The Purification and Characterization of Rat Gamma Interferon by Use of Two Monoclonal Antibodies

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SUMMARY

Two mouse monoclonal antibodies, designated DB-1 and DB-2, were isolated and used for the purification and characterization of recombinant rat interferon gamma (rRIF-γ) derived from Chinese hamster ovary (CHO) cells. The two antibodies belong to different classes (DB-1 is an IgG1 and DB-2 an IgA) and display similar epitope specificities as shown in competition binding experiments. Both antibodies, raised against rRIF-γ, exhibited high affinity for rat and mouse gamma interferon and efficiently neutralized the antiviral activity of both animal interferon species. Affinity chromatography analysis showed that a column with immobilized DB-1 was capable of complete binding of rat and mouse gamma interferon, both natural and recombinant DNA-derived. As visualized by SDS–polyacrylamide gel electrophoresis and Western blot analysis, the purified rRIF-γ preparation consisted of at least seven molecular forms with $M_r$ values ranging between 14 000 and 25 000, with a relative abundance of a 18 000 $M_r$ protein. Gel permeation chromatography of crude rRIF-γ gave coincident peaks of rRIF-γ proteins (all different forms) and interferon activity corresponding to a $M_r$ value of 45 000. The results suggest that the molecular heterogeneity was due to differential glycosylation and was not the consequence of a proteolytic degradation process.

INTRODUCTION

Interferons (IFNs) are proteins synthesized by vertebrate cells after exposure to viruses, double-stranded RNA or other inducers (Fuller & Marcus, 1980; Ho, 1984). Although initially defined as antiviral proteins, IFNs have multiple biological effects including immunity-modulating and anti-tumour activity, both in vitro and in vivo. During the almost 25 years since the discovery of IFN, studies have indicated that within an animal species different types of IFNs can be induced with different physicochemical and antigenic specificities. Three major antigenic types have been defined: IFN-α, -β and -γ (Stewart, 1980). Whereas IFN-α and IFN-β are produced by a variety of cell types, IFN-γ is produced only by T lymphocytes upon induction with mitogens, or upon antigenic stimulation of sensitized cells (Valle et al., 1975; Marcucci et al., 1981). Earlier studies suggested that IFN-γ may have biological properties distinct from those of the other two species but due to its scarcity and problems during isolation it was always questionable whether the observed properties were solely due to IFN-γ and not to other lymphokines in the semi-purified preparations. Nowadays, pure IFN-γ is available and it has been shown to be an important immunoregulator (Basham & Merigan, 1983).

Knowledge about IFN-γ has increased greatly, mainly as a result of recent cloning of the human, mouse and rat IFN-γ genes in appropriate host/vector systems (Gray & Goeddel, 1982, 1983; Dijkema et al., 1985). Screening of the respective gene libraries revealed in all cases a single chromosomal gene, and sequence analysis of the cloned genomic DNAs predicted single polypeptides with overall lengths of 146, 137 and 136 amino acids for human, rat and mouse IFN-γ, respectively. All three IFN-γ gene sequences indicate two potential glycosylation sites.
However, up to now no substantial differences in biological activity have been reported between IFN-γ with and without sugar moieties.

In a previous publication we described the isolation of the RIF-γ structural gene from the genomic DNA of a rat gene library (Dijkema et al., 1985). The coding sequence was inserted into a vector under the control of the simian virus 40 early promoter and transfected into dihydrofolate reductase-defective (DHFR-) Chinese hamster ovary (CHO) cells. Initial DHFR+ transformants secreting moderate levels of rat IFN activity were passaged in progressively higher concentrations of methotrexate (MTX) and several stable MTX-resistant cell lines were selected which secreted rRIF-γ at 20000 to 100000 U/ml per day. In order to study the biochemical and immunochemical properties of this recombinant product, we used the hybridoma technique of Köhler & Milstein (1975) for the isolation of a collection of different monoclonal antibodies (MAbs) specific for RIF-γ. This paper describes two of these MAbs, one of which was successfully employed in an affinity chromatography system for the purification of rRIF-γ. By using the same affinity column, it was also possible to obtain pure mouse gamma interferon with the same high recovery from crude preparations.

**METHODS**

*Interferon preparations and assay.* rRIF-γ was produced by a CHO DHFR+ transformant which constitutively secretes 80000 U/ml per day into its culture medium (Dijkema et al., 1985). This cell line is routinely maintained at 37 °C in a humidified atmosphere containing 5% CO₂. Cell monolayers were grown in Dulbecco's modification of Eagle's medium (DMEM; Flow Laboratories, cat. no. 12-332-54) supplemented with 10% foetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM-glutamine and 100 μM-MTX. This medium is referred to as selective medium. When cells reached confluence, they were dispersed by trypsin and suspended in 10 ml selective medium (approx. 1-2 × 10⁶ cells/ml) and transferred to a 2 litre roller bottle (Falcon 3027). An additional volume of 90 ml selective medium was added to the cells. Cells were grown to confluence (usually in 4 days) and washed with two successive additions of 50 ml Hanks' balanced salt solution (HBSS; Flow Laboratories, cat. no. 18-104-54), after which 50 ml serum-free DMEM without MTX was added. The medium was changed every 24 h (for at least 7 days). The decanted media were pooled and centrifuged to remove residual cells. The supernatant is called crude rRIF-γ.

 Naturally derived preparations of rat and mouse gamma IFNs were prepared essentially as described by Johnson et al. (1981) using concanavalin A (Con A) as inducer. For the mouse system, 15 × 10⁶ spleen cells/ml (from CBA/Rij mice) were induced with 5 μg/ml Con A. For the rat (Wag/Rij) these values were 20 × 10⁶ cells/ml and 20 μg/ml.

 Native rat IFN α/β (nRIF-α/β) with a specific activity of 2 × 10⁵ laboratory units/mg protein was obtained from a transformed rat embryonic cell line (Ratec) growing in monolayer cultures after exposure to Sendai virus (van der Meide et al., 1986).

 Recombinant rat IFN-α (rRIF-α) was produced in Escherichia coli and had a specific activity of 6 × 10⁵ laboratory units/mg protein (Dijkema et al., 1984).

 Recombinant mouse IFN-γ (rMIF-γ) produced in CHO cell cultures was kindly supplied by Professor W. Fiers (Ghent University, Belgium). Its activity was 6000 IU/ml (calibrated against an international mouse IFN-α/β preparation, NIH cat. no. G002-904-511).

 Recombinant human IFN-γ (rHuIFN-γ) from bacterial cells (E. coli) was obtained from Biogen (Geneva, Switzerland) and was more than 98% pure, containing 8 × 10⁵ IU/ml. Partially purified native human IFN-γ (nHu-IFN-γ) from induced leukocytes was obtained from Meloy Laboratories (Springfield, Va., U.S.A.), containing 10⁴ IU/ml (Braude, 1983a, b).

 For estimation of the antiviral activity, the cytopathic effect reduction assay described by Armstrong (1971) was used. For rat IFNs we used Ratec cells, for mouse IFNs L929 cells and for human IFNs HEp-2 cells. In all assays vesicular stomatitis virus (VSV) was used as challenge virus. All titres from human and mouse IFNs are expressed in reference units, whereas rat IFNs are calibrated against a laboratory standard preparation.

*Preparation of hybridomas.* Recombinant rat IFN-γ produced by CHO cells was partially purified by controlled pore glass (CPG) adsorption/desorption techniques (Dijkema et al., 1985) and used as an immunizing agent. Sp2/O-Ag-14 (Shulman et al., 1978) mouse myeloma cells were fused with spleen cells from immunized mice as described previously (van der Meide et al., 1985). Positive cultures were determined by ELISA testing and cloned by limiting dilution. The subclasses of monoclonal antibodies were determined by immunoelectrophoresis using rabbit antibodies to specific subclasses of mouse immunoglobulins. Ascites tumours were induced in the peritoneal cavity of BALB/c mice by injection of 10⁶ hybridoma cells.

*Polyclonal antiserum to rRIF-γ.* Culture medium from CHO cells secreting high levels of rRIF-γ was concentrated by lyophilization and partially purified by CPG adsorption/desorption. This material was subjected...
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to SDS-PAGE under reducing conditions. After staining of the gel with Coomassie Brilliant Blue the most prominent band attributable to rRIF-γ protein (Mr 18000) was cut from the gel. Gel slices containing approximately 50 μg of protein were washed for 2 days in 96% ethanol containing 0.1 M sodium acetate at 4 °C with several changes. The ethanol solution was decanted and 2 ml of sterile phosphate-buffered saline (PBS) was added to the gel slices. After 10 min at room temperature, the re-swollen slices were homogenized and injected subcutaneously without adjuvant into a rabbit weighing 5 kg. Biweekly immunizations with 50 μg of protein were continued for 10 weeks. Subsequently a booster injection of 200 μg of 18K protein was given. For this last injection, the protein was removed from the gel slices by electroelution using an Isoe electrophoretic sample concentrator (Allington et al., 1978). Eight days thereafter the rabbit was bled from the marginal ear vein.

**SDS–PAGE, Western blot analysis and protein determination.** SDS–PAGE was performed in 5 to 30% gradient gels using the Pharmacia vertical gel electrophoresis system under conditions described by the manufacturer. Proteins run on 2.0 mm-thick gradient gels were electrophoretically transferred onto nitrocellulose as described by Towbin et al. (1979) and Burnette (1981) at 100 mA (approx. 8 V) for 18 h in 0.192 M-glycine, 25 mM-Tris-HCl pH 8.3, 20% methanol utilizing a Pharmacia destaining apparatus. Protein concentrations were determined by the method described by Bradford (1976) utilizing the Bio-Rad micro-protein assay system.

**Preparation of monoclonal antibody affinity column.** Partially purified DB-1 immunoglobulins were obtained by precipitation with half-saturated ammonium sulphate and coupled to cyanogen bromide-activated Sepharose CL-4B (Pharmacia) as described by the manufacturer with some modifications. Briefly, 35 g of Sepharose was swollen in 1 mM-HCl for 15 min and washed with 5 litres of coupling buffer (0.1 M-NaHCO₃ pH 8.5, 0.5 M-NaCl). The MAbs (300 mg of IgG in a total amount of 750 mg of protein) were dissolved in coupling buffer at 6 mg protein/ml and mixed with the resin to a final volume of 250 ml. This solution was rotated overnight at 4 °C. The sites of the gel not occupied by antibody were subsequently blocked by incubating the gel in 300 ml 0.1 M-NaHCO₃ pH 8.5, 1.0 M-ethanolamine for 90 min at 4 °C. The gel was extensively washed with, successively, 0.1 M-sodium acetate/1 M-NaCl pH 4.0, PBS/1 M-NaCl pH 7.5, PBS/4 M-urea pH 4.0 and PBS pH 7.4. The Sepharose gel was poured into a column (20 x 2.6 cm) and equilibrated with PBS pH 7.4. A flow rate of 2 ml/min was used for the purification of mouse and rat IFN-γ.

**Affinity chromatography of rRIF-γ.** Culture medium (600 ml) of CHO cells secreting rRIF-γ and containing about 60 000 units/ml was lyophilized and resuspended in one-tenth of the original volume. The suspension was centrifuged for 15 min at 30 000 g and the clarified supernatant was applied to the affinity column. The column was washed with three bed volumes of PBS pH 8.0, 0.5 M-NaCl. Proteins bound to the immobilized DB-1 antibodies were eluted with 0.2 M-phosphate buffer pH 12.0, 0.5 M-NaCl. The rRIF-γ-containing fraction was collected in a tube already containing 0.2 vol. 1 M-phosphate buffer pH 7.0. The purified rRIF-γ was dialysed against PBS pH 8.5 with several changes for 48 h and lyophilized in the presence of glucose (0.2 g/10 ml). All steps were performed at 4 °C.

**RESULTS**

**Generation and characterization of anti-rat IFN-γ monoclonal antibodies**

Spleen cells of BALB/c mice, immunized with partially purified rRIF-γ, were fused with mouse myeloma cells. The resulting hybrid cell lines were screened for reactivity with pure rRIF-γ by ELISA. Twelve out of 680 hybrid culture supernatants contained anti-RIF-γ activity. These 12 primary cultures were subcloned by limiting dilution and seven stable clones were obtained. All seven monoclonal antibodies were characterized by immunoelectrophoresis using specific antisera to mouse immunoglobulins (details to be described elsewhere). Two MAbs of different (sub)classes, DB-1 (IgG1) and DB-2 (IgA), were chosen for characterization of rRIF-γ and application in affinity chromatography procedures.

**Affinity chromatography**

The culture supernatant of one of the seven subclones gave an especially intense staining in ELISA. The MAb (DB-1) of this cell line was selected for immunoaffinity purification of rRIF-γ. Ascitic fluid was prepared in BALB/c mice and the immunoglobulins were partially purified by 50% (saturation) ammonium sulphate precipitation. The immunoglobulins were coupled to CNBr-activated Sepharose according to the procedure described under Methods. Crude rRIF-γ was passed through a column containing immobilized DB-1. No activity could be detected in the flowthrough fraction whereas more than 99% of the retained activity could be eluted with 0.2 M-phosphate buffer pH 12 (see Table 1). To study whether this column had affinity for naturally derived RIF-γ (nRIF-γ), the same procedure was followed for a RIF-γ preparation from Con A-
<table>
<thead>
<tr>
<th>Type of IFN</th>
<th>Input (total units $\times 10^{-4}$)</th>
<th>Sp. act. of input (U/mg) $\times 10^{-4}$</th>
<th>Flowthrough (total units $\times 10^{-4}$)</th>
<th>High salt wash (total units $\times 10^{-4}$)</th>
<th>Eluate pH 12 (total units $\times 10^{-4}$)</th>
<th>Sp. act. of eluate (U/mg)</th>
<th>Recovery (%) in pH 12 eluate</th>
</tr>
</thead>
<tbody>
<tr>
<td>rRIF-γ (CHO)</td>
<td>300</td>
<td>6 $\times 10^5$</td>
<td>0.5</td>
<td>2.0</td>
<td>296</td>
<td>4 $\times 10^6$</td>
<td>99</td>
</tr>
<tr>
<td>nRIF-γ (Con A)</td>
<td>10</td>
<td>90</td>
<td>0.01</td>
<td>0.02</td>
<td>9.7</td>
<td>ND†</td>
<td>97</td>
</tr>
<tr>
<td>rMIF-γ (CHO)</td>
<td>30</td>
<td>800</td>
<td>0.5</td>
<td>0.05</td>
<td>30.5</td>
<td>ND†</td>
<td>100</td>
</tr>
<tr>
<td>nMIF-γ (Con A)</td>
<td>1.8</td>
<td>140</td>
<td>0.01</td>
<td>0.06</td>
<td>1.5</td>
<td>ND</td>
<td>83</td>
</tr>
<tr>
<td>rHuIFN-γ (E. coli)</td>
<td>10</td>
<td>500</td>
<td>0.8</td>
<td>7.8</td>
<td>0.9</td>
<td>ND</td>
<td>9</td>
</tr>
</tbody>
</table>

* The different IFN species were applied to a column with a bed volume of 12 ml.
† ND, Not determined.
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stimulated rat spleen cells. A similar purification was achieved with almost 100% recovery of the applied activity (see Table 1).

CHO cell-produced rMIF-γ and a nMIF-γ preparation obtained from Con A-stimulated mouse spleen cells were also applied to this column. In these cases also, the flowthrough fractions contained negligible amounts of activity and more than 99% (rMIF-γ) to 80% (nMIF-γ) of the applied activity was recovered at pH 12. Low binding efficiency was found for rHuIFN-γ; 10% of the applied activity was found in the flowthrough fraction, 80% was eluted with high salt and the remaining 10% was recovered at pH 12 (Table 1).

SDS–PAGE and Western blot analysis

The immunoadsorbent-purified rRIF-γ was analysed by SDS–PAGE and revealed a dense 18000 Mr (18K) band together with at least five minor bands, two of which migrated to positions between 18K and 20K and the other three to between 14K and 17K. A number of faint bands were found in the region between 23K and 25K (see Fig. 1 and 6). Western blot analysis of pure rRIF-γ indicated that not only the 18K main band but also the minor bands showed antigenicity towards MAb DB-2 (see Fig. 2). In this assay DB-1 was less effective. The immunoblot of a crude rRIF-γ preparation (not shown) exhibited no alterations of the relative proportions of the different rRIF-γ proteins, indicating that the different forms were not a consequence of degradation or modification during the purification procedure. No reactivity could be observed with the MAb towards the weak bands at 23K to 25K positions. However, by using a highly specific polyclonal anti-rRIF-γ serum which was raised against the main 18K band (see Methods), the results showed that these weak bands were also immunoreactive (Fig. 2).

Neutralization of the antiviral activity of MIF-γ and RIF-γ by MAbs DB-1 and DB-2

To determine the potency of the two MAbs in neutralizing the antiviral action of rat and mouse IFN-γ, we incubated a constant amount of IFN-γ with twofold serial dilutions of ascites fluid. The antiviral activity of natural and recombinant RIF-γ was efficiently neutralized by both DB-1 and DB-2, although DB-1 was about twice as effective as DB-2 (Table 2). We also found a strong neutralizing activity of DB-1 for natural and recombinant DNA-derived MIF-γ preparations. DB-2 also neutralized the antiviral activity of MIF-γ. If, however, we compare the potency of DB-1 and DB-2 towards RIF-γ and MIF-γ, the neutralizing activity differed significantly between these animal IFN species, in favour of MIF-γ. This is in contrast with the neutralizing activity of the anti-rRIF-γ polyclonal antiserum which showed less activity against rMIF-γ. As shown in Table 2, DB-1 and DB-2 had no measurable neutralizing activity towards human IFN-γ.

Cross-species antiviral activity of MIF-γ and RIF-γ

Because of the close protein homology (Dijkema et al., 1985) we also expected a significant antiviral cross-reactivity between MIF-γ and RIF-γ on rat and mouse cells. As presented in Table 3 a strong antiviral activity was observed with RIF-γ on L929 mouse cells, but in contrast MIF-γ was only marginally active on rat cells. The high activity of rat IFN-γ on mouse L929 cells is remarkable in comparison with the homologous combination. This phenomenon is most likely attributable to differences in the specific activities of MIF-γ and RIF-γ (see Discussion). Naturally derived RIF-γ showed some activity on human HEp-2 cells. At present, it is not clear whether nRIF-γ itself is responsible for this antiviral effect.

Determination of epitope specificity

To study whether DB-1 and DB-2 recognize distinct epitopes, a competition binding experiment was performed. Constant amounts of each type of MAb labelled with biotin were mixed with increasing concentrations of unlabelled DB-1 or DB-2. Excess amounts of each unlabelled MAb were then assayed for their ability to block the binding of labelled MAb to rRIF-γ adsorbed to the wells of a microtitre plate. Fig. 3 shows that DB-1 and DB-2 mutually
Fig. 1. SDS-PAGE of immunochromatographically purified rRIF-γ produced by CHO cells. (a) pH 12 column eluate. (b) Culture supernatant before application to affinity column. The positions of reference proteins are indicated at the right.

Fig. 2. Visualization of rRIF-γ proteins by Western blot analysis using MAb DB-2 (a) and a rabbit polyclonal antiserum (b) specific for RIF-3. Fifty μg of affinity-purified rRIF-γ was subjected to SDS-PAGE and transferred to nitrocellulose paper as described previously (van der Meide et al., 1985). The positions of reference proteins are indicated at the right.

blocked each other’s binding, indicating that they bind close to, or at the same (overlapping) site on rRIF-γ.

Gel filtration of rRIF-γ

The molecular weight of human and mouse IFN-γ, estimated by gel filtration chromatography, varies between 40K and 50K (Yip et al., 1981, 1982; Osborne et al., 1979; Gribaudo et al., 1984). Because the molecular weight on a reducing SDS-polyacrylamide gel is substantially
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Fig. 3. Competition assay between monoclonal antibodies DB-1 and DB-2. One-hundred ng pure rRIF-γ was coated on each well of a microtitre plate. Constant amounts (500 ng) of each type of MAb labelled with biotin (van der Meide et al., 1985) were mixed with increasing concentrations of unlabelled DB-1 or DB-2. The mixtures were added to the wells and incubated for 30 min at 37 °C. After washing, the wells were filled with 200 μl of a solution (2 μg protein) containing complexes of streptavidin with biotinylated alkaline phosphatase polymer. This complex was prepared as described by Leary et al. (1983). After an incubation time of 1 h at 37 °C, the wells were extensively washed and filled with 200 μl p-nitrophenyl phosphate (500 μg/ml). Substrate conversion was determined after 30 min (37 °C, dark) by absorbance at 405 nm. Labelled DB-1 (a) and DB-2 (b) competed with unlabelled DB-1 (□) and DB-2 (■).

Table 2. Neutralizing activity of monoclonal antibodies DB-1 and DB-2 compared with an anti-rRIF polyclonal antiserum to rat, mouse and human IFNs*

<table>
<thead>
<tr>
<th>Type of IFN</th>
<th>Neutralizing activity (NU/ml)†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MAb (IgG) DB-1</td>
</tr>
<tr>
<td>rRIF-γ (CHO)</td>
<td>30 700</td>
</tr>
<tr>
<td>nRIF-γ (Con A)</td>
<td>20 000</td>
</tr>
<tr>
<td>rRIF-α/β (E. coli)</td>
<td>&lt; 50</td>
</tr>
<tr>
<td>nRIF-α/β (Ratec)</td>
<td>&lt; 50</td>
</tr>
<tr>
<td>rMIF-γ (CHO)</td>
<td>126 000</td>
</tr>
<tr>
<td>nMIF-γ (Con A)</td>
<td>84 000</td>
</tr>
<tr>
<td>rHultIFN-γ (E. coli)</td>
<td>&lt; 30</td>
</tr>
<tr>
<td>nHultIFN-γ (PHA)</td>
<td>&lt; 30</td>
</tr>
</tbody>
</table>

* 0.5 ml IFN preparations containing 5 to 20 units of IFN were incubated with equal volumes of serial twofold dilutions of hybridoma ascites fluid or serum (polyclonal antibodies) in DMEM containing 1% FCS for 1 h at room temperature. One-hundred μl mixtures were then added to cultures of HEp-2 (HultIFN-γ), L929 (MIF-γ) or Ratec (RIF-γ) cells in microtitre plates and challenged with VSV.

† One neutralizing unit (NU) is defined as the amount of antibody sufficient for the neutralization of one unit of antiviral activity.

ND, Not determined.

Table 3. Cross-species antiviral activity of rat, mouse and human gamma IFNs on different cell lines

<table>
<thead>
<tr>
<th>Type of IFN</th>
<th>Activity on cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HEp-2 (human)</td>
</tr>
<tr>
<td>rHultIFN-γ (E. coli)</td>
<td>100</td>
</tr>
<tr>
<td>nHultIFN-γ (leukocytes)</td>
<td>100</td>
</tr>
<tr>
<td>rMIF-γ (CHO cells)</td>
<td>&lt; 0.5</td>
</tr>
<tr>
<td>nMIF-γ (spleen cells)</td>
<td>&lt; 2</td>
</tr>
<tr>
<td>rRIF-γ (spleen cells)</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>nRIF-γ (spleen cells)</td>
<td>12</td>
</tr>
</tbody>
</table>

* The values are the activities expressed in percentages of those found for the homologous combinations. The values are determined by a c.p.e. reduction assay using VSV as challenge virus (see Methods).
Fig. 4. (a) Molecular weight determination of rRIF-γ by gel filtration over a column of Fractogel TSK HW-50(s) coupled to an FPLC System (Pharmacia). rRIF-γ was concentrated 15-fold by lyophilization and dialysed against 20 mM-triethanolamine pH 8.5, 200 mM-NaCl. One-half ml concentrated rRIF-γ (clarified by centrifugation) was applied to a column (27 x 1.5 cm) and chromatographed at a flow rate of 0.5 ml/min and a column pressure of 0.5 bar (5 x 10^4 Pa). The eluate was monitored at 280 nm (continuous trace) and fractions of 0.5 ml were collected and assayed for antiviral activity (histogram). For calibration of the column, the following standard proteins were run under identical conditions. A, thyroglobulin [M, 669000, effluent volume (e.v.) 12.5 ml]; B, aldolase (M, 158000, e.v. 14.3 ml); C,
less, it has been suggested that under native conditions IFN-γ is composed of two or three monomers. To estimate the apparent molecular weight of rRIF-γ under non-denaturing conditions, a crude sample of rRIF-γ was subjected to gel permeation chromatography. The elution profile from a TSK HW-50(s) (Merck, Darmstadt, F.R.G.) gel permeation column is presented in Fig. 4(a). More than 95% of the applied activity was recovered at a position corresponding to an apparent mol. wt. of 45K. The IFN active fractions from the column were concentrated by lyophilization and applied to an SDS-polyacrylamide gel. The results in Fig. 4(b) show that after staining, all activity was recovered in association with the different forms of rRIF-γ which were also present in immunoadsorbent-purified material.

Post-translational modification of rRIF-γ

To find out whether the observed heterogeneity of rRIF-γ is due to differentially glycosylated polypeptides or is caused by other post-translational processes such as proteolytic degradation, we examined by SDS-PAGE and Western blot analysis the presence of the different forms at various times during synthesis of rRIF-γ in CHO cell cultures. The results (Fig. 5) show that in the first 4 h of culture, a predominant protein was produced with a Mₐ value which was significantly less than the products found after 18 h of incubation. Later, the degree of heterogeneity markedly increased and the additional forms showed higher molecular weights. These findings can be best explained by assuming that in the first 4 h, a major unglycosylated form is produced. Later on, this same polypeptide is modified by the addition of glycosyl groups before or during secretion. The different forms are presumably the result of heterogeneity of the sugar moieties.

DISCUSSION

Our initial aim was the isolation and characterization of MAbs to rRIF-γ for use in immunoaffinity chromatography and the study of the biological and physicochemical properties of RIF-γ. In practice, we have found it difficult to remove all traces of contaminating proteins from rRIF-γ preparations produced in CHO cell cultures. Conventional procedures such as salt fractionation, ion-exchange chromatography and gel permeation usually suffered from low protein recovery and often more than 90% of the biological activity was lost, which was paralleled by a significant decrease in molecular weight of the different molecular forms of rRIF-γ (see below). Because rRIF-γ preparations are extremely labile at pH values below 8 (Dijkema et al., 1985), all purification steps must be performed at pH > 8, which further limits the application of certain techniques and buffer systems. When such conventionally purified rRIF-γ preparations were subjected to SDS-PAGE, the Coomassie Brilliant Blue-stained gel revealed several bands with a prominent species at 18K (Fig. 6). A series of minor bands were visible in the 14K to 20K and 22K to 25K regions, and it was not certain whether these additional bands were contaminating proteins, degradation products of rRIF-γ or other forms of rRIF-γ such as have been found for human and mouse IFN-γ preparations (Rinderknecht et al., 1984; Gribaudo et al., 1985a). A strong indication that we were dealing with different forms of rRIF-γ came from gel permeation experiments. These experiments revealed that more than 95% of the applied activity of a crude rRIF-γ preparation was recovered at the 45K position, suggesting that rRIF-γ proteins form complexes with each other or with an unknown component. These active fractions were subjected to SDS-PAGE, revealing multiple bands between 14K and 20K, in accordance with the gel pattern obtained with rRIF-γ after a multistep conventional purification scheme (Fig. 6). Furthermore, Western blot analysis with a rabbit polyclonal antiserum raised against the main 18K protein species which had been electroeluted

bovine serum albumin (Mₐ, 67000, e.v. 15:5 ml); D, egg albumin (Mₐ, 45000, e.v. 16:7 ml); E, chymotrypsinogen A (Mₐ, 25000, e.v. 20:3 ml); F, proteinase K (Mₐ, 18500, e.v. 24:7 ml); G, lysozyme (Mₐ, 14500, e.v. 31:4 ml). (b) SDS-PAGE of crude rRIF-γ fractionated by gel permeation chromatography. One-half ml fractions from the TSK HW-50 column (a) containing more than 90% of the recovered IFN activity were lyophilized, resuspended in sample buffer and subjected to gel electrophoresis.
Fig. 5. Kinetics of rRIF-γ production in CHO cell cultures. Cell monolayers were grown in selective medium in a 2 l roller bottle (see Methods). After reaching confluence they were washed twice with HBSS and 50 ml serum-free DMEM without MTX was added (time zero). The bottle was rotated for 24 h at 37 °C in an atmosphere with 5% CO₂. (a) Samples were assayed periodically for IFN activity. (b) Western blot analysis of crude rRIF-γ obtained at various times during synthesis. At 4, 16 and 24 h samples were withdrawn and subjected to SDS–PAGE. Subsequently, the proteins were electrophoretically transferred to nitrocellulose sheets. The blots were incubated successively with MAb DB-2 and sheep anti-mouse total immunoglobulin–alkaline phosphatase conjugate.

from SDS–polyacrylamide gel slices, indicated that at least seven minor bands had antigenicity towards this antiserum. However, at that time it could not be excluded that tiny amounts of contaminating proteins in the gel-eluted material were responsible for these additional immunoreactive bands. Only MAbs would give us conclusive information on this. A number of previous studies have described MAbs to mouse and human IFN-γ (Gribaudo et al., 1985b; Schreiber et al., 1985; Liang et al., 1985; Le et al., 1984), but so far no MAbs against RIF-γ have been reported. This paper describes two MAbs specific for RIF-γ of which one (DB-1) was used for an immunosorbent affinity chromatography procedure, and the other (DB-2) for Western blot analysis. These MAbs were generated by the injection of recombinant DNA-derived RIF-γ into BALB/c mice. The MAb DB-1 immobilized to a Sepharose 4B column strongly adsorbed mouse and rat IFN-γ and also showed some affinity for human IFN-γ. Neither mouse nor rat IFN-γ activity could be eluted with 0.5 M NaCl, but it was readily eluted with 0.2 M phosphate buffer pH 12. The recovery was almost quantitative (> 98%). rRIF-γ protein thus obtained had a specific activity of 4 × 10⁶ laboratory units per mg protein, SDS–PAGE and Western blot analysis of affinity-purified rRIF-γ revealed a pattern of multiple bands similar to that found with the conventional purification scheme, confirming the existence of different forms of IFN-γ.
in CHO cell-derived preparations. The experiments in Fig. 5, showing that during synthesis of rRIF-γ the heterogeneity increases concomitantly with the molecular weight of the additional forms, and the fact that no activity loss was observed during purification by affinity chromatography make it likely that the heterogeneity originates from the addition of sugar residues to rRIF-γ protein before or during secretion into the culture medium and is not a consequence of proteolytic degradation process during synthesis and purification. However, at present it cannot be excluded that the observed heterogeneity is partly due to exopeptidase digestion during synthesis as suggested for mouse IFN-γ (Gribaudo et al., 1985a). More experiments are needed to clarify the mechanism which generates the heterogeneity of rRIF-γ.

At the onset of the preparation of monoclonal antibodies against rRIF-γ, it was expected that this protein injected into mice would be a weak immunogen because of the strong amino acid homology between MIF-γ and RIF-γ (Dijkema et al., 1985). Distinct epitope-specific sites on both proteins would be scarce and it was reasonable to expect that MAbs would be isolated which were directed against dissimilar structures. However, the two MAbs described in the present study are not only immunoreactive with RIF-γ but also show a high binding efficiency towards MIF-γ. The results of the immunoabsorbent experiments using DB-1 with the two animal IFN species give us no information about affinity differences between MIF-γ and RIF-γ, which might be indicative of structural differences. In order to trace possible differences in antigenic structure, we determined the specificity of DB-1 in a neutralization assay. In this assay it was found that the antiviral activity of each animal IFN species was neutralized to a different extent: MIF-γ was neutralized about four times more effectively than RIF-γ. These results can be explained by assuming that we are dealing with structural differences in binding sites, but it is
more likely that the difference is at least partly due to variation in the specific activity of RIF-γ and MIF-γ. Pure rRIF-γ has a specific activity of $3 \times 10^6$ to $4 \times 10^6$ units/mg protein based on a laboratory standard determined by a c.p.e. reduction assay. The MIF-γ interferon units were calibrated against a NIH mouse IFN-α/β international standard preparation and we do have strong indications that pure rMIF-γ derived from CHO cells has a specific activity of about $2 \times 10^7$ reference units per mg. Taking this last point into account, the results suggest that we are dealing with a structurally highly conserved epitope.

In the neutralization assay we also tested the potency of DB-2 towards mouse and rat IFN species. The neutralizing capacity of DB-2 also varied significantly between MIF-γ and RIF-γ, in favour of MIF-γ. The only difference found between DB-1 and DB-2 was that the latter is half as effective, which might be attributed to differences in the immunoglobulin content of the ascites preparations. These experiments suggest that both antibodies are associated with a region involved in eliciting the antiviral activity.

To evaluate the possibility that the epitopes recognized by DB-1 and DB-2 are (partially) identical, we performed competition experiments in which unlabelled MAb was assayed for its ability to block the binding of labelled MAb to rRIF-γ. These studies indicated that both antibodies bind close to, or at the same site on rRIF-γ.

Because of the high specificity of DB-1 and DB-2 towards MIF-γ, the results might indicate that the two B cell clones produce antibodies which specifically react with an antigenic determinant on a self protein. This would indicate that B cell clones directed against an epitope present on MIF-γ can be activated and recovered by somatic cell hybridization, demonstrating the existence of precursors of anti-MIF-γ reactive antibody producers in mice. However, this statement must be interpreted with caution, because virtually all autoantibodies are IgMs and have low binding affinities to their respective self-antigens (Dighiero et al., 1985). Moreover, the observed high specificity of these two antibodies is in contrast with the usually broad reactivity of autoantibodies towards different antigens.

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Purification of rat IFN-γ


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