20. Bon, S., Cartaud, J. & Massoulié, J. *Eur. J. Biochem.* **85**, 1–14 (1978).
21. McMahan, U. J., Sanes, J. R. & Marshall, L. M. *Nature* **271**, 172–174 (1978).
22. Lyles, J. M. & Barnard, E. A. *FEBS Lett.* **109**, 9–12 (1980).
23. Rieger, F., Koenig, J. & Vigny, M. *Devl Biol.* **76**, 358–365 (1980).
24. Ellman, C. L., Courtney, K. D., Andres, V. & Featherstone, R. M. *Biochem. Pharmac.* **7**, 88–95 (1961).
25. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. *J. biol. Chem.* **193**, 266–275 (1951).
26. Padykula, H. A. & Herman, F. *J. Histochem. Cytochem.* **3**, 170–195 (1955).
27. Guth, L. & Samaha, F. J. *Expl Neurol.* **25**, 138–152 (1969).
28. Koenig, J. & Rieger, F. *Devl Neurosci.* **4**, 249–257 (1981).

A rat monoclonal antibody against mouse α and β interferon of all molecular weight species

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Monoclonal antibodies have proven to be invaluable reagents for the characterization and purification of human interferons (IFN). Hybridoma lines secreting monospecific antibodies against human IFN-α and IFN-β have been isolated, and these antibodies have shown no cross-reactivity between the two interferons[1–4]. Murine interferons, when analysed by polyacrylamide gel electrophoresis, have two major migration patterns, one in the 28,000–35,000 (28–35 K) molecular weight (M_r) region and the second in the 22 K region[5–8]. Based on some homology of the N-terminal amino acid sequence with human interferons, the 28 K and 35 K species are classified as mouse IFN-β and the 22 K species as mouse IFN-α[9]. Minor species have also been described but their relationship to IFN-α and IFN-β is unknown[10,11]. We have now established a stable hybridoma line secreting rat antibodies to mouse virus-induced interferon. The monoclonal antibodies are of the IgG class, with binding activity to all the different M_r specied, as demonstrated in an enzyme-linked immunosorbent assay (ELISA) and by affinity chromatography. In addition, the antiviral activity of all the M_r species was neutralized by the antibody but the neutralizing activity was much weaker than the binding activity. This indicates the presence of a common antigenic site for all mouse α and β interferon species.

The interferon used for immunization was elicited with Newcastle disease virus (NDV) in monolayer cultures of Swiss mouse C-243 cells as described elsewhere[10]. Purification was performed by two-step affinity chromatography on poly(U)–Sepharose and anti-interferon antibody-coupled Affigel-10 columns. The antibody used for this purification consisted of the globulin fraction of serum from a goat immunized with NDV-induced C-243 cell mouse interferon. The specific activity of the purified interferon was 2.4×10^9 U per mg protein (all interferon units are expressed as international reference units) and its electrophoretic purity was assessed on 15% polyacrylamide slab gels in the presence of SDS in a Tris-glycine buffer system[8]. The biological activity of interferon preparations was determined by measuring the protection from the cytopathic effect of vesicular stomatitis virus on L929 cell monolayers.

An 8-week-old female rat of the LOU strain received 5×10^7 U of purified interferon in complete Freund's adjuvant subcutaneously (s.c.) and intraperitoneally (i.p.). This corresponds to 50 μg of interferon if calculated with a specific activity of 2.4×10^9 U (ref. 8). A second set of injections (s.c. and i.p.) with 2.5×10^7 U (25 μg) of purified interferon was given 3 weeks later in incomplete Freund's adjuvant. After 3 weeks the animal received 0.5×10^7 U (5 μg) of purified interferon i.p. on 3 consecutive days. The rat produced serum antibodies against interferon, with a neutralizing titre of 1/8,000 as measured against 4 interferon units. It was killed 2 days after the last injection and spleen and mesenteric lymph nodes were aseptically removed. The cells were fused with LOU-derived 210RCY3-Agl.2.3 rat myeloma cells[12] according to the protocol of Köhler and Milstein[13] modified by Fazekas *et al.*[14]. After fusion, the cells were cultured in Falcon Microtest plates at a density of 8×10^5 spleen cells per well on mouse peritoneal macrophage feeder layers in hypoxanthine–aminopterin–thymidine medium; 960 wells were seeded. Supernatants of growing hybridoma colonies were tested for binding activity using an ELISA. Microelisa plates (Dynatech, M 129B) coated with purified interferon were filled with 50 μl of hybridoma supernatant and kept at 37 °C for 30 min. After washing, horseradish peroxidase-conjugated rabbit anti-rat immunoglobulin preparations (Cappel Laboratories) were used as indicator antibody in 1 : 200 dilution in phosphate-buffered saline (PBS) containing 3% bovine serum albumin (BSA) and 1‰ Tween 20. After 30 min incubation at 37 °C the wells were washed and for the colour reaction, 100 μl of 25 μg *o*-phenylenediamine per ml of 10 mM phosphate buffer, *p*H containing 0.02% H_2O_2 (30%) were added. The reaction was terminated after 20–30 min with 50 μl of 3M HCl and absorption was read at 492 nm. Of the 960 wells seeded, 250 were positive for hybridoma growth, and the supernatants of 17 of these gave a positive ELISA reaction (twice the background reading), but only one (clone C5A) was found to be stable after two subclonings. Cells from wells containing supernatant active in ELISA were immediately subcloned by limiting dilution and supernatants from subclones tested. In this way a stable hybridoma line secreting monoclonal antibodies binding to electrophoretically pure interferon was established. The antibody belongs to the rat IgG class, as determined by immunoprecipitation, gel filtration and electrophoresis on 15% polyacrylamide gel in the presence of 0.1% SDS and 1% β-mercaptoethanol. The secretion of antibodies was checked regularly and the cell line found to be stable for over 8 months.

For mass production of antibodies, adult male rats (LOU) were injected i.p. with 2 ml of Pristan mineral oil and 1 week

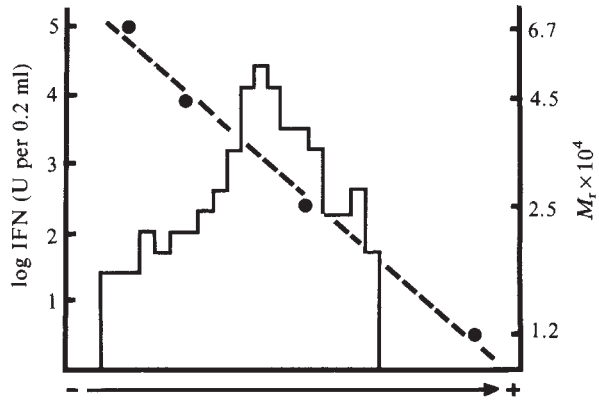

Fig. 1 Electrophoretic profile of interferon activity eluted from polyacrylamide gels after affinity chromatography on monoclonal antibodies fixed to Affigel-10 (Biorad). To prepare the column, ammonium sulphate-precipitated antibodies derived from 50 ml ascites fluid was coupled to the Affigel in 100 mM phosphate buffer, *p*H 7, at 4 °C. Free binding sites were saturated with ethanolamine and the column washed with a cycle of 100 mM phosphate buffer *p*H 7 and then with a cycle of 100 mM citrate buffer, *p*H 2.2. Interferon purified on poly(U)–Sepharose (7.7×10^5U) was applied to the column. No interferon activity was recovered in the flowthrough. After a washing step, the bound interferon was desorbed with citrate buffer, *p*H 2.2. The peak fractions were pooled, dialysed against 125 mM Tris buffer, *p*H 6.8, containing 1% SDS then concentrated on an Amicon PM10 membrane and boiled with 1% β-mercaptoethanol for analysis on a 15% polyacrylamide gel in the presence of 0.1% SDS. Molecular weight markers consisted of BSA (67,000), ovalbumin (45,000), chymotrypsinogen (25,000) and cytochrome *c* (12,500). For the determination of interferon activity, 2-mm slices were cut and eluted overnight at 4 °C.

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Table 1 Neutralization of the antiviral activity of the different M_r species of murine interferon

$M_r(\times 10^3)$	log IFN titre Control	+ Antibody
65	3.28	2.68
35	5.38	4.78
28	4.48	3.88
22	3.28	2.98
15	2.38	2.07
Unfractionated IFN	5.88	5.28

Interferon purified on poly(U)–Sepharose[8] was electrophoretically separated on 15% polyacrylamide gels in the presence of 0.1% SDS and 1% β-mercaptoethanol. 1-mm slices of the gel were eluted overnight in 0.2 ml of Tris-SDS 0.1%. The antiviral activity of the eluates was determined in the presence or absence of 2.5 μl of antibody-containing ascites fluid at each dilution of the samples and expressed as $\log_{10}$ of the interferon titre. Comparable results were obtained when the antibody was prepared from supernatants of hybridoma cultures. The results of four different neutralization experiments indicated no significant difference in the degree of neutralization of the various M_r species. Supernatants of myeloma cultures, concentrated and treated like the hybridoma supernatants, were without effect.

later with $5–10 \times 10^6$ hybridoma cells, also i.p. One to two weeks later ascites fluid was recovered from the peritoneal cavity and IgG purified by ammonium sulphate precipitation, sometimes followed by gel filtration on Sephacryl-S300. The interferon-binding activity in ELISA from the ascites fluid was 1,000-fold higher than that from culture supernatants.

When crude mouse interferon, consisting of a mixture of all M_r forms, was tested in the presence of purified monoclonal antibody, the antiviral activity was significantly reduced. However, the neutralizing titre was considerably lower than the binding titre, since an antibody preparation giving a positive ELISA reaction at a dilution of 1/12,800 completely neutralized 4 U of mouse interferon only at a dilution of 1/32, or lower.

Anti-interferon antibodies from culture supernatant were retained by protein A–Sepharose in binding conditions via the Fc part of the IgG molecule. Crude interferon was applied to the column after extensive washing, antigen–antibody complexes were desorbed. The elution pattern of interferon activity desorbed at *p*H 3, after electrophoresis on 15% polyacrylamide gel in the presence of SDS, shows that the protein A–Sepharose coated with the monoclonal antibodies retained both IFN-α (22 K) and IFN-β (28–35 K). Insufficient interferon was used in this experiment to allow detection of the 65 K and 15 K species. Additional evidence for binding to all interferon species was then obtained by affinity chromatography on monoclonal antibodies bound to Affigel-10. In these conditions, there was complete retention of NDV-induced C-243 interferon and the bound interferon could be desorbed by lowering the *p*H with 0.1 M Na-citrate buffer. Because the major α and β interferon species are present in about equivalent amounts in our crude C-243 cell interferon preparation, as shown by neutralization with anti-mouse IFN-β serum, total retention by the column is indicative of affinity for all M_r forms. This was confirmed by an electrophoretic analysis of the interferon desorbed from the column which showed that the major M_r forms were present in the desorbed material (Fig. 1). The minor 15 K species was not recovered from the gel, but affinity of the antibody for this species was demonstrated in the following experiments.

To determine the capacity of the antibody to neutralize all the different mouse interferon species, a preparation of NDV-induced C-243 interferon, prepared as described previously[10], was purified and concentrated on poly(U)–Sepharose. An aliquot of the peak fraction, with a titre of 2.4×10^7 U, was dialysed overnight against sample buffer (0.125 mM Tris *p*H 6.8, β-mercaptoethanol 1%, SDS 0.1%) and after boiling for 2 min, electrophoresed on a 15% polyacrylamide slab gel as described previously[8]. The gel was cut into 1-mm slices which were eluted overnight in 0.2 ml of Tris-SDS 0.1% per slice. Titration of the interferon activity of all fractions in the presence or absence of themonoclonal antibody revealed the presence of the two major peaks, one at 28–35 K (murine IFN-β) and one at 22 K (murine IFN-α) and the two minor peaks of 65 K and 15 K. The antiviral activity of all the fractions was reduced by a factor of two to four in the presence of the antiserum: the results for the peak fractions are summarized in Table 1. Affinity of the antibody for all the M_r forms was then confirmed in an ELISA assay, in which a 50-μl aliquot of each fraction was left to adsorb to Microelisa plates (Dynatech M129) overnight at room temperature. The reaction was then carried out as described above, using antibody obtained from ascites fluid. A positive reaction was scored in all the wells that had received the fractionated interferon, from 65 K to 15 K down.

All previously described monoclonal antibodies against human interferon show specificity for either IFN-α or IFN-β. Recently, a monoclonal rat antibody against mouse IFN-β has been obtained that did not bind mouse IFN-α[15]. Therefore, it is rather surprising that the first monoclonal antibody we obtained after immunizing a rat showed no specificity in binding to a particular mouse interferon species but instead binds to all of them. Antigenic differences between mouse IFN-α and IFN-β have been described by Yamamoto and Kawade[16], who immunized rabbits against the fast- and slow-moving components of NDV-induced mouse L-cell interferon. The antisera they obtained showed some cross-reactivity as measured by binding of interferon to immobilized antibody columns and the authors attributed this to possible cross-contamination of the interferons used for immunization.

The results with the monoclonal antibody presented here suggest that the antisera of Yamamoto and Kawade may well have contained cross-reacting antibodies. Tryptic digests of the high M_r species (40 K, corresponding to mouse IFN-β) and the low M_r species (24 K, corresponding to mouse IFN-α) of mouse L-cell interferon have revealed the presence of a common polypeptide structure[17] and regions of homology have also been found in the amino acid sequence of human α and β interferons[18]. As indicated by the results of the binding and neutralization experiments with the rat monoclonal antibody that we have obtained, at least one antigen site is shared by all M_r forms of mouse interferon. This may be a highly conserved region of some importance for the activity of the different interferon species.

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1. Secher, D. S. & Burke, D. C. *Nature* **285,** 446–450 (1980).
2. Hochkeppel, H. K., Menge, U. & Collins, J. *Nature* **291,** 500–501 (1981).
3. Montagnier, L., Laurent, A. G. & Gruest, J. *C.r. hebd. Séanc. Acad. Sci., Paris* D**291,** 893–896 (1980).
4. Staehelin, T. *et al. Proc. natn. Acad. Sci. U.S.A.* **78,** 1848–1852 (1981).
5. Stewart II, W. E., Legoff, S. & Wiranowska-Stewart, M. *J. gen. Virol.* **37,** 277–284 (1977).
6. Yamamoto, Y. & Kawade, Y. *J. gen. Virol.* **33,** 225–236 (1976).
7. Kawakita, M. *J. biol. Chem.* **253,** 598–602 (1978).
8. De Maeyer-Guignard, J., Tovey, M. G., Gresser, I. & De Maeyer, E. *Nature* **271,** 622–625 (1978).
9. Taira, H. *et al. Science* **207,** 528–530 (1980).
10. Cachard, A. & De Maeyer-Guignard, M. *Annl Virol. Inst. Pasteur* E **132,** 307–312 (1981).
11. Stewart II, W. E., Chudzio, T., Lin, L. S. & Wiranowska-Stewart, M. *Proc. natn. Acad. Sci. U.S.A.* **75,** 4814–4818 (1978).
12. Galfrè, G., Milstein, C. & Wright, B. *Nature* **277,** 131–133 (1979).
13. Köhler, G. & Milstein, C. *Nature* **256,** 495–497 (1975).
14. Fazekas de St Groth, S. & Scheidegger, D. *J. immun. Meth.* **35,** 1–21 (1980).
15. Trapman, J. *et al.* in *The Biology of the Interferon System* (eds De Maeyer, E., Galasso, G. & Schellekens, H.) 77–80 (Elsevier, Amsterdam, 1981).
16. Yamamoto, Y. & Kawade, Y. *Virology* **103,** 80–88 (1980).
17. Iwakura, Y., Yonehara, S. & Kawade, Y. *Biochem. biophys. Res. Commun.* **84,** 557–563 (1978).
18. Taniguchi, T. *et al. Nature* **285,** 547–549 (1980).