production and screening of cell hybrids producing a monoclonal antibody to human interferon-α

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(accepted 13 november 1980)

summary

mice and rats were immunized with human lymphoblastoid interferon, a mixture of human leukocyte (interferon-α) and human fibroblast (interferon-β) interferons, and their serum anti-interferon levels measured. anti-interferon activity was detected in all animals, although the mouse sera had higher titres than the rat sera. the spleens of two mice were removed and used to prepare hybrid myelomas. anti-interferon activity in tissue culture supernatants was measured using a direct neutralization assay and a clone (nk2) producing antibody to human interferon-α (leukocyte) was isolated. the anti-interferon activity of this monoclonal antibody was confirmed using a new indirect immunoprecipitation (iip) assay, which shows some advantages over the direct neutralization assay. the antibody did not neutralize human interferon-β (fibroblast) or mouse interferon.

introduction

two major classes of human type i interferons (ifns), ifn-α (leukocyte) and ifn-β (fibroblast) may be distinguished serologically and are the products of distinct structural genes (cavalieri et al., 1977; taniguchi et al., 1980). the raising of specific antisera to the interferons has been complicated by a lack of purified interferon for immunizing animals. this problem can now be overcome by using the technique of köhler & milstein (1975) to construct a hybrid myeloma producing a monoclonal antibody directed against interferon. this technique requires a suitably immunized mouse or rat and a good assay for screening the hybrid cells for the production of an anti-interferon antibody. for such a screen we chose to use a direct bioassay in which the interferon remaining un-neutralized after interaction with the immunoglobulin produced by the hybrid myeloma clone, was assayed for antiviral activity. this assay led to the isolation of a hybrid myeloma termed nk2, producing an antibody to human interferon-α (huifn-α, using the nomenclature described by stewart et al., 1980) (secher & burke, 1980). in this paper we describe the procedures used to immunize the animals with interferon and to screen for anti-interferon activity. we also describe some of the properties of the nk2 monoclonal antibody.

methods

interferon. the interferon used as immunogen for the first series of mice inoculations was human lymphoblastoid interferon [huifn-α (ly); stewart et al., 1980]. this was produced in namalwa cells by induction with sendai virus after pretreatment with sodium butyrate as described by baker et al. (1980). the interferon was then concentrated by precipitation with ammonium sulphate to yield a product which contained 2 × 10^3 reference research units/ml of interferon and had a specific activity of 5.3 × 10^3 reference research units/mg protein. this material was used as the immunogen for the first series of inoculations.
The immunogen for the second series of mice inoculations was partially purified HuIFN-α (Ly) (a generous gift from Drs K. Fantes and M. Johnston, Wellcome Laboratories, Beckenham, Kent). This material was prepared as described above and partially purified by immunochromatography. Two batches were used: the first, which was used for primary immunization (code 600/13), contained $7.2 \times 10^6$ reference research units/ml and had a specific activity of $2.4 \times 10^7$ units/mg protein; the second, used for the final intravenous inoculation (602/10), contained $6.0 \times 10^6$ reference research units/ml, and had a specific activity of $9.3 \times 10^7$ units/mg protein. These figures were supplied by Dr K. Fantes.

Interferon-β (fibroblast) was prepared for the precipitation experiment by superinduction of human diploid fibroblasts after induction with poly(rI).poly(rC) (Havell & Vilcek, 1972), and for the column experiment, by treatment of MG-63 cells (Billiau et al., 1977) with NDV strain F as described by Meager et al. (1979). The material made in MG-63 cells was partially purified by chromatography on Blue Sepharose CL-6B (Knight et al., 1980) to give a product containing $4 \times 10^4$ reference research units/ml with a specific activity of $4.5 \times 10^5$ units/mg protein. Mouse interferon was prepared by treatment of L cells with NDV strain F (Cooper et al., 1979). The product contained $2 \times 10^6$ reference research units/ml and had a specific activity of $10^5$ units/mg protein.

Immunization procedure. One group of six Balb/c mice (female, 18 to 22 g; Olac, ‘76’ Ltd.) and a group of three rats (one Lou, two BA × Lou) were injected. The mice were injected once every 2 weeks intraperitoneally with about $3 \times 10^4$ units interferon in 0.16 ml (first series) or once a week with about $10^6$ units interferon in 0.16 ml (second series) emulsified in complete Freund’s adjuvant, and then at 2-week intervals for up to 12 weeks with a similar dose of interferon in incomplete Freund’s adjuvant. The rats were injected in the footpads (intradermally) and in the hind leg (intramuscularly) or in multiple subcutaneous sites. About $10^6$ units emulsified in complete Freund’s adjuvant (total vol. 0.6 ml) was injected/rat at approx. 2-week intervals as indicated in Fig. 1 (a). The mice and rats were bled from the tail vein in order to follow the production of antibodies against interferon by incubating serial 0.5 log₁₀ dilutions of the serum with 10 units lymphoblastoid interferon at 37 °C for 1 h, before assay of the residual interferon titre as described below. The anti-interferon titre was defined as the reciprocal of that dilution of serum that reduced the interferon titre by 50%.

Cell fusion and growth of hybrid cells. The mouse with the highest anti-interferon titre in both series was injected intravenously with $1 \times 10^5$ units interferon (first series) or $2.2 \times 10^6$ units interferon (second series) and 4 days later the mouse was bled and the spleen removed for fusion with NS1 myeloma cells. Following fusion, cells were grown in 48 2 ml cultures (24-well Linbro plates, Flow Laboratories, no. 76-033-05) and hybrid cells were selected and cloned as previously described (Galfrè et al., 1977). One to 3 weeks after the fusion, growth of hybrid cells was seen in all 48 wells, and the culture supernatants were assayed for anti-interferon activity.

Interferon assay. Interferon was routinely assayed by the inhibition of nucleic acid synthesis (INAS) method (Atherton & Burke, 1975), in which the effect of interferon pre-treatment upon subsequent viral RNA synthesis is measured. The cells used for assay were bovine turbinate cells (a gift from Dr A. W. McClurkin, National Animal Disease Center, Ames, Iowa, U.S.A.), cat lung cells (a gift from Dr J. Desmyter, Rega Institute, Leuven, Belgium), EBTr cells (a gift from Dr J. Vilcek, New York University, N.Y., U.S.A.), several strains of human fibroblasts (GM 258, HEF-9 and HFF) all of which have been described previously (Meager et al., 1979), L6/1 rat cells, L-929 mouse cells and Madin–Derby bovine kidney (MDBK) cells. In some experiments interferon was also assayed by reduction in cytopathic effect (c.p.e.), yield reduction or plaque reduction assays (Finter, 1973). All human interferon titres are quoted in reference research units using the HuIFN-α reference research standard 69/19.
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Fig. 1. (a) Production of anti-interferon activity in three rats (▲, ●, ■) injected with lymphoblastoid interferon at different times after starting the injections. Interferon was injected at the times indicated by the arrows, and the antibody titre against α-interferon determined as described in Methods. (b) Production of anti-interferon activity in four mice (●, □, ▼, ◇) injected with lymphoblastoid interferon at 14-day intervals after starting injections.

Anti-interferon assay. Supernatant fluids from cell hybrids were assayed either directly for anti-interferon activity, or indirectly by an immunoprecipitation (IP) procedure. In the direct procedure, supernatant fluids (0.5 ml) were mixed with an equal volume of Dulbecco’s modified Eagle’s medium (DMEM), containing 20% foetal calf serum (Gibco-Biocult) containing about 2 units HuIFN-α (Ly). After incubation at 37 °C for 1 h, the fluids were assayed for residual interferon by the INAS assay in HFF cells, using 2 × 0.5 ml vol. for duplicate cultures in small glass vials (1 cm diam.). This dose of interferon produces a 50% depression of virus RNA synthesis; i.e. 1 HFF interferon unit is equivalent to 2 reference research units. When other cell systems were used for assay, the amount of interferon was adjusted to give 50% depression of virus RNA synthesis in those cells. Anti-interferon activity is shown by an increase in the level of 3H-uridine incorporation (due to virus RNA synthesis) above that due to interferon alone. Subsequently, an IP assay was developed, in which dilutions of supernatant fluid, mouse ascites fluid or IgG in PBS (10 µl) were incubated with about 720 units HuIFN-α (10 µl) at 37 °C for 5 h. Antigen–antibody complexes were then precipitated by the addition of 5 µl normal mouse serum as carrier, followed by 75 µl of sheep mouse immunoglobulin antiserum (slight antibody excess). After incubation at 37 °C for 30 min and at 4 °C for 16 h, the samples were centrifuged at 8000 g for 5 min to remove the antibody–antigen precipitate, and the supernatant was assayed for its residual interferon content by the INAS method in HFF cells.

RESULTS

Animal immunizations

Two groups of mice and one group of rats were immunized with two different preparations of lymphoblastoid interferon. Testing of the serum demonstrated that interferon was immunogenic in both species. In the rats there appeared to be a primary and then a short secondary response (Fig. 1 a). We were not able to boost the level of antibody after a period of rest and no rat has been used for fusion. The response of the mice varied from one animal to another, but the titres generally increased until a plateau was reached, which was little affected by further immunization. Fig. 1 (b) shows the response to the first series of injections. The antibody titres were similar in the second series. Considerable variation in response was seen between different mice. No difference was seen in the anti-interferon titres produced.
Table 1. Anti-interferon activity of supernatant fluids from other hybrid myelomas and dilutions of mouse anti-HuIFN-α (Ly) serum*

<table>
<thead>
<tr>
<th>Fluid</th>
<th>Virus RNA synthesis (ct/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Control</td>
<td>12258</td>
</tr>
<tr>
<td>(2) Interferon alone</td>
<td>5550</td>
</tr>
<tr>
<td>(3) Interferon + supernatant 1</td>
<td>5019</td>
</tr>
<tr>
<td>(4) Interferon + supernatant 2</td>
<td>5179</td>
</tr>
<tr>
<td>(5) Interferon + supernatant 3</td>
<td>4846</td>
</tr>
<tr>
<td>(6) Interferon + mouse serum 1/50</td>
<td>9678</td>
</tr>
<tr>
<td>(7) Interferon + mouse serum 1/500</td>
<td>9015</td>
</tr>
<tr>
<td>(8) Interferon + mouse serum 1/5000</td>
<td>7686</td>
</tr>
<tr>
<td>(9) Interferon + mouse serum 1/50000</td>
<td>7342</td>
</tr>
</tbody>
</table>

* Human lymphoblastoid interferon [HuIFN-α (Ly)] was tested for its capacity to reduce virus RNA synthesis (lines 1 and 2). It was also tested after mixing with the supernatants from three rat hybrid myelomas (lines 3 to 5), one producing antibody against human complement (YB2/42) (supernatant 1, Lachmann et al., 1980), one of unknown specificity (YM2/25) (supernatant 2, M. Spitz, B. W. Wright & C. Milstein, personal communication) and one against a mouse IgG (YA2/40) (supernatant 3, Galfré et al., 1979), or with different dilutions of the serum of the immune mouse whose spleen was used for the fusion (lines 6 to 9).

when either crude or partially purified interferon was used as the immunogen (data not shown).

Similar observations have been made by others in mice (Dalton et al., 1978). Spleen cells from the mouse with the highest serum anti-interferon titre in each group were used for fusion.

**Detection of anti-interferon activity produced by hybrid cells**

Anti-interferon activity was initially screened by using the direct method. The test system was first checked for its ability to detect small amounts of anti-interferon by assaying a series of coded samples which were supernatants from other cell fusions, using spleen cells from mice injected with irrelevant antigens. Alternatively, dilutions of the serum from the mouse used for the fusion were used. The results (Table 1) indicate that the effect of dilutions of up to 1/5000 of the mouse serum could be detected by the increase in 3H-uridine incorporation. The irrelevant supernatant fluids had a small effect upon the assay, apparently increasing the antiviral effect of the interferon, possibly because of a non-specific effect on virus RNA synthesis. When the supernatant fluids from the hybrid cells obtained from the mice which had anti-interferon antibodies were tested, several cultures from both series showed low levels of activity. In the assays from the second series, only one culture (well no. 13) was reproducibly active when culture fluids harvested over a period of 2 weeks were assayed (Fig. 2). Even with this culture, the effect was very small, the radioactivity being increased by no more than 3% of the virus controls. Some cultures (e.g. well no. 6) showed a reduction in incorporation of radioactivity below that due to interferon alone, probably due to substances in the medium which non-specifically inhibited virus RNA synthesis. To eliminate this effect, all radioactivity in subsequent assays was expressed as a percentage of the corresponding virus control, i.e. the radioactivity after incubation of a mixture of culture fluid and fresh medium rather than a mixture of interferon and fresh medium. When the cells from the cultures which showed low levels of anti-interferon activity were cloned in soft agar, a number of clones were isolated which showed anti-interferon activity. This activity was shown against human interferon-α (leukocyte) when assayed on either human or bovine turbinate cells, and also when virus synthesis was measured by either c.p.e., yield or plaque formation as well as by the routine INAS method. The culture fluids had no effect on virus yield in mouse or rat cells or on the effect of mouse or rat interferon in the homologous cells. However, the majority of the clones showing this apparent anti-interferon activity did not secrete detectable amounts of IgG and it was concluded that
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Fig. 2. Screening of cell culture supernatant fluids for anti-interferon activity by the direct method. Culture fluid was mixed with about 2 reference units of lymphoblastoid interferon added to HFF cells which, after overnight incubation, were challenged with Semliki Forest virus. Virus growth was measured as $^3$H-uridine incorporation into intracellular virus RNA. The incorporation in the presence of interferon alone is shown by the dotted line, and the incorporation in the absence of interferon (the virus control) was double this value. The lines show the effect on virus RNA synthesis of mixing with cell culture fluids harvested 13 days (top panel), 16 days (middle panel) and 20 days (bottom panel) after the fusion.

the anti-interferon effect was not always due to anti-interferon antibody but sometimes to some other unidentified factor. The clones obtained from the first fusion were abandoned since none of the clones which showed anti-interferon actually released IgG, and while the same effect was shown by clones obtained from the second fusion, use of the reverse
plaque assay to test for the presence of secreted mouse immunoglobulin made it possible to identify those clones secreting immunoglobulin. In this way, a clone from well 13, clone 35, subclone 6 (clone 13.35.6) referred to as NK2, was isolated (Secher & Burke, 1980). The NK2 cells were incubated in medium containing $^{14}$C-lysine, and the culture supernatant analysed by SDS-polyacrylamide gel electrophoresis (Cotton et al., 1973). The autoradiograph of the dried gel indicated that the major product of NK2 cells was an immunoglobulin whose heavy chain had the mobility of a $\gamma$-chain and whose light chain had a mobility slightly greater than that of X63 light chain (Fig. 3). No trace of the X63 light chain produced by the NS1 myeloma parent was detected in the NK2 supernatant.

**Anti-interferon activity of clone NK2**

The cells of well 13 from the second fusion were cloned twice to yield a clone secreting anti-interferon activity. This activity was also shown by the IgG secreted by the clone, and was readily detected by the IIP anti-interferon assay (Secher & Burke, 1980). When the extent of neutralization, measured as percentage of the added interferon that remained in the supernatant fluid, was plotted against the immunoglobulin concentration the neutraliza-
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Fig. 4. Neutralization of IFN-α by NK2 IgG as measured by either the direct anti-interferon assay (○) or the indirect immunoprecipitation assay (●). The interferon remaining in the supernatant after neutralization is shown as a percentage of the original titre.

Fig. 5. Neutralization of IFN-α by NK2 IgG (▲) or by W6/32 IgG (●), and of IFN-β by NK2 IgG (●) or by W6/32 IgG (○). W6/32 is an IgG directed against a human HLA antigen produced by another hybrid cell (Barnstable et al., 1978).

Specificity of the antibody

Human interferon-α (leukocyte) and human interferon-β (fibroblast) are readily distinguishable antigenically. It was therefore important to determine whether this monoclonal antibody showed any activity against HuIFN-β. A precipitation experiment in which different concentrations of NK2 IgG were incubated with preparations of the HuIFN-α and HuIFN-β showed that the antibody had little effect on the activity of human interferon-β preparation which had been purified to remove all human interferon-α, except at the highest IgG concentration used (500 μg/ml), suggesting comparatively weak neutralization of interferon-β. Using the same conditions, the antibody was very effective against the HuIFN-α (Fig. 5). A similar result was obtained by use of an affinity column consisting of the NK2 IgG attached to a Sepharose support: 60% of the interferon-β passed straight through the column and only 0.6% was retained and then eluted by the pH 2 buffer, using conditions known to be effective for HuIFN-α. Mouse interferon consists mainly of interferon-β plus a small amount (about 1%) of an interferon-α which cross-reacts with human α-interferon (Stewart & Havell, 1980). It was therefore not surprising that the antibody column failed to retain any mouse interferon (about $3 \times 10^{-4}$%).
DISCUSSION

Work described in this and a previous paper (Secher & Burke, 1980) has resulted in the isolation of a hybrid myeloma making an antibody directed against human interferon. The initial detection of this hybrid cell was made by determining the interferon activity remaining after incubation of lymphoblastoid interferon with the products secreted by a large number of clones. However, spurious effects in the assay and the low neutralizing effect of the monoclonal antibody to interferon made choice of the appropriate clones difficult, and it was only through continued assessment of a number of potentially positive clones that a correct choice could be made. This choice was confirmed when the indirect assay was developed. This assay could also have been used for the initial screening, as shown by the results of assays of a number of hybrid cell supernatant fluids which had been stored at -20 °C. The screening assay conditions were chosen so as to make the screen as sensitive as possible: the test fluids were incubated with a small amount of interferon before assay on sensitive cells using an assay where it was easy to detect small differences in interferon titre. It is not surprising that, under these conditions, a number of false positives (clones releasing an anti-interferon activity which was not IgG for example) were detected. The assay could have set up with a much lower limit of sensitivity, e.g. the ability to completely neutralize 10 units interferon. Such a screen would probably not have produced false positive clones, but would probably have failed to detect the clone (NK2) that was ultimately isolated.

So far, only one hybrid myeloma has been isolated that produces antibody-neutralizing leukocyte interferon activity, although other clones present may have remained undetected by the screening procedure used.

The NK2 antibody is an important advance over antibodies produced by conventional methods. The latter, while strongly neutralizing interferon activity, comprise a range of immunoglobulin molecules directed against various antigenic sites on the interferon molecule. They also contain antibodies to the impurities present in the interferon preparations used to immunize the animals, and this has limited their use in interferon purification. The monoclonal antibody, on the other hand, while not showing a high neutralizing titre against the biological activity of interferon, has many advantages because of its specificity and the ease of production of large amounts of antibody in mice injected with the hybrid myeloma.

The monoclonal antibody produced by the hybrid myeloma has already been used to purify interferon-α (Secher & Burke, 1980) and to develop a radioimmunoassay for interferon (D. Secher, unpublished results). The antibody appears to be specific for interferon-α; there was little reaction with human interferon-β, and none with mouse interferon, which is mainly a β-interferon. Thus, although both human interferons α and β protect human cells, and these both interact with a common or similar receptor on the cell surface, this common site is not involved in the antigenic site recognized by the NK2 antibody. Finally, the monoclonal antibody will allow the establishment of new and reliable interferon standards, and could itself be used as a standard antibody preparation for calibrating other anti-interferons.

We thank Dr C. Milstein for encouragement and advice and J. Flint, H. E. Graves, R. King, L. T. Davies, D. P. Wright and P. Gregory for technical assistance, the Medical Research Council for financial support and Drs K. Fantes and M. Johnston, the Wellcome Laboratories, Beckenham, Kent for a generous gift of partially purified lymphoblastoid interferon for immunization.

REFERENCES


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(Received 18 August 1980)