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INTERFERON-NEUTRALIZING OR ENHANCING ACTIVITIES IN HYBRIDOMA CELL FLUIDS AFTER *IN VITRO* IMMUNIZATION

D. STANČEK[1], M. STANČEKOVÁ[2], J. K. PIMENTA D. REIS[3]

[1]Institute of Preventive and Clinical Medicine; [2]Faculty of Natural Sciences, Commenius University, Bratislava, Czechoslovakia; [3]Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte, Brazil

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Summary. - Several hybridomas supernatants capable of interferon beta (IFN-beta) or "IFN epsilon" ("IFN-eps") neutralizing or enhancing activities were obtained after *in vitro* immunization of BALB/c and C57 mice spleen cells and their fusion with Sp2/0 plasmacytoma cells. Besides rather low anti-IFN-beta or "eps" antibody secretion several cloned hybridoma fluids contained a factor potentiating anti-viral activity of the both IFNs. It is speculated that this activity is due to production by some hybridomas of another lymphokine.

Key words: human amniotic membrane interferons; lymphokine secretion by hybridomas; interferon-neutralizing or enhancing activity

Introduction

Both *in vivo* and *in vitro* immunization were successfully used for monoclonal antibody production against different types of IFNs (Morser *et al.*, 1981; Novick *et al.*, 1983; Chang *et al.*, 1984; Liang *et al.*, 1985; Alfa *et al.*, 1987; Echtenacher *et al.*, 1987; Sugi *et al.*, 1987; Kontsek *et al.*, 1988). Reis *et al.* (1989) described a production of an IFN induced in human amniotic membranes (HAM) which seems antigenically unrelated to any one of so far known IFNs ("IFN-eps"). According to our data (Stanček, unpublished data) we were not able to obtain a clear-cut proof of the presence of "IFN-eps" in HAM cells by means of most progressive separation techniques. An attempt was thus made to produce hybridomas by fusion of Sp2/0 myeloma cells and mouse spleen cells immunized with the two IFNs and compare the outcome of such fusion. The immunization was carried out either *in vitro* with partially purified IFN-beta and "eps" induced in HAM cells or by *in vivo* "priming" followed then by *in vitro* immunization using the same antigens.

Materials and Methods

Interferons. IFN-beta and "IFN-eps" were produced in HAM cells as described by Reis *et al.* (1989). "IFN-eps" is released by induced HAM cells together with IFN-beta but it cannot be neutralized by antibodies against IFN-alpha, IFN-beta or IFN-gamma. Supernatants from HAM cultures induced by parainfluenza 1 virus (Mill Hill strain) or Sendai virus were concentrated by Millipore ultrafiltration system. Blue Sepharose chromatography, anti-human albumin Sepharose affinity chromatography and anti-IFN-beta affinity chromatography were applied for further purification. Final product contained 4-5x10^5 U of "IFN-eps"/ml with specific activity 10^5 - 3x10^6 U of "IFN-eps"/mg protein. In the case of IFN-beta purification the achieved values were 2-7x10^5 U of IFN-beta/ml with specific activity 3-10x10^5 up to $\geq$ 5x10^7 IFN-beta/mg protein.

Immunization. Suspension of spleen cells from 4-8 weeks old BALB/c and C57 female mice were used either dirrectly for *in vitro* immunization or pre-immunized *in vivo* for 12 days ("priming") and then immunized *in vitro* according to our modification of methods introduced by Borrebaeck (1983) and Echtenacher *et al.* (1987). 2.5x10^8 spleen cells obtained from seven BALB/c and five C57 4-8 weeks old female mice were resuspended in 30 ml of RPMI-1640 medium supplemented with HT medium, thymocyte conditioned medium (TCM, BALB/c thymocyte culture supernatants) or HYBRI-MAX medium, 2 % foetal calf serum, gentamicine (20 μg/ml) and 2-mercaptoethanol (50 mmol/l). The cultures were treated with 1.5x10^5 U of semipurified "IFN-eps", transfered in 15 ml volumes into two 75 cm^2 Falcon plastic flasks and incubated as desribed further. After 3 days of incubation 10^4 U of "IFN-eps" antigen was added again with partial exchange of the old media for fresh one and the cultures were kept for another 2 days. The infected spleenocytes were then harvested, washed twice and used for fusion with Sp2/0 myeloma cells as described below. In the case of *in vivo* "priming" followed by *in vitro* immunization 4x10^4 U of IFN-beta in 0.2 ml were injected intraperitoneally with complete Freund's adjuvant to five BALB/c and five C57 mice at the day 0 and 6. On the 12th day after the first injection mice were killed, spleen cell suspension prepared and seeded into two 75 cm^2 Falcon flasks. Further steps were the same as described for *in vitro* immunization with "IFN-eps" including the dosage of IFN-beta antigen.

Hybridomas production and maintenance. Fusion of IFN antigen-immunized spleen cells with Sp2/0 myeloma cells was usually carried on in 1:1 or 2:1 ratio mixing 1.7-7x10^7 washed spleen cells with either the same or half amount of Sp2/0 cells. 47 % PEG 1500 and 3 % DMSO were applied according to commonly used procedure (Köhler and Milstein, 1975; Borrebaeck, 1983). The fused cells were seeded in 24 well plastic plates kept in "HT non-selective" medium for 24 hr either in the presence or absence of mice macrophages "feederlayer" and then replaced by "HAT hybridoma-selective" medium containing lymphokines from BALB/c mouse thymocytes prepared by ourselves or supplied by Sigma (HYBRI-MAX). Cultures were kept at 37 °C with 5 % CO_2 humidified atmosphere for 3-8 weeks and then cloned by limiting dilutions or kept in liquid nitrogen for further passages. BALB/c or C57 mouse peritoneal macrophages were randomly used also for cellular debris-clearing purposes.

Detection of IFN-neutralizing or enhancing activities. The cultures of IFN-beta or "eps" induced hybridomas were passaged in 6, 24 or 96 well plastic plates or in 75 cm^2 Falcon plastic flasks for several weeks. The hybridoma supernatants were regulary checked for IFN-neutralizing or IFN-antiviral-enhancing activities. The tests were performed as originally described by Reis *et al.* (1989). Twofold serial dilutions of low titered IFN-beta or "eps" (about 10 U) were incubated with supernatant fluids of hybridoma cells for 1 hr at 37 °C in 5 % CO_2 humidified atmosphere and distributed into 96 well microplates with Vero cells (5x10^4 cells/well). The cultures were incubated for 24 hr and challenged with 50-150 CPD_{50} of VSV. Cytopathic effect of VSV was measured 24-48 hr later either spectrophotometrically or under light microscope after fixation and staining with crystal violet solution.

Results

Efficiency of hybridoma formation and survival and detection of IFN-beta or "eps" blocking or potentiating activities in sellected hybridomas fluids

Efficiency of hybridomas survival 3 months after the fusion was 25 % for "IFN-eps", 54 % or 60 % for IFN-beta independently on the presence or absence of mouse macrophage "feeder-layer" (Table 1). Inoculation of 11 hybridomas into peritoneal cavity of BALB/c mice and *in vitro* recultivation of 3 hybridomas was successfully realized and productive clones obtained. The capability of surviving hybridomas to produce IFN-neutralizing factor 8–($\geq$ 20) days after fusion is demonstrated in Table 2. Some of the >20 days-old hybridomas were cloned at least twice.

In contrary to our expectations some hybridoma fluids, when tested in Vero cells showed IFN-enhancing rather than IFN-neutralizing effect. Cumulative results on IFN-neutralizing or enhancing factors present in hybridoma fluids are demonstrated in Table 3. Mean CPE value ($\bar{x}$) was obtained by spectrophotometrical or microscopical estimation of CPE of EMC virus in Vero cell cultures treated with "positive" hybridoma fluids in IFN-neutralization assays.

As can be seen from Table 3 both IFNs when mixed with fluids from certain cloned hybridoma cultures expressed several times higher either IFN-neutralizing or enhancing activities in comparison with the antiviral potential of the IFNs alone.

The same results were obtained when some of the cloned hybridomas were inoculated into peritoneal cavity of BALB/c mice and ascites fluids later checked for the presence of IFN-neutralizing or potentiating activities; no increased activities were seen (results not shown).

To answer the question whether the above described IFN activities are not a result of the presence of a factor in the hybridoma-free media, various checking

Table 1. Efficiency of fusion and hybridomas survival

Days after fusion	IFN antigen used for immunization:		
	„IFN-eps"	IFN-beta plus macrophages	IFN-beta minus macrophages
20	8/24*(33 %)	24/24 (100 %)	9/10 (90 %)
50	7/24 (29 %)	24/24 (100 %)	8/10 (80 %)
75	7/24 (29 %)	22/24 (92 %)	8/10 (80 %)
$\geq$ 90	6/24 (25 %)	13/24 (54 %)	6/10 (60 %)

* $\frac{\text{number of surviving hybridoma cultures}}{\text{number of cell cultures shortly after fusion}}$

Table 2. Presence of IFN-neutralizing activity in hybridoma fluids

Days after fusion	IFN antigens used for: a) screening: b) immunization: "IFN-eps" "IFN-eps"	IFN-beta IFN-beta
8	1*(12.5 %)	10 (43 %)
15	2 (25 %)	4 (17 %)
≥ 20	7 (87.5 %)	5 (22 %)

* number of hybridoma cultures with IFN-neutralizing activity

Table 3. IFN-beta or "eps" inhibition or potentiation in Vero cells treated with IFNs only or with hybridoma fluids mixed with either of them

IFN-beta or "IFN-eps" dilutions	Mean CPE value of EMC virus in Vero cells treated with:* IFN-beta control	Hybridoma fluids and IFN-beta		"IFN-eps" control	Hydridoma fluids and "IFN-eps"	
		a**	b***		a**	b***
1:2	< 0.01****	< 0.01	2.48	< 0.01	< 0.01	3.16
1:4	< 0.01	< 0.01	3.66	< 0.01	< 0.01	3.80
1:8	0.60	< 0.01	4.00	1.00	< 0.01	4.00
1:16	2.60	0.37	4.00	2.00	< 0.01	4.00
1:32	3.40	1.12	4.00	3.33	0.28	4.00
1:64	4.00	2.87	4.00	3.66	1.14	4.00
1:128	4.00	4.00	4.00	4.00	3.14	4.00
1:256	4.00	4.00	4.00	4.00	4.00	4.00

* CPE $\bar{x}$ value in control untreated cells was < 0.01
CPE $\bar{x}$ value in control EMC-treated cells was 4.0
** hybridomas secreting IFN-beta or "eps" enhancing factor
*** hybridomas secreting IFN-beta or "eps" neutralizing factor
**** values based on $\bar{x}$ calculations from titrations of 26 IFN-beta or "IFN-eps" reactive hybridoma fluids

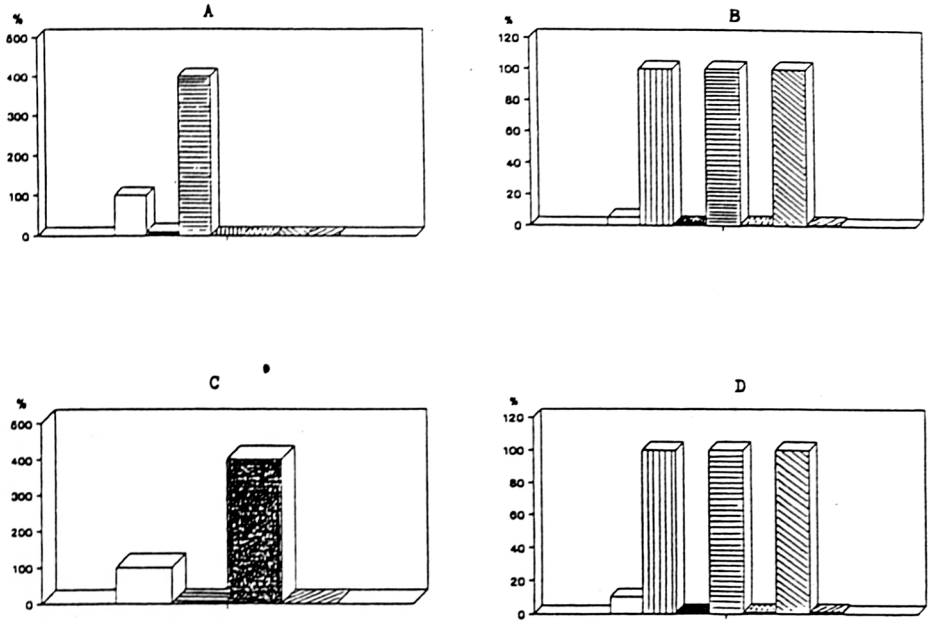

Fig. 1

Fig. 1-A. IFN-beta neutralizing or enhancing activity in hybridoma fluids
☐ IFN-beta control; ■ IFN-beta plus A4 h.f.; ▤ IFN-beta plus B2 h.f.; ▤ IFN-beta plus IFN-beta-ab plus B2 h.f.; ▩ IFN-beta plus IFN-beta-ab; ▧ IFN-beta-ab control; ▨ EMC virus control

Fig. 1-B. Influence of medium used for hybridoma cultivation on IFN-beta activity
☐ HAT; ▥ HAT plus IFN-beta; ■ Sigma medium; ▤ Sigma medium plus IFN-beta; ▩ RPMI-1640; ▧ RPMI-1640 plus IFN-beta; ▨ EMC virus control

Fig. 1-C. "IFN-eps" neutralizing or enhancing activity in hybridoma fluids
☐ "IFN-eps" control; ▤ "IFN-eps" plus D4 h.f.; ■ "IFN-eps" plus 1A h.f.; ▩ EMC virus control

Fig. 1-D. Influence of medium used for hybridoma cultivation on "IFN-eps" activity
☐ HAT; ▥ HAT plus "IFN-eps"; ■ Sigma medium; ▤ Sigma medium plus "IFN-eps"; ▩ RPMI-1640; ▧ RPMI-1640 plus "IFN-eps"; ▨ EMC virus control

1A, A4, B2, D4 - hybridomas; ab - antibody; h.f. - hybridoma fluid

was carried out. Fig. 1A-D summarize the obtained results. The results confirmed those demonstrated in Table 3. No activities were found in the hybridoma cultures treated with several types of hybridomas-free media or their additives (Fig. 1A-D). The only exception was HAT solution which had a slight IFN-like inhibitory effect on CPE of EMC virus.

Discussion

The existence of a new type of IFN family i.e. "IFN-eps" as reported by Reis *et al.* (1989) remains an open question. In spite of our effort to purify IFNs induced in HAM cultures by most progressive techniques we were able to immunize BALB/c and/or C57 mice spleen cells with final products far not sufficient for full scale *in vivo* or efficient *in vitro* immunization. The reason was either insufficient quantity or quality of the obtained IFN products. Though in the case of IFN-beta the final achievable purity was close to presumed absolute value ($\geq 5x10^7$), the available product was not sufficient for more effective *in vivo* or *in vitro* immunization. In the case of "IFN-eps" purification our results (not shown) point on the possible configuration changes on the surface of the IFN-beta protein molecule during purification procedures rather than on basic differences in the antigenic characteristics of two different IFN molecules (Kontsek *et al.*, 1990).

Small quantities of IFN-beta or "IFN-eps" neutralizing activities present in some cultural fluids of the unselected or cloned hybridomas, reported in this paper, are certainly not sufficient for more specified IFN studies. Further effort is necessary to obtain highly productive anti-"IFN-eps" hybridoma clone for such studies. In agreement with other authors (Borrebaeck, 1983; Echtenacher *et al.*, 1987), prevailing majority of our *in vitro* induced hybridoma clones produced anti-IFN antibodies of IgM rather than IgG class as can be presumed on the basis of their short-time secretion.

The explanation of IFN-beta or "IFN-eps"-enhancing activities present in several hybridomas fluids remain an open question. It seems possible that a cytokine-like activity may influence anti-EMC IFN-beta and "eps"activities. Several cytokines were reported to have either IFN inhibiting (interleukin-1, epidermal growth factor, platelet-derived growth factor) or IFN-potentiating activities (tumour necrosis factor) (Daiguji *et al.*, 1988; Kohase *et al.*, 1987, 1988; Reis *et al.*, 1988). Some of the antiviral activity may be contributed to HAT medium and namely to its aminopterin component.

Hybridomas producing IFN-enhancing factor may be employed for further studies concerning lymphokine network interrelations and its role in antiviral or immunomodulation mechanisms in general.

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