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PREPARATION OF HYBRIDOMAS PRODUCING MONOCLONAL ANTIBODIES AGAINST HUMAN INTERFERON

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Summary. — To prepare hybridomas secreting monoclonal antibodies (MoAb) against human α-interferon (α-IFN), BALB/c mice were immunized with IFN produced in Namalwa cells. Native α-IFN, as well as partially purified or on cellulose adsorbed α-IFN preparations were used for immunization. Seven hybridomas continuously secreting IgG against human α-IFN were prepared by fusion of splenocytes from immunized donors with the mouse myeloma cells. MoAb reacted in ELISA as well as in neutralization test with human lymphoblastoid, leukocytic and recombinant α-IFN.

Key words: lymphoblastoid interferon; preparation of hybridomas; testing of hybridoma antibodies

Introduction

Interferons (IFN) are the most studied proteins among lymphokins. They were first identified by their ability to protect cells against viruses. In addition to the antiviral activity, IFN also possess immunomodulating and antiproliferative properties. They are more and more extensively employed for treatment of patients. It should be noted, however, that the synthesis of IFN preparations in human cell cultures by the use of viruses or other inducers yields a mixture of proteins in which the portion of IFN accounts for only 0.1—1%. Because contaminating proteins may exert adverse effects, native IFN preparations are purified from contaminating proteins by using a variety of techniques. One of these is the binding of IFN to monoclonal antibodies (MoAb) followed by their elution (Secher and Burke, 1980).

In addition to IFN purification for clinical purposes, MoAb are used in IFN assay (Scott *et al.*, 1985) and also to discriminate between different IFNs showing different properties (Allen *et al.*, 1982). This paper presents data on the preparation and analysis of a series of hybridomas producing MoAb to human α-IFN.

Materials and Methods

Interferons. Lymphoblastoid IFN was prepared in Namalwa cells induced with Newcastle disease virus (NDV). The cell suspension was cultured in RPMI-1640 medium in the presence of 7% calf serum treated with polyethylene glycol and 50 μg/ml of gentamycin. In order to in-

crease the IFN yield, the cells were cultured for 48 hr prior to induction in the medium containing 0.001 mol/l of sodium butyrate. The inducer (NDV) was added in the form of infected chick embryo allantoic fluid (70 EID_{50} per cell).

After 90 min the cell suspension was diluted with RPMI-1640 to a concentration of $1.0-1.5\times$ $\times 10^6$ cells per ml and then 2% calf serum was added. After 20 hr the resulting culture fluid was clarified by low-speed centrifugation and used as native IFN. IFN was purified and concentrated according to the procedure of Cantell and Hirvonen (1978) based on protein precipitation with ethanol at acid pH.

Human leukocyte IFN was a commercial preparation produced by N.F. Gamaleya Institute of Epidemiology and Microbiology (U.S.S.R. Academy of Medical Sciences).

Recombinant IFN-α- was used in the form of a commercial drug Reaferon manufactured by Biopreparat (U.S.S.R.).

Immunization of mice. Female Balb/c mice were immunized using two techniques:

1. The animals were administered i.p. 1.0 ml of native lymphoblastoid IFN (6.4×10^3 IU/ml) for 3 subsequent days. After one month the mice were given i.v. for 3 subsequent days 1.0 ml of lymphoblastoid IFN preparation purified according to Cantell and Hirvonen (1978), having an activity of 1.02×10^5 IU/ml and a specific activity of 2×10^5 IU per 1 mg of protein.

2. A sorbent was prepared on the basis of dialdehyde cellulose according to the method of Lekhtsind and Gurvitch (1981). The purified lymphoblastoid IFN was dialysed against carbonate-bicarbonate buffer, pH 9.0; the IFN preparation containing 10 mg of the protein was conjugated with 100 mg of oxidated cellulose. The quantity of the bound protein was determined with the help of bromphenol blue using a standard curve: it appeared to be 14 μg per 1 mg of dry sorbent weight. The resulting immunosorbent was suspended in 10 ml of saline; 0.5 ml of the suspension was administered s.c. to mice. After 1 month the mice were reimmunized with purified lymphoblastoid IFN. On the 1st day 0.1 ml of IFN was injected i.v., then 0.2 ml by i.p. and s.c. routes on days 2 and 3.

Preparation of hybrid cells. Three days after the last immunization the spleen was removed from mice to prepare a splenocyte suspension. NSO mouse myeloma cells in the logarithmic growth phase were mixed with splenocytes in a ratio 1 : 5. A 50% solution of polyethylene glycol for gas chromatography was used as fusion agent (molecular weight 1000, Merck, F.R.G.). The cells were fused according to the method described by Kennett (1980).

The cell mixture to be fused was placed into 96-well plastic plates covered with the feeding layer of nonimmune mouse splenocytes (3×10^5 cells per well were plated 1 day before the experiment). The cultivation was carried out in HAT medium for 20 days.

Identification of hybridomas producing antibodies against IFN. Culture fluid from growing hybrid cultures was tested for antibody production against IFN-α with the use of solid-phase enzyme-linked immunosorbent assay (ELISA). The 96-well plates (Nunc Immuno Plate I, Denmark) were sensitized with IFN preparation (a specially selected batch purified according to the procedure of Cantell and Hirvonen (1978) which did not cause any background staining) at 4 °C for 72 hr. They were then rinsed with running water and three times with phosphate buffered saline (PBS), pH $7.2-7.3$ containing 0.5% of bovine serum albumin (BSA) and 0.05% Tween-20 (PBS-Tween-BSA). Nonspecific sites were blocked with 1% BSA for 1 hr and then rinsed 3 times with PBS-Tween-BSA. Culture fluids tested were diluted 1 : 3 with PBS-Tween--BSA containing 5% horse serum and added to the wells (50 μl). Adsorption of antibodies continued for 1 hr at 37 °C. The plates were then washed as described above and 50 μl of peroxidase--labelled donkey IgG serum fraction against mouse IgG (Cappel Laboratories) was added (at a 1 : 1000 dilution) for 30 min at 37 °C. After washing of plates with the above mentioned solution, 50 μl of the substrate (0.04% orthophenylenediamine and 0.001% H_2O_2 in acetate--citrate buffer, pH 5.0) was added. The reaction was stopped by adding 4N H_2SO_4.

Preparation of ascitic fluids. Female BALB/c mice $2-2$ 1/2 month old were administered i.p. 0.5 ml of Pristane (Koch Light, Great Britain). After 7 to 14 days the animals were inoculated by the same route with $(4-5\times 10^6)$ hybrid cells, washed with PBS and resuspended in PBS with addition of 10 units/ml of heparin. After 10 to 14 days the ascitic fluid was collected from abdominal cavity of mice.

Neutralization of IFN antiviral activity by MoAb. For determination of IFN-neutralizing activity of MoAb human embryo lung diploid cells (strain L-68) and bovine embryo kidney cells (MDBK line) were grown in test tubes. Double dilutions of ascitic fluid in Eagle's medium were prepared and 0.5 ml were mixed with IFN (16 units activity in 0.5 ml). The mixtures

Table 1. Production of hybridoma cells

No. of experiment	Material for immunization of mice		No. of plated cultures (wells)	No. of cultures with the indicated number of colonies								Hybridomas produced
	primmary immunization	reimmunization		1	2	3	4	5	6	7	≧8	
1	native IFN	purified IFN	960	289	118	34	8	5	2	1	0	457 (47.6%)
2	purified IFN with sorbent	purified IFN	768	155	116	107	54	47	43	16	115	653 (85.02%)

were stirred in a shaker for 1 hr at 37 °C, and one ml was then added into each tube with the cell cultures. By 24 hr later the samples were removed and 100 $TCID_{50}$ of vesicular stomatitis virus (VSV) was added. The results were read after 48 hr according to the cytopathic effect of the virus.

Classification of hybridoma immunoglobulins. Class identification of MoAb immunoglobulins was performed by the help of enzyme-immunoassay using mouse ascitic fluid. The plates were sensitized with IFN as described above. The Ig tested was then layered over it and treated with rabbit serum against mouse IgM or IgG heavy chains. Peroxidase-labelled donkey IgG against mouse IgG and the substrate were finally added.

Results

To prepare the hybridomas, mice were immunized with lymphoblastoid IFN. Two immunization techniques were employed: one used nonadsorbed IFN and the other — IFN immobilized on cellulose sorbent. Two mice were taken for each test. Table 1 shows the results of preparation of hybrid cells. The efficiency of hybridoma formation was higher in test 2 than in test 1; they occurred in 85% and 47.6% of the wells, respectively.

Contemporarily, to prepare hybridomas for further work, it was necessary to use single isolated cell colonies; these appeared to be more numerous in test 1.

Hybridoma cells from separate colonies were plated in HAT medium into the 24-well plates with the feeder layer from nonimmune mouse splenocytes. The medium was changed on days 5 or 6 in culture. The hybridoma cells were grown until they almost entirely covered the bottom of the well. This usually lasted for 10 to 14 days.

After that culture fluid was collected and tested for antibodies against IFN-α by ELISA. The results are presented in Table 2. Two hybridization experiments yielded 7 hybrid clones continuously secreting antibodies against human IFN-α. Antibody titre of the culture fluid was not very high (1 : 8 to 1 : 128). Immune ascitic fluids collected from mice inoculated with hybrid cells had an antibody titre of 400 to 4000.

We tested the ability of MoAb to neutralize the antiviral activity of different human α-IFN, namely the lymphoblastoid, leukocytic and recombinant α-IFN. The tests were carried out in diploid human embryo lung and bovine embryo kidney cells. Antibody titre of ascitic fluids was 1 : 4 to 1 : 128 (Table 3) and of the hybridoma culture supernatants it ranged between 1 : 4 to 1 : 16 depending on the IFN used.

Table 2. Detection of hybridomas secreting MoAb to α-IFN by ELISA

No of experiment	No. of cultures tested	Cultures producing antibodies to α-IFN	No. of hybridoma clones continuously secreting MoAb
1	191	17 (8.9%)	5
2	96	16 (16.6%)	2

Table 3. Ability of MoAb to neutralize antiviral activity of α-IFN in L-68 and MDBK cell cultures (dilution reciprocals)

Designation of hybridoma	IFN			
	lymphoblastoid	leukocytic	recombinant	
	L-68	MDBK	L-68	MDBK
C_9	32	64	n.t.	n.t.
F_2	16	128	8	32
G_8	16	64	4	8
B10	32	64	n.t.	n.t.
D6	16	128	n.t.	n.t.
39C	4	32	4	64
47C	16	64	8	8
MoAb to monkey pox virus	<4	<4	<4	<4
normal ascitic fluid	<4	<4	<4	<4

Note. MoAb G_8 was tested after the ascitic fluid was kept for 6 months at −20 °C
n.t. — not tested

All hybridomas were found to secrete class G immunoglobulins as evidenced by ELISA using rabbit sera to mouse IgG and IgM.

Discussion

Two immunizations of two mice each with lymphoblastoid human IFN-α yielded 7 hybridoma cultures continuously secreting antibodies against IFN-α. The available literature offers, as a rule, very prolonged schedules for IFN immunization either in mice or in rats before subsequent preparation of hybridoma cells. The immunization lasted sometimes for a few months (Staehilin *et al.*, 1981; Männel *et al.*, 1982; Norvick *et al.*, 1983). We describe a short immunization schedule lasting for 1 month. The results of hybridoma production under such conditions appeared to be quite satisfactory. Apparently, high serum titres of anti-IFN antibodies are not obligatory and a long immunization course is not needed. The efficiency of hybridoma cell production was found to be unrelated to the titre of the serum antibodies in donors (Schönherr and Houwink, 1984). It is important that the booster immunization should be carried out 2 or 3 days before removal of the spleen when it contains many blast cells which are more efficient for fusion with myeloma cells than the resting immunocytes.

It has also been shown that IFN preparations used for immunization need not to be highly purified. Native IFN prepared in Namalwa cells was used for first immunization. Some of the resulting hybridoma cells will most likely produce MoAb to IFN contaminating proteins, e.g. to ovalbumin present in the cell cultures together with the inducer (NDV), or to bovine serum proteins present in the medium. This does not hamper, however, the detection of MoAb against IFN, provided that the tests are carried out with purified IFN preparation.

During immunization of animals IFN is often injected in combination with complete or incomplete Freund's adjuvant (Secher and Burke, 1980; Männel *et al.*, 1982). On our experiments IFN was conjugated with oxidated cellulose which appeared to increase the quantity of the resulting hybridoma clones.

The IFN-neutralizing titre of MoAb appeared to be higher when tested in MDBK cells than in human diploid L-68 cells. This is in good accord with the data that IFN from Namalwa cells had a higher affinity for MDBK cells than for human cells (Feinstein *et al.*, 1985). Our MoAb reacted in neutralization test both with lymphoblastoid α-IFN as well as with leukocytic and recombinant α-IFN. They apparently bound to epitopes common for all these α-IFN types. It can be noted, however, that recombinant α-IFN was much weaker neutralized with the MoAb than lymphoblastoid or leukocytic α-IFN. This indicates the significant differences between recombinant α-IFN and the two others.

Since the MoAb produced reacted with epitopes common for different α-IFNs they probably may be suitable for purification of all the three clinically employed α-IFN. To test this possibility the avidity of antibodies produced with respect to α-IFN has to be assessed: it must be high enough to provide adequate adsorption of IFN to the immunosorbent but not too high in order not to hamper the elution of IFN from the column.

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