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MONOCLONAL ANTIBODIES NEUTRALIZING THE ANTIVIRAL AND THE ANTIPROLIFERATIVE ACTIVITIES OF HUMAN INTERFERON GAMMA

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Summary. — Spleen cells from BALB/c mice immunized with human leukocyte interferon gamma (HuIFN-gamma) were fused with mouse NSO myeloma cells. Nine hybridoma lines were found secreting monoclonal antibodies (MoAb) which were able to neutralize the antiviral activity of HuIFN-gamma. All nine MoAb inhibited also the antiproliferative activity of HuIFN-gamma. The ability of tested MoAb to inhibit both the antiviral and antiproliferative activities of HuIFN-gamma supports the suggestion that a common domain on HuIFN-gamma molecule might be responsible for both biological activities. However, ELISA and/or RIA proved unsuitable for detection of these specific MoAb.

Key words: monoclonal antibodies; human interferon gamma; antiviral and antiproliferative activities

Introduction

Interferon (IFN) comprises a family of proteins exerting a broad spectrum of biological activities on both normal and transformed cells of various origin (Stewart, 1979). Among the three major types of IFN (alpha, beta, gamma), IFN-gamma have been reported to have the strongest biological action on human tumour cells (Rubin and Gupta, 1980). It has marked antiviral and antiproliferative effects (Crane *et al.*, 1978) together with the high immunomodulating activity, different from other IFN types (Dolei *et al.*, 1983; Friedman and Vogel, 1983). Much progress has recently been made in the study of HuIFN-gamma, and MoAb have proved extremely useful for unambiguous identification, characterization and purification of the HuIFN-gamma molecule (Oleszak *et al.*, 1983; Sedmak *et al.*, 1985; Feit *et al.*, 1986; Miyata *et al.*, 1986).

We report here generation and characterization of several stable mouse hybridoma lines secreting MoAb with the ability to inhibit the antiviral and antiproliferative effects of natural human leukocyte IFN-gamma.

Materials and Methods

Interferon. Purified human leukocyte IFN-gamma (HuIFN-gamma a gift of Dr. H. L. Kayquinen from the Finnish Red Cross Blood Transfusion Service, Helsinki, Finland, Lot G-8510) was used for immunization of mice and for primary screening of hybridomas.

Immunization. Eight-week-old female BALB/c mice (Institute of Virology, Bratislava) were used. Mice were injected intraperitoneally with $2.5 \cdot 10^5$ units of HuIFN-gamma emulsified with Freund's complete adjuvants and 7 weeks later, one of the mice was boosted intraperitoneally with 10^5 units of HuIFN-gamma in buffered saline. After further three days the mouse was sacrificed by cervical dislocation and the spleen was removed and used for hybridoma preparation.

Cell lines. Mouse myeloma cell line NSO, the immunoglobulin non-secreting variant NS-1, was used for fusion. The cells were propagated in Dulbecco's modified minimal Eagle medium (DMEM, Sigma) containing 15 % horse serum (Elan clone, Czechoslovakia), 2 mmol/l L-glutamine (ÚSOL), 4.5 g/l glucose (Spofa), 400 units/l insuline (Spofa), 0.029 mmol/l 2-mercaptoethanol (Serva), 50 mg/l gentamycine (M. A. Bioproducts). This medium was designated as growth medium (GM).

Human cell line L-41 was granted by dr. A. Duks from Institute of Microbiology, Latvian Academy of Sciences, Riga, U.S.S.R. Cells were maintained in Eagle's minimal medium (MEM, ÚSOL) supplemented with 10 % calf serum, 2 mmol/l l-glutamine and 50 mg/l gentamycine, and used for antiviral IFN-assay.

Human cell line HL-60 was cultivated in RPMI-1640 (Serva) supplemented with 10 % horse serum, 2 mmol/l l-glutamine and 50 mg/l gentamycine. This cells have the appearance of promyelocyte cells and were used for antiproliferative IFN-assay.

Production of hybridomas. Spleen cells ($2 \cdot 10^8$) were mixed with myeloma cells ($4 \cdot 10^7$) and fused in 1 ml of 50 % polyethylene glycol (PEG, MW 1550, Serva) prepared in DMEM supplemented with 0.015 ml dimethylsulfoxide (Koch Light) and 0.1 ml of 7.5 % $NaHCO_3$ (ÚSOL) according the method of Fazekas de St. Growth and Scheidegger (1980). After 90 sec at room temperature, the PEG was slowly diluted with 15 ml of DMEM. The cells were then spun at 500 g for 10 min. The pellet was resuspended in GM supplemented with 0.1 mmol/l hypoxanthine (Sigma), 0.004 mmol/l aminopterine (Sigma) and 0.016 mmol/l thymidine (Sigma).

The treated cell suspension was plated in 0.2 ml amounts into 96-well microculture plates (Koh-i-noor, Hardtmuth) at concentration of $2 \cdot 10^5$ spleen cells per well. The plates were incubated at 37 °C in a humidified 6 % CO_2 atmosphere for 14 days and then culture supernatants from wells containing hybridomas were collected for screening.

Assay of antiviral interferon activity. Antiviral activity of HuIFN-gamma was assayed by measuring the inhibition of cytopathic effect (CPE) caused by encephalomyocarditis virus (EMC) in monolayers of human L-41 cells cultured in 96-well tissue culture plates. Wells with appropriately diluted IFN samples (0.1 ml per well) were seeded with $3 \cdot 10^4$ of L-41 cells in 0.1 ml of MEM with 10 % calf serum (CS). Following overnight incubation, the medium was replaced by 0.1 ml MEM + 10 % CS containing 100-1000 $TCID_{50}$ (50 % tissue culture infectious dose) of EMC and CPE was observed after the next 40 hr incubation. The HuIFN-gamma concentration inducing 50 % protection of CPE on L-41 cells corresponded approximately to 1 unit of NIH HuIFN-gamma standard Gg 23-901-530.

Primary screening by IFN neutralization assay. 0.15 ml of hybridoma culture supernatant was distributed in 0.05 ml aliquots into three wells of a microtitre plate and 0.05 ml of MEM containing 8, 4, 2 units of serialy diluted HuIFN-gamma was added gradually. The test mixture was incubated at 37 °C for 1 hr and then $3 \cdot 10^4$ L-41 cells were added to wells as in usual IFN assay. By 24 hr later the cells were challenged with EMC and were scored visualy for virus-induced CPE after another 40 hr.

Test for inhibition of antiproliferative activity of interferon. The ability of MoAb to inhibit the antiproliferative activity of HuIFN-gamma was determined on human HL-60 cells. The test was performed in 96-well plates. Briefly: 0.05 ml of hybridoma culture supernatant was mixed with 0.05 ml of RPMI-1640 containing 5 units of HuIFN-gamma and immediately $5 \cdot 10^4$ HL-60 cells in 0.1 ml were added to each well. Plates were incubated for 72 hr in 6 % CO_2 atmosphere at 37 °C and the cell concentrations per well were determined microscopically using Bürker chamber for enumeration. Each MoAb and IFN mixture was plated in triplicate. Culture supernatants from NSO and from clone 1 B (producing MoAb against human serum albumine) were used as control. Cell viability was determined by trypan blue exclusion.

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

No.	Hybridoma line	Chromosome number[a]	Ig type[b]	Neutralization of HuIFN-gamma antiviral activity[c] (IFN-units)	antiproliferative activity[d] (% from untreated cells)
1.	A6	71.6	G2a, k	4	78
2.	A8	75.6	G1, k	8	90
3.	A11	74.2	M, k	16	102
4.	B13	66.6	G3, k	16	103
5.	C5	102.0	G3, k	16	105
6.	C10	74.1	G1, k	8	83
7.	D10	68.3	G2, k	16	99
8.	E2	72.5	G1, k	8	89
9.	F3	73.3	M, k	16	100
	Control				
	NSO	42.6	0	0	64
	1B	74.4	G1, k	0	61

[a] — Twenty mitoses analysed at each clone
[b] — Mouse Ig type detected by enzyme immunoassay
[c] — Antiviral activity of indicated amount of HuIFN-gamma in 0.05 ml neutralized by mixing with 0.05 ml of hybridoma culture supernatant.
[d] — Antiproliferative activity of 5 units of HuIFN-gamma in 0.05 ml inhibited by mixing with 0.05 ml of hybridoma culture supernatant. Data are the mean of 3 replicate wells.

Cloning of hybridomas. Cloning of selected hybridomas was performed by the limiting dilution technique (Goding, 1980) on feeder layer from BALB/c mouse spleen cells prepared 48 hr in advance.

Determination of immunoglobulin types. Immunoglobulin (Ig) types of anti-HuIFN-gamma MoAb were determined by the usual sandwich method of enzyme immunoassay (EIA) using swine anti-mouse Ig and anti-isotype sera against mouse Ig conjugated with horseradish peroxidase (Serotec).

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

Results and Discussion

The serum from immunized mouse used for hybridoma preparation was able to neutralize the antiviral activity of 8 units of leukocyte HuIFN-gamma in dilution 1 : 800. Spleen cells from this mouse were fused with myeloma cells in the presence of PEG and, 14 days later, the growing hybridomas were detected. Hybridoma clones plated from 864 wells were found in 286 wells (33 %). The culture supernatants from these wells were collected, and assayed for neutralization of the antiviral activity of HuIFN-gamma. Nine hybridoma clones secreting MoAb with an IFN neutralizing capacity for at least 2 units of HuIFN-gamma were selected as positive. These hybridomas were subcloned resulting in establishment of stable hybridoma cell lines. Chromosomal analysis confirmed the hybrid character of the prepared cell lines (Table 1).

The Ig types of the anti-HuIFN-gamma MoAb were identified from the results of EIA using a panel of antibodies to a different mouse Ig (Table 1). The most of them were IgG types (3xG1, G2, G2a, 2xG3) and two were IgM types. All tested MoAb contained κ-type light chain.

The maximal neutralizing capacity of the culture supernatants of positive hybridomas against antiviral effect of HuIFN-gamma was determined. As shown in Table 1, 0.05 ml of hybridoma supernatant inhibited the antiviral activity of HuIFN-gamma in the range of 2—16 units per 0.05 ml.

Screening for specific anti-HuIFN-gamma MoAb by EIA or RIA has proved surprisingly unreliable. As test antigen we used HuIFN-gamma (500 units per well) containing also human serum albumine (HSA). From the nine neutralizing MoAb only six were detected as positive in both EIA and RIA. However, all six had approximately the same binding ability to HSA. We did not find plausible explanation of these results. On the other hand, Oda *et al.* (1986) detected by EIA clones producing MoAb specific for recombinant HuIFN-gamma with no binding to HSA.

Supernatants which were able to neutralize the antiviral effect of HuIFN-gamma were assayed to determine their effect on the antiproliferative activity of HuIFN-gamma. Human HI-60 cells were cultured for 3 days in the presence of 5 units of HuIFN-gamma mixed with the culture supernatants and then the cell concentrations were counted. The viability of cells after IFN and MoAb treatment was more than 95 %. Table 1 shows that control culture supernatants (from NSO cells or IB clone producing anti-HSA MoAb) caused reduction in cellular proliferation to 64—67 % from IFN-untreated cells. All anti-HuIFN gamma MoAb or completely inhibited (A11, B13, C5, F3) or reduced (A6, A8, C10, D10, E2) the antiproliferative effect of HuIFN-gamma. The results indicate a strong correlation between inhibition of antiproliferative and inhibition of antiviral activities. MoAb with higher neutralizing capacity of antiviral activity of HuIFN-gamma were found also more potent in neutralization of antiproliferative activity.

These data convincingly demonstrated the ability of given MoAb to inhibit two biological activities of HuIFN-gamma. Our findings support the results of Ziai *et al.* (1986) who found two anti-HuIFN-gamma MoAb to inhibit the antiviral and antiproliferative activities of HuIFN-gamma and to enhance the HLA antigen expression. Rubin *et al.* (1982) and Feit *et al.* (1986) also reported that MoAb to HuIFN-gamma completely blocked the antiproliferative and the antiviral effects of partially purified HuIFN-gamma.

The mechanism(s) by which MoAb inhibit the two IFN-gamma functions is not clear. A plausible explanation suggests a single active domain on the HuIFN-gamma molecule which might be responsible for both biological activities.

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