Hyporesponsiveness to Dog Interferon Induction in vitro

By SHAW C. TSAI AND MAX J. APPEL*

James A. Baker Institute for Animal Health, Department of Veterinary Microbiology, New York State College of Veterinary Medicine, Cornell University, Ithaca, New York 14853, U.S.A.

(Accepted 9 May 1983)

SUMMARY

Sera from dogs that were hyporesponsive to interferon (IFN) induction blocked virus induction of IFN in spleen cells in vitro but not IFN action in dog kidney cells. Similarly, macrophages or their culture fluids were found to block IFN induction in lymphoid cells in vitro. Lymphoid cells deprived of phagocytic cells secreted IFN continuously for 4 days, whereas lymphoid cells in the presence of phagocytic cells produced IFN for only 1 day. It was speculated that the 'hyporeactivity factor' in the sera from hyporesponsive dogs was macrophage-derived.

INTRODUCTION

Interferon (IFN) is a small protein with potent antiviral and anti-tumour cell activity. Research for the therapeutic use of IFN has been approached in two ways: (i) production of pure exogenous IFN for inoculation, and (ii) development of potent non-toxic IFN inducers. Although production of exogenous IFN has been greatly improved by genetic engineering (Derynck et al., 1980; Goeddel et al., 1980; Hitzeman et al., 1981; Weissbach et al., 1981), it would be more desirable to have a non-toxic IFN inducer which would induce substantial amounts of natural IFN in vivo. One of the major barriers in using an IFN inducer is the development of a refractory state or 'hyporesponsiveness' to IFN induction, which prevents repeated induction of IFN within a short period of time (Breining et al., 1975; Ho & Kono, 1963; Ho et al., 1965; Youngner & Stinebringer, 1965; Stringfellow & Glasgow, 1974; Stringfellow et al., 1977; Tsai & Appel, 1979; Tsai, 1980).

Hyporesponsiveness to IFN induction has been demonstrated in dogs. When dogs were inoculated with virus or synthetic IFN inducers, IFN was released in the serum within 24 h, and not thereafter. When the inoculation was repeated 2 days after the first, IFN production was impaired or not detected. This hyporesponsive condition was not inducer-specific and lasted for almost 2 weeks after the first inoculation (Tsai & Appel, 1979). The 'hyporeactivity factor' has been reported to be an in vivo phenomenon, and previous attempts at in vitro study of the cellular origin of this factor have been unsuccessful (Ho & Postic, 1967; Ho et al., 1976; Tarr et al., 1978).

In the present study, hyporesponsiveness to IFN induction was examined in vitro in dog lymphoid cells. Serum from hyporesponsive dogs and macrophages and their secretions were found to block the induction of dog IFN.

METHODS

Preparation of lymphoid cells. The lymphoid cells used to produce IFN were obtained from spleen and peripheral blood. Preparation of the cells has been described elsewhere (Tsai & Appel, 1983). The spleens were aseptically removed from 2- to 6-week-old specific pathogen-free (SPF) pups, minced to 1 mm³ size, and suspended in Medium 199 with Hanks' balanced salt solution (HBSS, Gibco), 0.5% lactalbumin hydrolysate and antibiotics (100 U penicillin/ml and 50 μg streptomycin/ml). Suspensions were stirred at room temperature for 1-5 to 2 h, then filtered through gauze and centrifuged at 400 g for 20 min. Cell pellets were washed once with the same medium, then resuspended in RPMI 1640 culture medium (Gibco) supplemented with 20% foetal calf serum (FCS).

For the preparation of blood lymphoid cells, 10 to 20 ml blood was drawn from the jugular vein of an SPF dog and defibrinated in 125 ml Erlenmeyer flasks by swirling with 10 to 15 glass beads at 200 rev/min on a Model G-2 shakerplate (New Brunswick Scientific, New Brunswick, N.J., U.S.A.) for 10 min. Defibrinated blood was
diluted by adding an equal amount of HBSS. Five ml of this suspension was overlaid onto 2 ml of Ficoll–Isopaque in 100 × 10 mm polystyrene tubes, and centrifuged at 500 g for 40 min at room temperature. The Ficoll–Isopaque was prepared by dissolving 9.56 g Ficoll 400 (Pharmacia) in 130.4 ml deionized distilled water. Ficoll was sterilized by filtration, and 20 ml 75% (w/v) sterile Isopaque (Nyegaard, Oslo, Norway) was added, which gave a refractive index of 1.3563 or osmolarity from 305 to 320 milliosmoles. The lymphocyte band at the HBSS–Ficoll interface was collected and washed (400 g, 10 min) with HBSS, then resuspended in culture medium.

Removal of phagocytic cells. Phagocytic cells were removed from lymphoid cells as described by Böyum (1977). To every 10 ml of cell suspension or defibrinated blood, 0.2 g carbonyl iron powder (grade 420; GAF, New York, N.Y., U.S.A.) was added; cells were then incubated at 37 °C for 45 min with shaking every 5 min. The cell suspension was then centrifuged at 400 g for 10 min. The supernatant fluid was discarded and the upper three-quarters of packed cells were collected into RPMI 1640 medium supplemented with 20% FCS when spleen cells were processed. From defibrinated blood, the supernatant serum was removed and the top 2 ml of packed cells was collected into 3 ml HBSS for every 10 ml of defibrinated blood processed. The 5 ml blood suspension was then overlaid onto 2 ml Ficoll–Isopaque in a 100 × 10 mm polystyrene tube for isolation of mononuclear cells, as described. Approximately 25% of mononuclear cells were removed after this process, including all adherent cells.

Preparation of subcutaneous macrophages (SCMP). Adult dogs vaccinated with canine distemper virus were used for preparation of SCMP. Procedure was as described by Ryan & Spector (1970). Thermaxon plastic coverslips (no. 1–1/2, 15 mm round or 22 × 60 mm, Lux Scientific Co., Newbury Park, Ca., U.S.A.) were surgically implanted into the subcutaneous spaces on both sides of the flank area, and were withdrawn 4 days later. Coverslip pieces with approximately 10° cells per slide were placed into 24-well Linbro tissue culture plates. One ml Eagle's minimal essential medium with 0.5% lactalbumin hydrolysate, 10% FCS, was supplemented in each culture well. Some macrophage cultures were infected with Newcastle disease virus (NDV) at a multiplicity of infection (m.o.i.) of 1 for the production of IFN or a hyporeactivity factor. Fluids harvested from these cultures were treated like IFN samples.

IFN induction in lymphoid cells. Induction of IFN in dog lymphoid cells has been described elsewhere (Tsai & Appel, 1983). Usually, approx. 1 × 10° lymphoid cells in 2 ml culture medium were incubated at 37 °C, 5% CO₂, in a humidified incubator for various time periods. Unless specifically stated, NDV, B-1 strain, at an m.o.i. of 1 was used as the virus inducer. Culture fluids were removed at 24 h intervals for IFN assay. When fluid changes were made, cells were washed once, then resuspended in 37 °C prewarmed culture medium. Harvested samples were ultracentrifuged at 100000 g for 2 h, tested, and found to be free of virus inducer. Samples were stored at −20 °C. Cells were tested for viability with 0.4% trypan blue.

Cocultivation of lymphocytes and macrophages was done by adding 5 × 10° peripheral lymphocytes in 1 ml culture medium to 1 × 10° macrophages attached to coverslips in 24-well tissue culture plates, as described. Control lymphocytes without macrophages were incubated in different wells.

IFN assay. A 50% plaque reduction assay in secondary dog kidney (DK) cells with vesicular stomatitis virus (VSV) was used for IFN titrations as described elsewhere (Tsai & Appel, 1979). Each sample was assayed in duplicate and samples from each experiment were titrated with the same batch of DK cells.

Test of hyporeactivity factor to IFN induction. The effect of different samples on hyporeactivity to IFN induction was tested in both spleen cells and peripheral blood lymphoid (PBL) cells. Samples that were selected for a hyporeactivity test included culture fluids from NDV-infected or uninfected SCMP, and sera from dogs during a hyporesponsive state to IFN induction. Sera were taken from dogs that had been intravenously inoculated with 0.25 mg/kg poly(IC)LC [a mixture of Poly(IC), L-lysine and carboxymethylcellulose] 31 h earlier. At the time of serum collection, dogs were no longer responsive to IFN induction and IFN was not detected in sera (Tsai & Appel, 1979).

Spleen and PBL cells were exposed to medium containing different concentrations of hyporeactivity factor-containing samples for 24 h. After one washing with culture medium these cells were infected with NDV at an m.o.i. of 1 for IFN production. Normal dog serum, FCS, and/or culture medium alone were included as controls.

Test of hyporeactivity factor on IFN activity. The effect of sera from hyporeactive dogs on IFN activity was tested on DK cells. These cells were cultivated with various dilutions of serum samples (as described in the previous section) for 24 h either before or after they were treated with IFN preparations for 24 h. Cell cultures were then challenged with VSV as described. Cells were washed three times with phosphate-buffered saline between different exposures.

RESULTS

Effect of a serum hyporeactivity factor on IFN induction in dog spleen cells

The sera collected from dogs 31 h after IFN induction with poly(IC)LC greatly reduced IFN production in dog spleen cells when compared with IFN production in cells exposed to normal
Table 1. Effect of a hyporeactivity factor derived from dog serum on NDV-induced* IFN production in dog spleen cells

<table>
<thead>
<tr>
<th>Treatment of dog spleen cells</th>
<th>IFN titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% Dog serum no. 1†</td>
<td>20 ± 2‡</td>
</tr>
<tr>
<td>50% Dog serum no. 2†</td>
<td>40 ± 3</td>
</tr>
<tr>
<td>50% Normal dog serum</td>
<td>413 ± 6</td>
</tr>
<tr>
<td>20% FCS</td>
<td>447 ± 21</td>
</tr>
</tbody>
</table>

* NDV was used at an m.o.i. of 1.
† Sera collected from dogs 31 h after intravenous injection with 0·25 mg poly(IC)LC/kg body weight.
‡ Results are the average of data from two experiments. IFN titres are in units per 0·3 ml ± standard error of the means.

Table 2. Effect of a hyporeactivity factor derived from dog serum on IFN activity in DK cells

<table>
<thead>
<tr>
<th>Treatment of DK cells</th>
<th>IFN titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>First incubation</td>
<td>Second incubation</td>
</tr>
<tr>
<td>(24 h)</td>
<td>(24 h)</td>
</tr>
<tr>
<td>Dog serum no. 1*</td>
<td>IFN</td>
</tr>
<tr>
<td>Normal dog serum</td>
<td>IFN</td>
</tr>
<tr>
<td>IFN†</td>
<td>Dog serum no. 1</td>
</tr>
<tr>
<td>IFN</td>
<td>Normal dog serum</td>
</tr>
</tbody>
</table>

* Sera collected from dogs 31 h after intravenous injection with 0·25 mg poly(IC)LC/kg body weight.
† Results are the average of data from two experiments. IFN titres are in units per 0·3 ml ± standard error of the means.
‡ IFN in sera from dogs collected 8 h after intravenous injection with 1·5 mg poly(IC)LC/kg body weight.

Dog serum or 20% FCS (Table 1). Sera containing a hyporeactivity factor were not toxic to spleen cells when tested by dye exclusion, and did not contain IFN.

Effect of a serum hyporeactivity factor on IFN activity

The hyporeactivity factor in dog serum that affected IFN production did not influence IFN activity greatly. Interferon titres expressed in DK cells that were treated with the hyporeactivity factor for 24 h either before or after IFN exposure differed only slightly from IFN titres produced in DK cells that were treated with normal dog serum (Table 2).

Influence of macrophages on IFN production

The secretion of NDV-induced IFN from dog spleen cells without phagocytic cells continued after 24 h and reached a peak 4 days after incubation (Fig. 1), whereas IFN production from spleen cells with phagocytic cells ceased and the titre decreased slightly after 24 h. At day 4, the IFN titres produced by cells without phagocytic cells were 2843 ± 47 units per 0·3 ml and 1925 ± 212 units per 0·3 ml in two individual experiments. IFN produced by spleen cells containing phagocytic cells were 864 ± 34 units per 0·3 ml and 835 ± 40 units per 0·3 ml respectively. Like spleen cells, peripheral lymphocytes deprived of phagocytic monocytes produced significantly (P < 0·01) higher IFN titres than cells with phagocytic monocytes, when both cell populations were adjusted to the same concentration (Table 3).

Effect of SCMP on IFN production

SCMP in culture produced little, or no IFN upon induction with NDV. In contrast, peripheral blood lymphocytes produced substantial amounts of IFN after NDV stimulation (Table 4). When peripheral lymphocytes were cocultivated with SCMP at a ratio of 5 : 1 and infected with NDV, IFN production never began.

Culture fluids from SCMP were tested for their effect on IFN induction by NDV in peripheral blood lymphocytes and spleen cells. IFN production was greatly impaired in cells
Fig. 1. Effect of phagocytic cells on NDV-induced IFN production in dog spleen cells. NDV, B-1 strain, was used at an m.o.i. of 1; dog spleen cells were used at a concentration of $1 \times 10^7$ cells/ml. Experiment 1, with phagocytic cells (••••); experiment 2, with phagocytic cells (○○○○); experiment 1, without phagocytic cells (•--••); experiment 2, without phagocytic cells (○--○○).

Table 3. Effect of culture fluids from SCMP on NDV-induced IFN production by lymphoid cells*

<table>
<thead>
<tr>
<th>Cell source</th>
<th>Phagocytic monocytes</th>
<th>Culture fluid</th>
<th>Amount of IFN induced</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBL</td>
<td>+</td>
<td>RPMI 1640 + 20% FCS</td>
<td>404 ± 48†</td>
</tr>
<tr>
<td>PBL</td>
<td>+</td>
<td>NDV-SCMP†</td>
<td>0</td>
</tr>
<tr>
<td>PBL</td>
<td>−</td>
<td>RPMI 1640 + 20% FCS</td>
<td>1671 ± 200</td>
</tr>
<tr>
<td>PBL</td>
<td>−</td>
<td>NDV-SCMP</td>
<td>427 ± 16</td>
</tr>
<tr>
<td>PBL</td>
<td>−</td>
<td>Control-SCMP†</td>
<td>400 ± 79</td>
</tr>
<tr>
<td>Spleen cells</td>
<td>+</td>
<td>RPMI 1640 + 20% FCS</td>
<td>1124 ± 61</td>
</tr>
<tr>
<td>Spleen cells</td>
<td>+</td>
<td>NDV-SCMP</td>
<td>373 ± 29</td>
</tr>
<tr>
<td>Spleen cells</td>
<td>−</td>
<td>RPMI 1640 + 20% FCS</td>
<td>2475 ± 131</td>
</tr>
<tr>
<td>Spleen cells</td>
<td>−</td>
<td>Control-SCMP</td>
<td>822 ± 101</td>
</tr>
</tbody>
</table>

* Final cell concentrations were adjusted to $1 \times 10^7$ cells per vial for cultures with or without phagocytic monocytes. NDV was used at an m.o.i. of 1. IFN was tested 24 h after induction.
† Culture fluids from NDV-infected SCMP (NDV-SCMP) or uninfected SCMP (control-SCMP).
‡ Results are the average of data from two experiments. IFN titres are in units per 0.3 ml ± standard error of the means.

Table 4. Production of IFN by SCMP and peripheral lymphocytes in dogs

<table>
<thead>
<tr>
<th>Cell source</th>
<th>NDV*</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCMP†</td>
<td>2 ± 0†</td>
<td>0</td>
</tr>
<tr>
<td>Peripheral lymphocytes§</td>
<td>264 ± 70†</td>
<td>3 ± 0</td>
</tr>
<tr>
<td>Combined</td>
<td>2 ± 0†</td>
<td>0</td>
</tr>
</tbody>
</table>

* NDV, B-1 strain, was used at an m.o.i. of 1.
† $1 \times 10^6$ cells/culture.
‡ Results are the average of data from two experiments. IFN titres in units per 0.3 ml ± standard error of the means.
§ $5 \times 10^6$ cells/culture.
incubated in culture fluids from SCMP, infected or uninfected with NDV, as compared to IFN production in cells incubated in control medium RPMI 1640 with 20% FCS (Table 3).

**DISCUSSION**

It appears that macrophages are the cells responsible for early termination of IFN production by dog lymphocytes. When phagocytic cells were removed from spleen cells, the production of IFN continued over a period of 4 days (Fig. 1). This concept was further supported by direct *in vitro* cocultivation of SCMP with peripheral blood lymphocytes. The production of IFN from lymphocytes was completely blocked by the cocultivation (Table 4). The interference probably was mediated by soluble factors secreted from macrophages rather than cell-to-cell contact because culture fluid from macrophages alone impaired the IFN production of lymphocytes either from spleen or peripheral blood (Table 3). Since IFN was not detected in macrophage culture fluids, the previous possibility that IFN itself may be involved in hyporesponsiveness induction could be eliminated. Culture fluids from macrophages also impaired IFN production in DK cells, indicating that the action was not limited to lymphoid cells. Culture fluids from both NDV-infected and uninfected SCMP had the same modulating effect on IFN production. Activation of macrophages may be required in order to secrete the modulating factor. The macrophages used in these experiments were obtained from implanting coverslips subcutaneously and had been activated.

The production of a 'hyporeactivity factor' by canine macrophages appears to be in contrast with reports of IFN production in macrophages in other species (Smith & Wagner, 1967; Smith *et al.*, 1973; Rudenko & Smorodintsev, 1969). There may really be species differences, or it might be that different macrophage populations in various stages of activation or maturation may respond in different ways. Further search for the 'hyporeactivity factor' in macrophages from other species may be warranted, therefore.

It remains unresolved whether the 'hyporeactivity factor' in the serum from hyporesponsive dogs was macrophage-derived. However, the data obtained *in vitro* with macrophages and their products make it highly probable.

This work was supported by Public Health Service Grant NS 14342 from the National Institutes of Health. The assistance of Mary Beth Metzgar and Ann Signore is greatly appreciated.

**REFERENCES**


(Received 5 January 1983)