the expression of spontaneous IFN in mouse peritoneal cells by means of monoclonal antibodies (MAbs) and, using different experimental approaches, we demonstrate that both α and β MuIFNs are expressed in peritoneal cells from normal young mice. We also provide experimental evidence indicating that both α and β IFNs are produced by mouse peritoneal cells after induction with Newcastle disease virus (NDV) or lipopolysaccharide (LPS), and that MuIFN-β is the major antigenic component of macrophage IFNs. The predominance of MuIFN-β in bone marrow-derived macrophage cultures induced by NDV was also recently reported by Brehm & Kirchner (1986).

METHODS

Mice. Male and female DBA/2, Swiss, C57BL/6, C3H/HeN and BALB/c mice 5 to 8 weeks old were obtained from Charles River Breeding Laboratories, Italia S.p.A., Milan, Italy.

Reagents. RPMI 1640 medium (Microbiological Associates Bioproducts, Walkersville, Md., U.S.A.) was supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 2 mM-glutamine and foetal calf serum (FCS) (Gibco) at a final concentration of 10% (v/v). All tissue culture reagents were purchased as 'endotoxin-free', as assessed by the Limulus amoebocyte assay.

Interferon. Amounts of IFN are expressed as experimental units (Proietti et al., 1986). One of these units as expressed in the text is the equivalent of 8 IFN reference units.

Antibodies to interferons. All sera were decomplemented and extensively absorbed on murine cells. The immunoglobulin fractions were separated by ammonium sulphate precipitation (protein concentration varied between 10 and 33 mg/ml) and shown to be devoid of any toxicity for mouse peritoneal macrophages.

Sheep anti-mouse α/β globulin (sheep no. R5/4; Proietti et al., 1986) had a neutralizing titre of 1 × 10^{-5} against 4 to 8 units of MuIFN-α/β. Rat MAb to MuIFN-γ (kindly provided by Dr E. A. Havell (Trudeau Institute Inc., New York, N.Y., U.S.A.; Spitalny & Havell, 1983) had a neutralizing titre of 5 × 10^{-4} against 4 units of MuIFN-γ. The origin of hybridomas producing rat anti-MuIFN-α (clone 4E-A1) and anti-MuIFN-β (clone 7F-D3) MAbs has been described elsewhere (Kawade & Watanabe, 1987). A different hybridoma cell line (clone 9B-D12) producing non-neutralizing rat MAbs to MuIFN-α (Kawade & Watanabe, 1987) was also used. This MAb ('control antibody') bound MuIFN-α from L cells but it neutralized only a minor part [α1 and α2 subtypes, kindly provided by Drs J. Trapman and E. Zwarthoff (Rotterdam, The Netherlands) (Kawade & Watanabe, 1987)].

Cells were passaged in ascitic form in BALB/c nude mice. Ascitic fluids were concentrated by ammonium sulphate precipitation. The MAb to MuIFN-α (clone 4E-A1) had a titre of 2 × 10^{-3} against 4 units of MuIFN-α. In some experiments we used an antibody preparation of anti-MuIFN-α purified by DEAE-cellulose chromatography and concentrated to a titre of 5 × 10^{-4} against 4 units of MuIFN-α. The MAb to MuIFN-β had a titre of 2.5 × 10^{-5} against 4 units of MuIFN-β. The non-neutralizing antibody to MuIFN-α (control antibody) had a titre of 1.25 × 10^{-4} against 4 units of MuIFN-α. Monoclonal antibodies to MuIFN-α and to MuIFN-β did not exhibit any cross-reactions against each other.

Titration of antibodies to IFN was performed as follows. Serial dilutions of antibodies were performed in RPMI medium with 2% FCS in 96-well culture microplates (Falcon). Fifty μl of each antibody dilution was added to 50 μl of IFN (4 to 8 experimental units) in a different 96-well culture microplate. For MAbs to MuIFN-α either purified MuIFN-α from L cells (Kawade & Watanabe, 1987) or MuIFN-α from CHO cells (kindly provided by Drs J. Trapman and E. Zwarthoff) were used; for MAbs to MuIFN-β, we used electrophoretically pure MuIFN-β from NDV-induced C-243 cells, purified by affinity chromatography using a Sepharose column with MAbs to MuIFN-β (kindly provided by Dr J. Trapman through the courtesy of Dr G. B. Rossi, Rome, Italy). IFN and antibodies were incubated at 37°C for 90 min before the addition of L cells (20000 cells/well in 100 μl of RPMI with 2% FCS). After 24 h at 37°C, L cell monolayers were infected with VSV (0.1 p.f.u./cell) and c.p.e. was observed 24 h later.

Seeding of peritoneal macrophages in culture dishes. Mice were injected intraperitoneally (i.p.) with various globulins or test substances. At different times thereafter, mice were killed and the peritoneal cavity was washed with 2.5 ml of nutrient medium (RPMI 1640 medium containing 10% FCS). Peritoneal cells from each mouse were seeded in two wells of a 24-well plastic plate (Nunc), each well containing approximately 0.5 × 10^6 cells in 1 ml. Cells were allowed to attach to the plastic culture dish at 37°C for 3.5 h, and non-adherent cells were discarded. Approximately 5 × 10^4 cells remained firmly adhered in each well after several washings. There was no significant difference in the number of cells recovered from the peritoneal cavities of mice injected with the different antibody preparations or in the number of cells adherent to the culture wells.

Mice were incubated at 37 °C for 90 min before the addition of L cells (20000 cells/well in 100 μl of RPMI with 2% FCS).
Expression of IFNs in macrophages

The experiments to be described were undertaken only with peritoneal cells firmly adherent to the culture wells after vigorous washing. The cells could be detached by trypsin only with some difficulty. Over 95% of the cells were stained for non-specific esterase using techniques previously described (Belardelli et al., 1984) and were positive in immunofluorescence studies using a rat MAb (F4/80) specific for mouse macrophages (provided by S. Gordon and A. B. Ezekowitz, Oxford, U.K.).

**Determination of virus multiplication in peritoneal macrophages: virus yield.** In each well, 0.2 ml of a virus dilution was added to peritoneal macrophages (m.o.i. approx. 0.2 p.f.u. per cell). After 1 h of incubation at 37 °C the cell sheet was washed thoroughly and 1 ml of nutrient medium containing 10% FCS was added. After incubation for 18 h at 37 °C in a 5% CO₂/air incubator, virus yields were harvested, centrifuged and the supernatant was titrated on a monolayer of L929 cells.

**Preparation of donor mouse cells**

*Total peritoneal cells.* The peritoneum was washed with 2.5 ml of RPMI 1640 nutrient medium containing 10% FCS; cells were centrifuged, resuspended in RPMI medium with 10% FCS and used as donor cells in cocultivation experiments with target cells.

*Mice peritoneal macrophages.* Mouse peritoneal cells (10⁶ cells per ml) were allowed to attach to a plastic Petri dish (Nunc) in 10 ml of RPMI nutrient medium containing 10% FCS for 20 min at 37 °C. The monolayer was washed three times with RPMI medium without serum to remove non-adherent cells, the remaining cell monolayer was trypsinized, the cells were recovered by using a rubber policeman, counted and viability was determined. Macrophages were washed with RPMI containing 10% FCS before use as donor cells.

**Preparation of target cells.** For preparation of virus-permissive macrophage monolayers, peritoneal washings were prepared as described above and seeded in 24-well plastic plates (Nunc), each well containing approximately 10⁶ cells per ml. Cells were allowed to attach to the plastic culture dish at 37 °C for 3 h and non-adherent cells were discarded. The criteria used to define these firmly attached cells as macrophages have been previously described (Belardelli et al., 1984). Macrophages were cultivated in vitro for 4 to 5 days, at which time they became permissive for VSV and EMCV and were used as target cell monolayers. Monolayer cultures of 'aged' macrophages showed the same sensitivity as monolayer cultures of L929 cells to the antiviral action of MuIFN-α/β.

**Assay for induction of antiviral state in target cell monolayers.** VSV yield assay. A 0.1 ml sample of medium containing freshly harvested total peritoneal cells (usually 2 × 10⁶) was first mixed with 0.2 ml of soft agar (Bacto-agar (Difco) 0.33% in RPMI 1640 with 5% FCS) to prevent the donor peritoneal cells from adhering to the underlying target cell monolayers. After solidification for 5 min at 4 °C, a second layer of soft agar was applied. Antibodies to IFNs were incorporated in both layers of soft agar. After incubation for 24 h at 37 °C, agar and donor peritoneal cells were discarded, and the remaining monolayer was washed three times with medium (RPMI 1640 with 2% FCS). A 0.2 ml sample of a virus dilution (m.o.i. approx. 0.2) was added to each well. After 1 h of incubation at 37 °C the cell sheet was washed thoroughly, and 1 ml of nutrient medium was added. After incubation for 18 h at 37 °C in a 5% CO₂/air incubator, the cell-free nutrient medium was assayed for virus in mouse L929 cells.

**RESULTS**

**Effect of injection of mice with MAbS to IFNs on the multiplication of VSV in peritoneal macrophages**

Injection of mice with sheep polyclonal antibody to MuIFN-α/β or MAbS to MuIFN-α or -β 4 days before harvesting rendered peritoneal macrophages permissive for VSV (Belardelli et al., 1984; Table 1), whereas VSV failed to multiply or multiplied to only a slight extent in macrophages from control-injected mice.

It was necessary to inject mice at least twice with 10⁴ to 10⁵ neutralizing units of these MAbS to render peritoneal macrophages permissive for VSV, whereas a single injection of 10³ to 10⁴ neutralizing units of a sheep polyclonal antibody to MuIFN-α/β was sufficient to render peritoneal macrophages permissive (data not shown). Injection of mice with MAbS to both MuIFN-α and to MuIFN-β was more effective than either antibody alone (Table 1; expt. 2). In contrast, a different MAb to MuIFN-α (which binds to but does not neutralize MuIFN-α) and a rat MAb to MuIFN-γ did not exert any significant effect on the capacity of VSV to replicate in peritoneal macrophages (Table 1; expt. 2).
Table 1. Effect of inoculation of mice with antibodies to IFNs on the multiplication of VSV in peritoneal macrophages in vitro

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>log$_{10}$ Mean VSV yield/0.2 ml$^\dagger$</th>
<th>Expt. 1$^\ddagger$</th>
<th>Expt. 2$^§$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS (1)</td>
<td>1.5 ± 0.2</td>
<td>0.7 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Normal sheep globulin (2)</td>
<td>1.7 ± 0.3</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Sheep antibody to MuIFN-α/β (3)</td>
<td>3.6 ± 0.4</td>
<td>3.5 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>MAb to MuIFN-α (4) (clone 4E-AL)</td>
<td>3.4 ± 0.3</td>
<td>2.2 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>MAb to MuIFN-β (5) (clone 7F-D3)</td>
<td>-</td>
<td>2.5 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>MAbs to MuIFN-α (4) and to MuIFN-β (5)</td>
<td>-</td>
<td>3.2 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>MAb to MuIFN-α (6) (clone 9B-D12, non-neutralizing)</td>
<td>-</td>
<td>≤ 0.5</td>
<td></td>
</tr>
<tr>
<td>MAb to MuIFN-γ (7)</td>
<td>-</td>
<td>1.0 ± 0.4</td>
<td></td>
</tr>
</tbody>
</table>

* Treatments as indicated by numbers in parentheses. (1) One injection at -4 days; (2) one injection (diluted 1:5 with PBS) at -4 days; (3) injected with 1.6 × 10$^4$ neutralizing units (n.u.) at -4 days; (4) injected with 10$^4$ n.u. at -4 and -1 days; (5) injected with 5 × 10$^4$ n.u. at -4 and -1 days; (6) injected with 1.25 × 10$^4$ n.u. at -4 and -1 days; (7) injected with 10$^4$ n.u. at -4 and -1 days.

† Results are given ± S.E.M. The significant values (P < 0.001 against control treated mice) have been underlined.

‡ Seven-week-old DBA/2 mice were injected i.p. with different antibody preparations as indicated. Virus yield per 0.2 ml was determined 18 h after infection of peritoneal macrophages with VSV. There were five mice in each group.

§ Six-week-old male Swiss mice were injected i.p. with 0.2 ml of different antibody preparations as indicated. Virus yield per 0.2 ml was determined 18 h after infection of peritoneal macrophages in vitro with VSV. There were five mice in each group.

Effect of monoclonal and polyclonal antibodies to IFNs on the decay of the antiviral state to VSV in peritoneal macrophages during cultivation in vitro

VSV does not multiply in the majority of peritoneal macrophages freshly explanted from 4- to 8-week-old male or female mice. However, when peritoneal macrophages were cultivated in vitro for 3 to 5 days these cells became permissive for virus replication (Proietti et al., 1986).

Fig. 1 shows the effects of sheep polyclonal antibody to MuIFN-α/β or MAb to MuIFN-α on the decay in vitro of the antiviral state in peritoneal macrophages during cultivation in vitro. Addition of either antibody resulted in an enhanced decay of the antiviral state in macrophages (i.e. VSV titres increased when antibodies to IFN were added). This effect was greater in cultures incubated with sheep polyclonal antibody to MuIFN-α/β than in cultures with MAb. Fig. 2 shows the effects of different MAbs to MuIFNs on VSV multiplication in peritoneal macrophages after 2 days of culture in vitro. Both MAbs to MuIFN-α or -β were effective in increasing virus replication in VSV-infected macrophages. In contrast, the 'control antibody' to MuIFN-α, which binds part of the MuIFN-α from L cells without neutralizing it, and a MAb to MuIFN-γ did not exhibit any effect on VSV replication in peritoneal macrophages.

Effects of MAbs to MuIFNs on the transfer of the antiviral state from freshly harvested mouse peritoneal cells to target mouse macrophages 'aged' in vitro

Freshly harvested peritoneal cells from normal young mice transferred an antiviral state to syngeneic macrophages 'aged' in vitro permissive for virus replication (Proietti et al., 1986). This transfer of the antiviral state was completely abolished by the addition of a sheep polyclonal antibody to MuIFN-α/β in the co-culture medium (Proietti et al., 1986).

Different antibodies to MuIFNs were tested for their capacity to prevent the transfer of the antiviral state from freshly harvested mouse peritoneal cells to syngeneic target macrophages. MAbs to MuIFN-α or -β inhibited the transfer of the antiviral state by donor cells to target cell monolayers (Table 2). A mixture of both MAbs to MuIFN-α and -β completely inhibited the transfer of the antiviral state (Table 2, expt. 2 and 3). In contrast, neither the control non-neutralizing antibody to MuIFN-α nor the MAb to MuIFN-γ were effective in inhibiting the transfer of antiviral state.
Fig. 1. Effect of antibodies to MuIFNs on the in vitro decay of the antiviral state in mouse peritoneal macrophages. Peritoneal cells were harvested from the peritoneal cavity of 5- to 6-week-old male Swiss mice and incubated with or without different antibodies to IFN after separation as described in Methods. At different times of incubation, peritoneal macrophages were washed twice with medium and infected with VSV: virus yields were determined as described in Methods. There were three macrophage cultures for each experimental point. ○, Control macrophages; ●, + MAb to MuIFN-α (clone 4E-A1) (final titre, 5 × 10⁻² against 4 units of MuIFN-α); ■, + sheep polyclonal antibody to MuIFN-α/β (final titre, 5 × 10⁻² against 4 units of MuIFN-α/β).

Fig. 2. Effect of different MAbs to MuIFNs on VSV multiplication in peritoneal macrophages after 2 days of in vitro culture. Freshly explanted peritoneal macrophages from 6-week-old male Swiss mice were treated in vitro with different monoclonal antibodies and then infected with VSV. Virus yields were harvested and titrated as described in Methods. There were three virus-infected cultures for each experimental point. A, Control macrophages; B, + MAb to MuIFN-α (clone 9B-D12, non-neutralizing) (final titre, 1-25 × 10⁻³ against 4 units of MuIFN-α, from CHO cells); C, + MAb to MuIFN-β (clone 7F-D3) (final titre, 2.5 × 10⁻³ against 4 units); D, + MAb to MuIFN-α (clone 4E-A1) (final titre, 5 × 10⁻² against 4 units); E, + MAb to MuIFN-γ (final titre, 5 × 10⁻² against 4 units of MuIFN-γ).

Table 2. Effect of different antibodies to IFNs on the capacity of peritoneal cells to transfer an antiviral state in peritoneal macrophages permissive for VSV

<table>
<thead>
<tr>
<th>Donor peritoneal cells</th>
<th>Type of antibody present in co-culture*</th>
<th>log₁₀ Mean VSV yield/0.2 ml†</th>
<th>Expt. 1‡</th>
<th>Expt. 2‡</th>
<th>Expt. 3§</th>
</tr>
</thead>
<tbody>
<tr>
<td>−</td>
<td>−</td>
<td>4.5 ± 0.1</td>
<td>4.3 ± 0.3</td>
<td>4.3 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>−</td>
<td>2.3 ± 0.2</td>
<td>1.6 ± 0.1</td>
<td>2.0 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>+ Sheep antibody to MuIFN-α/β (1)</td>
<td></td>
<td>4.7 ± 0.2</td>
<td>6.4 ± 0.4</td>
<td>4.6 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>+ MAb to MuIFN-α (2) (clone 4E-A1)</td>
<td></td>
<td>4.3 ± 0.3</td>
<td>3.0 ± 0.4</td>
<td>2.8 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>+ MAb to MuIFN-α (3) (clone 9B-D12, non-neutralizing)</td>
<td></td>
<td>−</td>
<td>−</td>
<td>1.8 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>+ MAb to MuIFN-β (clone 7F-D3)</td>
<td></td>
<td>3.7 ± 0.2</td>
<td>3.6 ± 0.1</td>
<td>3.7 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>+ MAb to MuIFN-β (1)</td>
<td></td>
<td>−</td>
<td>5.6 ± 0.3</td>
<td>4.5 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>+ MAb to MuIFN-γ (2) and to MuIFN-β (4)</td>
<td></td>
<td>2.5 ± 0.4</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>+ MAb to MuIFN-γ (5)</td>
<td></td>
<td>−</td>
<td>−</td>
<td>−</td>
<td></td>
</tr>
</tbody>
</table>

* The neutralizing titres of antibodies to IFNs (final dilutions in the experimental test) were as follows: (1) 1 × 10⁻⁴ against 4 units of MuIFN-α/β, (2) 1 × 10⁻⁴ (expt. 1) and 2 × 10⁻³ (expt. 2 and 3) against 4 units of MuIFN-α; (3) 5 × 10⁻¹ against 4 units of MuIFN-α (from CHO cells); (4) 5 × 10⁻¹ (expt. 1) and 1.2 × 10⁻³ (expt. 2 and 3) against 4 units of MuIFN-β; (5) 2 × 10⁻³ against 4 units of MuIFN-γ.
† Results are given ± S.E.M. There were three VSV-infected macrophage cultures for each experimental condition.
‡ 2 × 10⁶ peritoneal cells freshly explanted from 5- to 6-week-old male Swiss mice were co-cultivated with syngeneic macrophage monolayers as described in Methods.
§ 2 × 10⁶ peritoneal cells freshly explanted from 6-week-old male DBA/2 mice were co-cultivated with syngeneic target macrophages.
Fig. 3. Inhibition of the transfer of the antiviral state by different amounts of antibody to IFNs. Peritoneal macrophages explanted from 6-week-old male DBA/2 mice were cultivated in vitro to render them permissive for VSV. After 4 days, they were co-cultivated with 2 x 10^6 peritoneal cells (freshly explanted from 7-week-old male DBA/2 mice) in the presence of different amounts of antibodies to IFNs. After 18 h at 37 °C, peritoneal cells were removed, and macrophage cultures were infected with VSV. There were three macrophage cultures for each experimental condition. Control MAb to MuIFN-α (non-neutralizing, clone 9B-D12); the neutralizing titre was determined using MuIFN-α subtype from CHO cells, MAb to MuIFN-α (clone 4E-A1); sheep polyclonal antibody to MuIFN-α/β (sheep R5/4).

Fig. 4. Relationship between the number of donor mouse peritoneal macrophages and their capacity to transfer an antiviral state in the absence and in the presence of MAbs to MuIFN-α and -β. Peritoneal macrophages, freshly explanted from 6-week-old male DBA/2 mice were treated with trypsin then suspended in agar-containing medium and co-cultivated with 4-day-old monolayer cultures of syngeneic macrophages in the absence or in the presence of MAbs to MuIFNs. After 18 h at 37 °C the donor cells in agar were removed, and the macrophage monolayers were washed and infected with VSV. There were three infected cultures for each point. Control, No antibodies; MAb to MuIFN-α (clone 4E-A1) (final titre, 4 x 10^{-2} against 4 units of MuIFN-α); MAb to MuIFN-β (final titre, 6 x 10^{-2} against 4 units of MuIFN-β).

Fig. 3 shows the relationship between the amount of neutralizing antibodies to MuIFN-α/β (sheep R5/4) or to MuIFN-α (rat MAb) in the co-culture and the extent of the antiviral state induced by donor peritoneal cells to target macrophages permissive for virus replication. One-thousand neutralizing units of rat MAbs to MuIFN-α were capable of significantly inhibiting the transfer of antiviral state, even though the same amount of sheep polyclonal antibody to MuIFN-α/β exerted a more marked neutralizing effect in this experimental system. In contrast the control rat MAb to MuIFN-α (clone 9B-D12), which had no neutralizing activity, did not significantly inhibit the transfer of the antiviral state by mouse peritoneal cells to target macrophages.

Fig. 4 shows the relationship between the number of donor mouse peritoneal macrophages and their capacity to transfer an antiviral state in the absence or in the presence of MAbs to MuIFN-α or to MuIFN-β. As few as 5 x 10^3 donor peritoneal macrophages were capable of inducing a more than 10-fold decrease of VSV yield in target cells, in agreement with previous results (Proietti et al., 1986) indicating that macrophages represent a very efficient cell population for the transfer of the antiviral state. At any ratio of donor macrophages to target
Expression of IFNs in macrophages

Fig. 5. Kinetics of interferon production after induction of peritoneal macrophages with NDV (a) and total peritoneal cells with LPS (b). (a) Peritoneal cells ($5 \times 10^5$) were explanted from 6-week-old male Swiss mice and separated as described in Methods. Peritoneal macrophages were infected with NDV (4 haemagglutination units per $5 \times 10^5$ cells). Virus was removed after 1 h at $37^\circ $C and macrophage monolayers were further incubated in fresh medium at $37^\circ $C. At different times after incubation cell supernatants were harvested, centrifuged and cell-free nutrient medium was titrated for IFN production as described in Methods. (b) Peritoneal cells ($10^6$) freshly harvested from 5- to 6-week-old male Swiss mice were first incubated for 1 h at $25^\circ $C in 1 ml of RPMI medium with $10\%$ FCS with LPS (20 µg/ml) (from *Escherichia coli*, serotype O26:B6) and subsequently incubated at $37^\circ $C without removing LPS. At different times after incubation cell supernatants were harvested, centrifuged and cell-free nutrient medium was titrated for IFN production as described in Methods.

cells both MAbs to MuIFN-α or to MuIFN-β were capable of inhibiting the transfer of the antiviral state. It is of interest that a complete inhibition of the transfer of antiviral state was observed only at lower concentrations (i.e. $5 \times 10^3$) of donor macrophages.

*Determinaton of the major and minor IFN components produced by macrophages and peritoneal cells after induction with NDV or LPS in vitro*

Fig. 5 shows the kinetics of IFN production after induction of peritoneal macrophages with NDV (a) and total peritoneal cells with LPS (b).

In NDV-induced macrophages, IFN activity was detected in the cell-free medium after 1 h of incubation of cells in fresh medium. Likewise IFN production was detected in the supernatant culture of LPS-treated peritoneal cells as early as 1 h after incubation at $37^\circ $C. Production of IFN was observed only after LPS treatment of total peritoneal cells. Addition of LPS to purified macrophage monolayers did not result in any detectable IFN production. However, it is of interest that LPS-induced IFN production by macrophages did occur, with kinetics similar to those shown in Fig. 5(b), provided that both macrophages and non-adherent peritoneal cells were incubated with LPS for at least 30 to 60 min before removal of non-adherent cells and LPS from the underlying macrophage monolayer (data not shown).
Table 3. Analysis of the major and minor antigenic components of IFNs produced by mouse peritoneal macrophages or cells after induction in vitro with NDV or LPS respectively*

<table>
<thead>
<tr>
<th>IFN samples</th>
<th>Antibody added</th>
<th>Apparent IFN titre</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDV-induced (macrophages)</td>
<td>None</td>
<td>178</td>
<td>100</td>
</tr>
<tr>
<td>NDV-induced (macrophages)</td>
<td>Anti-IFN-α</td>
<td>45</td>
<td>25</td>
</tr>
<tr>
<td>NDV-induced (macrophages)</td>
<td>Anti-IFN-β and anti-IFN-α</td>
<td>151</td>
<td>85</td>
</tr>
<tr>
<td>NDV-induced (macrophages)</td>
<td>Anti-IFN-β</td>
<td>14</td>
<td>8</td>
</tr>
<tr>
<td>LPS-induced (peritoneal cells)</td>
<td>None</td>
<td>89</td>
<td>100</td>
</tr>
<tr>
<td>LPS-induced (peritoneal cells)</td>
<td>Anti-IFN-β</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>LPS-induced (peritoneal cells)</td>
<td>Anti-IFN-α</td>
<td>67</td>
<td>75</td>
</tr>
<tr>
<td>LPS-induced (peritoneal cells)</td>
<td>Anti-IFN-β and anti-IFN-α</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>α1 subtype (CHO cells)</td>
<td>None</td>
<td>333</td>
<td>100</td>
</tr>
<tr>
<td>α1 subtype (CHO cells)</td>
<td>Anti-IFN-β</td>
<td>333</td>
<td>100</td>
</tr>
<tr>
<td>α1 subtype (CHO cells)</td>
<td>Anti-IFN-α</td>
<td>5.2</td>
<td>2</td>
</tr>
<tr>
<td>α1 subtype (CHO cells)</td>
<td>Anti-IFN-β and anti-IFN-α</td>
<td>5.2</td>
<td>2</td>
</tr>
</tbody>
</table>

*NDV- and LPS-induced IFNs were prepared as described in the legend to Fig. 5. Cell-free supernatants were harvested after 18 h at 37°C and IFNs were concentrated by ammonium sulphate precipitation. The apparent IFN titres were determined in the presence of antibody by the constant antibody method, as described by Kawade & Watanabe (1984). The neutralizing titres of antibodies to IFNs (final dilutions in the experimental test) were (i) MAb to MuIFN-β (clone 7F-D3), 4 × 10⁻³ against 4 units of MuIFN-β and (ii) MAb to MuIFN-α (clone 4E-AI), 3 × 10⁻³ against 4 units of MuIFN-α.

Both NDV- and LPS-induced IFNs from Swiss mice were harvested after 18 h and the IFNs produced were analysed using MABs to IFNs. As shown in Table 3, both LPS- and NDV-induced interferons were titrated in the presence or absence of anti-MuIFN-α and anti-MuIFN-β MABs (the constant antibody method; Kawade & Watanabe, 1984) and the results are expressed as the apparent IFN titres observed in the presence of antibody. Both IFN samples were largely neutralized by MABs to MuIFN-β. In fact, as shown in Table 3, in the presence of MABs to MuIFN-β the apparent IFN titres were reduced to 25% for NDV-induced IFN and to 15% for LPS-induced IFN, indicating that MuIFN-β is indeed the major antigenic component produced by macrophages or peritoneal cells after induction in vitro with NDV or LPS respectively. With anti-MuIFN-β at the same dilution, further addition of MABs to MuIFN-α caused an additional reduction of the IFN titres. By estimating this further reduction in the presence of both MABs it was possible to calculate that MuIFN-α represented approximately 17% of the NDV-induced IFN and 13% of the LPS-induced IFN (Table 3). Neither NDV- nor LPS-induced IFNs was neutralized by MAb to MuIFN-γ and both were acid-stable (data not shown). Similar results were obtained by analysing the antigenic components of NDV-induced macrophage or LPS-induced peritoneal cell IFNs from C57BL/6 and DBA/2 mice. No significant differences in the antigenic components were observed by analysing LPS-induced IFNs produced either by total peritoneal cells or by purified macrophages (from Swiss mice), after removal of non-adherent cells (data not shown).

DISCUSSION

The results of three different experimental approaches have suggested that resting peritoneal cells from normal pathogen-free mice are, under physiological conditions, in a MuIFN-α/β-induced antiviral state. First, injection of mice with polyclonal antibodies to MuIFN-α/β prevented this antiviral state in peritoneal cells and resulted in a decrease in the activity of the IFN-induced enzyme 2-5A synthetase (Belardelli et al., 1984; Gresser et al., 1985). Second, freshly harvested peritoneal cells or macrophages can transfer this antiviral state to susceptible target cells and this transfer can be blocked by incubation of polyclonal antibodies to MuIFN-α/β in the agarose containing the donor cells (Proietti et al., 1986). Third, the antiviral state of peritoneal macrophages decays with time in culture (Proietti et al., 1986) and this decay was accelerated when the same antibody was added to the cultures (Fig. 1).
As the evidence that endogenous IFN is responsible for this physiological antiviral state is indirect and rests on the use of different polyclonal antibodies to MuIFN-α/β (Belardelli et al., 1984; Gresser et al., 1985; Proietti et al., 1986), it was important to determine whether MAbs would exert similar effects and whether α and/or β MuIFNs were responsible. The results presented here showed clearly that MAbs to both MuIFN-α and -β were capable of preventing the antiviral state in peritoneal macrophages (Table 1), of inhibiting the transfer of the antiviral state (Table 2, Fig. 4) and of accelerating the decay of the antiviral state in culture (Fig. 1 and 2). A rat MAb to MuIFN-γ, as well as a MAb to MuIFN-α, which binds to MuIFN-α from L cells without neutralizing it (‘control antibody’), did not exert any of these effects on peritoneal macrophages. As the ‘control antibody’ was capable of neutralizing MuIFN-α1 and MuIFN-α2 subtypes (Kawade & Watanabe, 1987), our data would indicate that these MuIFN-α subtypes were not major components of the spontaneous IFN-α expressed in resting peritoneal cells.

In all our experiments we observed that a given amount of neutralizing units of sheep polyclonal antibodies to MuIFN-α/β was more effective than the corresponding amount of MAbs to MuIFN-α or MuIFN-β. The use of mixtures of both MAbs generally resulted in an additive neutralizing effect both in vivo (Table 1; exp. 2) and in vitro (Table 2; exp. 2 and 3). The most effective neutralizing effect of individual MAbs to MuIFN-α or MuIFN-β was observed using fewer peritoneal macrophages as donor cells (Fig. 4), in agreement with the general observation that MAbs exhibit a more marked neutralizing effect at endpoint dilutions of IFNs (Kawade & Watanabe, 1984, 1985).

Although the use of polyclonal and monoclonal antibodies to MuIFN-α and MuIFN-β has revealed the importance of these endogenous IFNs in inducing an antiviral state in peritoneal macrophages, we are not sure how these antibodies act. If these antibodies act by neutralizing small amounts of MuIFN-α and MuIFN-β (not detectable by our bioassays) why did we need several hundred to several thousand neutralizing units of antibody to observe an effect, and if both IFNs α and β were present why was the use of each antibody alone effective? It may be that the antibodies do not act by neutralizing free IFN, but by some other mechanism such as binding to IFN-like molecules on the macrophage surface; if such binding of antibody to macrophages occurs, it could prevent the development of the antiviral state.

Although it has been reported that macrophage IFNs induced with different agents are largely neutralized by polyclonal antibodies to MuIFN-α/β (Maehara & Ho, 1977; Maehara et al., 1977; Havell & Spitalny, 1983; Salo et al., 1985), the major and minor antigenic components have not been determined with precision using MAbs. Yamamoto (1981) reported that influenza virus-induced IFN produced by glass-adherent spleen cells was largely neutralized by polyclonal antibody to MuIFN-β. Brehm & Kirchner (1986) found that pure cultures of bone marrow-derived macrophages produced IFN-β exclusively after induction with poly(rI): poly(rC), whereas after induction with NDV they produced both IFNs α and β. The results presented here indicated that IFN-β was the major antigenic component (75 to 85%) and IFN-α the minor component (13 to 17%) produced by mouse peritoneal macrophages or cells after induction in vitro with NDV or LPS respectively (Table 3). The characterization of the LPS-induced peritoneal cell IFNs appears to be of particular interest as we have recently observed a direct correlation between the LPS response and transfer of the antiviral state by mouse peritoneal cells (Belardelli et al., 1987). At present, however, we do not know whether similar proportions of α and β IFNs are expressed spontaneously in mouse peritoneal cells.

Our studies do emphasize that endogenous IFNs α and β are both responsible for the antiviral state of resting peritoneal cells from normal mice. It seems to us likely that these low levels of endogenous IFNs may also exert other effects in the animal, such as restricting the growth of neoplastic cells. Thus injection of mice with antibody to MuIFN-α/β markedly enhanced the tumourigenicity of xenogeneic (Reid et al., 1981) or syngeneic (Gresser et al., 1983) tumour cells. These low levels of IFNs may also play some role in regulating the function of certain host cells, as well as in modulating the expression of surface antigens, such as histocompatibility antigens (Inaba et al., 1986) or antigens characteristic of the differentiated phenotype (Vogel & Fertsch, 1984).
REFERENCES


(Received 10 March 1987)