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MURINE MONOCLONAL ANTIBODIES AGAINST HUMAN FIBROBLAST (BETA) INTERFERON. CORRELATION OF THE NEUTRALIZATION OF ANTIVIRAL AND ANTIPROLIFERATIVE ACTIVITIES

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Summary. — Eight mouse hybridoma lines secreting monoclonal antibodies (MoAb) to human fibroblast interferon (HuIFN-beta) were established. All MoAbs were capable of neutralizing two different biological activities of HuIFN-beta: the antiviral activity and the antiproliferative activity. Positive correlation was demonstrated between the ability of hybridoma culture supernatants to neutralize the antiviral and antiproliferative effects of human fibroblast IFN. The neutralizing capacity of individual hybridoma culture supernatants depended on the concentration of MoAb in the sample. Only one of IFN-neutralizing MoAbs has shown binding capacity to human fibroblast IFN when used in enzyme immunoassay as solid-phase antigen. This MoAb was purified to homogeneity and its specific neutralizing activity against HuIFN-beta was calculated (3.5×10^4 I.U. per mg).

Key words: *monoclonal antibodies*; *human fibroblast interferon*; *antiviral and antiproliferative activity*

Introduction

Interferons (IFN) comprise a family of closely related naturally occurring proteins possessing antiviral, antiproliferative and various other biological activities (Stewart, 1979). Monoclonal antibodies (MoAb) proved to be effective in characterization, quantitation, and purification of various IFN types and subtypes which are available in partially purified form. Of three major types of human IFN (alpha, beta, and gamma) — "fibroblast" or beta IFN seems to be less frequently studied and used in therapeutic trials than the other two IFN-types. Also papers dealing with preparation of monoclonal antibodies against human IFN-beta are more seldom than the studies about generation of MoAbs to human IFN alpha or gamma.

In this paper we report on the preparation and characterization of a set of murine hybridomas secreting antibodies that were able to neutralize two basic biological effects of human fibroblast (beta) IFN, the antiviral and the antiproliferative action.

Materials and Methods

Interferon. Purified natural Human Fibroblast Interferon (BM 532) Lot No. L-0631, with a specific activity of 10^7 I.U./mg protein was kindly provided by Toray Ind. (Japan). This IFN was used for immunization and for screening of hybridomas.

Immunization. Human fibroblast IFN was emulsified in Freund's complete adjuvans and four-week-old female BALB/c mice (Institute of Virology, Bratislava) were immunized intraperitoneally (i.p.) with 2.5×10^5 units per animal. Four months later, one of the mice was boosted by i.p. route with 1.5×10^5 units of fibroblast IFN in buffered saline. Three days after the second immunization, the boosted mouse was bled and then sacrificed by cervical dislocation. Spleen was removed and the separated spleen cells were used for hybridoma preparation.

Preparation of hybridomas. The spleen lymphocytes from immunized mouse were fused with the mouse NSO line myeloma cells, at a 5 : 1 ratio, in polyethylene glycol 1550 (Serva), as described previously (Kontsek *et al.*, 1988). The cell suspension was distributed in 0.2 ml aliquots into 96-well microtitre plates (Koh-i-Noor, Hardtmuth, Czechoslovakia) at a concentration of 2.5×10^5 spleen cells per well. For selection of hybridomas, Dulbecco's modified Eagle's medium (Gibco, high glucose) supplemented with 0.1 mmol/l hypoxanthine (Sigma), 0.004 mmol/l aminopterine (Sigma), 0.016 mmol/l thymidine (Sigma), then with 2 mmol/l glutamine, 10 mg/l gentamycine, and 15% horse serum was used. Plated cells were grown at 37 °C in a humidified incubator with 5% CO_2 atmosphere for 14 days and then the culture supernatants from wells containing hybridomas were collected for screening.

Hybridomas from positive wells were cloned and recloned using the limiting dilution method on a feeder layer from BALB/c mouse spleen cells prepared 48 hr in advance.

IFN assay and neutralization test. The antiviral activity of human fibroblast IFN was determined by inhibition of the cytopathic effect (CPE) of vesicular stomatitis virus (VSV) on primary human fibroblasts in microtitre plates (Hardtmuth, Koh-i-Noor). The assay was calibrated against the NIH human fibroblast IFN reference standard G-023-901-527.

For screening of the neutralization activity of MoAbs, three 0.05 ml aliquots of hybridoma culture supernatants were incubated with serialy diluted fibroblast IFN (4—2—1 unit per 0.05 ml) in microtitre plates for 2 hr at 37 °C. The test mixture (0.1 ml) was transferred onto monolayers of primary human fibroblasts (3×10^4 cells per 0.1 ml) and 24 hr later the cells were challenged with 100 $TCID_{50}$ (50% tissue culture infectious dose) of VSV. The extent of virus-induced CPE was scored visually after another 40 hr.

Inhibition assay of the antiproliferative IFN activity. Screening and selection of MoAbs which inhibited the anti-proliferative activity of IFN was described previously (Kontsek *et al.*, 1988). The hybridoma culture supernatant (0.05 ml) was mixed with 0.05 ml of human fibroblast IFN

Table 1. Characterization of hybridoma lines secreting monoclonal antibodies to human fibroblast interferon

No.	Hybridoma line	Chromosome number[a]	Immunoglobulin type[b] secreted
1	Beta 1	83.8	G 1, k
2	Beta 6	76.8	G 2a, k
3	Beta 9	67.6	G 1, k
4	Beta 13	69.2	G 1, k
5	Beta 21	74.7	G 1, k
6	Beta 22	81.0	G 1, k
7	Beta 25	66.2	G 1, k
8	Beta 28	66.2	G 1, k

[a] — average chromosome number from twenty mitoses analysed at each clone. Sum of parental chromosome numbers — 82 (NSO cell 42, B cell 40).

[b] — mouse immunoglobulin type determined by enzyme immunoassay.

Table 2. Neutralizing capacity of hybridoma culture supernatants against human fibroblast interferon

Hybridoma culture supernatant	Titre of MoAb in the culture supernatant[a]	Neutralization of IFN-activity	
		Antiviral[b] (IFN-units)	Antiproliferative[c] (% of control cells)
Beta 1	128	4	82%
Beta 6	256	8	90%
Beta 9	512	16	95%
Beta 13	256–512	16	92%
Beta 21	1024	16	100%
Beta 22	32	16	98%
Beta 25	1024	16	96%
Beta 28	256	8	89%
A8[d]	512	0	73%
NSO[d]	0	0	72%

a — Immunoglobulin concentration determined by enzyme immunoassay (ELISA)
b — Units of HuIFN-beta in 0.05 ml neutralized by mixing with 0.05 ml of culture supernatant
c — Cell growth (in % from control IFN-untreated cells) after mixing 0.05 ml of IFN (100 units per ml) with 0.05 ml of hybridoma culture supernatant. Data are the mean of triplicate wells
d — Negative control: NSO — Ig nonsecreting myeloma cells
A8 — anti-HuIFN gamma MoAb secreting hybridomas

(100 units per ml) in a microtitre plate and immediately 2×10^4 of human HL 60 cells were added in 0.1 ml. The plates were incubated for 72 hr in 6% CO_2 atmosphere at 37 °C and the final cell concentration per well was determined microscopically in a Bürker-chamber. Each supernatant was assayed in triplicate and the cell viability was determined by trypan blue exclusion.

Determination of the immunoglobulin types. Mouse immunoglobulin (Ig) types of MoAbs in the hybridoma culture supernatants were determined by usual sandwich enzyme immunoassay (EIA) using swine antimouse Ig and anti-isotype sera against mouse Ig conjugated with horse-radish peroxidase (Serotec).

Quantitation of MoAbs in the culture supernatants. The antibody concentrations in these preparations were measured by an EIA. The plastic wells were coated with swine antimouse Ig (Sevac); a commercial peroxidase-conjugated swine antimouse Ig was used for the detection of MoAb.

Chromosomal analysis. The method of Rothfels and Siminovitch (1958) was utilized for karyological analysis of hybridomas. Twenty mitoses were evaluated in each analysed clone.

Purification of MoAb from ascitic fluid. Hybridoma clone Beta 6 was grown in ascitic fluid BALB/c mice. The IgG (G2a subclass) was precipitated with ammonium sulphate at 50% saturation and purified by Protein A-Sepharose column chromatography. The protein concentration in the preparation (IgG contents more than 95%) was determined by the Lowry method.

Results and Discussion

To obtain hybridomas secreting MoAb to human fibroblast IFN (HuIFN-beta) we used spleen cells from a mouse with serum antibodies which neutralized 8 units of fibroblast IFN in dilution 1 : 320 and which had an antiproliferative action against 100 units/ml of fibroblast IFN in dilution 1 : 50.

Following fusion, suspension of 2×10^8 spleen cells and 4×10^7 NSO cells was distributed into eight 96-well plates (768 wells seeded) and two weeks later the growth of hybridomas was observed in 281 wells (37%). At primary screening, hybridoma culture supernatants from 15 wells were shown to neutralize the antiviral activity of at least 1 unit of human fibroblast IFN. Cells from the positive wells were selected for further growth and characterization. After cloning and recloning procedure, eight stable hybridoma cell lines were established (Table 1). The hybrid nature of selected clones was confirmed by analysis of metaphase chromosomes. The clones had a significantly higher average chromosome number than any parental cells (spleen B-cells-40 and NSO cells 42 chromosomes), but the average chromosome number was 5 to 20% lower than the sum of the numbers of chromosomes in the parental cells. These findings confirmed our previous results of karyological analyses (Kontsek *et al.*, 1988*a*). Determination of mouse Ig isotype by EIA has sown that all eight MoAbs were IgGs with heavy chains G1, G2a, and with $\varkappa$-type light chains (Table 1).

Culture supernatants of hybridomas secreting anti-IFN MoAbs were collected for further characterization. The neutralizing capacity of these supernatants against two basic (antiviral and antiproliferative) activities of human fibroblast IFN are shown in Table 2 and Fig. 1. The tested supernatants (0.05 ml) were able to inhibit the antiviral activity of 2—16 units of fibroblast IFN. The same medium samples were assayed for antiprolifer-

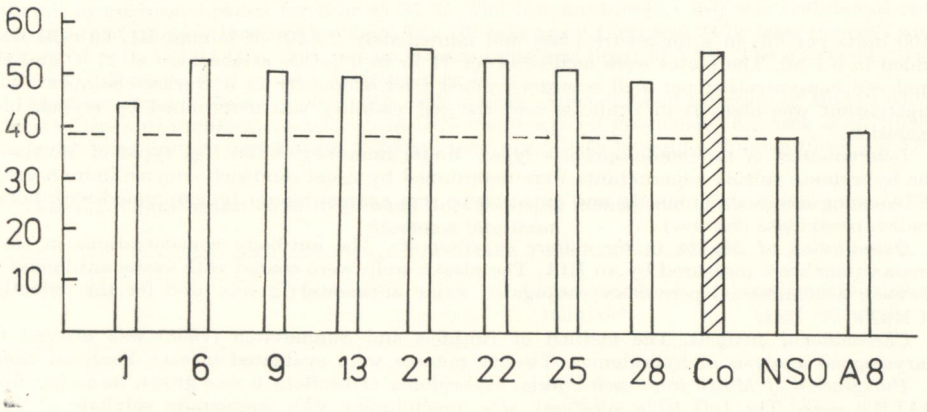

Fig. 1.

Neutralization of antiproliferative activity (cell growth inhibition) of human fibroblast IFN by MoAbs

Human HL-60 cells (2×10^4) were seeded in microculture wells containing either the medium alone (control), the medium containing 100 U/ml of HuIFN-beta (negative control: NSO — Ig nonsecreting myelomas, A8 — anti-HuIFN-gamma MoAb), or, the medium containing 100 U/ml of HuIFN-beta mixed with MoAbs from different Beta clones. Three days later, the cell number was determined.

Abscissa: supernatants from different Beta clones; ordinate: cell number ($\times 10^3$ cells per ml). Co = control.

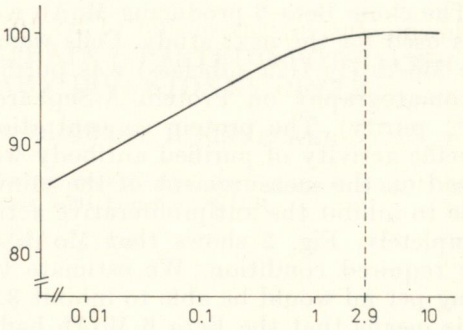

Fig. 2.

Neutralization of antiproliferative activity of HuIFN-beta at various concentrations of purified MoAb Beta 6

Human HL-60 cells (2×10^4) were seeded in microculture wells containing either the medium only (control), or the medium containing 100 U/ml of HuIFN-beta and mixed with various concentrations of MoAb Beta 6. Three days later, the cell number was counted in wells. Abscissa: concentration of MoAb (μg/ml); ordinate: cell number (% control).

ative activity of HuIFN-beta. In the assay, HL-60 cells (2×10^4 per well) were added to the mixture of hybridoma culture supernatants together with 100 units/ml of fibroblast IFN and incubated for 72 hr. Subsequently, the inhibition of cell proliferation was evaluated (Fig. 1). Culture supernatants from Ig-nonsecreting NSO cells and anti-HuIFN-gamma MoAb producing cells (A8) were included as negative controls. Table 2 and Fig. 1 show that negative controls did not inhibit the antiproliferative activity of IFN-beta which reduced the cell number in wells to 72—73 % ($38.3 \times 10^3 - 38.9 \times 10^3$ cells per ml) of IFN-non-treated culture (54×10^3 cells per ml). On the other hand, samples containing anti IFN-beta MoAb reduced or completely inhibited the antiproliferative activity of IFN and the cell growth reached 83—102 % ($44.1 - 54.4 \times 10^3$ cells per ml) of IFN-untreated microcultures.

A strong positive correlation was found between the ability of hybridoma culture supernatants to neutralize the antiviral and the antiproliferative activities of human fibroblast IFN. Culture supernatants with higher neutralizing antiviral activity were more efficient in inhibition of antiproliferative activity. The same relationship in characterization of neutralizing ability of anti-HuIFN-gamma MoAb was observed previously (Kontsek *et* al., 1988). The differences in the neutralizing capacity of culture supernatants seemed not be based on an inherent potency of secreted MoAb but, more probably, they reflect the variability of concentration of antibodies in the assayed samples. Data in Table 2 indicate a strong dependence of the biological activity of culture supernatants on immunoglobulin concentration (determined by EIA). The only exception, however, was Beta 22, probably reflecting the later consequence of genetic instability of this clone.

Neutralizing MoAbs were also tested in solid-phase immunoassay (data not shown). Plates were coated with fibroblast IFN (0.05 ml per well, 10^4 units per ml with specific activity 10^7 units per mg protein) and hybridoma culture supernatants were added to the wells. Only the MoAb (secreted by clone Beta 6) was found to be positive in this assay. Our results are in agreement with those of Novick *et al.* (1983) who also found that IFN-beta neutralizing MoAbs did not give satisfactory binding in solid-phase immunoassay.

The clone Beta 6 producing MoAb with neutralizing and binding activity was used for the next study. Cells were grown in BALB/c mice ascites and the MoAb (Ig G2a subclass) was purified from the ascitic fluid by affinity chromatography on Protein A-Sepharose to homogeneity (i.e. more than 95% purity). The protein concentration of this preparation and then the specific activity of purified antibody were determined. The calculation was based on the measurement of the minimal concentration of Ig, which was able to inhibit the antiproliferative activity of 100 units/ml of HuIFN-beta completely. Fig. 2 shows that MoAb at concentration of 2.9 μg/ml fulfil the required condition. We estimate that Beta 6 MoAb at concentration 1 mg per ml would be able to inhibit 3.5×10^4 units of HuIFN-beta per ml. This means that the Beta 6 MoAb had the specific neutralizing activity of 3.5×10^4 HuIFN-beta units per mg of protein. A detailed analysis of the specificity of this MoAb is beyond scope of this report and will be presented elsewhere.

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