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PREPARATION AND CHARACTERIZATION OF HYBRIDOMAS PRODUCING MONOCLONAL ANTIBODIES AGAINST HUMAN ALPHA INTERFERON

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Summary. — Hybridomas producing monoclonal antibodies against human alpha interferon (hu-IFNα) were constructed by fusion of NSO myeloma cells with spleen cells of BALB/c mice immunized with purified hu-IFNα. Altogether, 527 hybridomas were prepared in two separate experiments. From this cohort of hybridomas, 51 produced monoclonal antibodies against hu-IFNα. Seventeen out of fifty one hybridomas produced antibodies with neutralizing capacity for IFN while 34 hybridomas produced monoclonal antibodies with binding ability not accompanied with the neutralization of biological activity of IFN. The specificity of antibodies was determined with 3 types of tests: ELISA, ELISAN (modified ELISA) and neutralization test. Using isotype analysis, it has been found that 23 monoclonal antibodies were of IgM class, 20 were of IgG1 subclass, 4 were of IgG2b and 4 of IgG3 subclass. The average number of chromosomes in hybridomas was between 61.35 and 78.55. Their average doubling time was between 13.95 and 25.76 hrs.

Key words: monoclonal antibodies; human alpha interferon; hybridoma; isotypic and chromosomal analysis

Introduction

In comparison with other monoclonal antibodies hybridomas secreting antibodies against various types of human and/or mouse interferon (IFN) were obtained relatively late, i.e. when purified and potent IFN preparations were available for immunization of mice. The monoclonal antibodies proved useful both for characterization and purification of IFN (Secher and Burke, 1980, Staehelin *et al.*, 1981). However, not all monoclonal antibodies obtained are able to neutralize the biological activity of IFN. For this reason, the choice of the test for screening the antibody-producing hybridomas is of upmost importance. If the test employed is of "binding" type (ELISA, RIA), both i.e. binding and neutralizing antibodies will be detected (Staehelin *et al.*, 1981; Nannel *et al.*, 1982). However, a great deal of antibodies becomes lost in tests based on the neutralization of biological activity of IFN by antibodies.

In this study, we demonstrate the varying properties of monoclonal antibodies against IFN produced by hybridomas.

Materials and Methods

Antigen. Human lymphoblastoid (Namalva) IFN α with a specific activity of 7.4×10^7 units per mg of protein was a gift of Dr. K. Fantes (Wellcome Labs., G. Britain). The human blood leucocyte IFN with spec. activity of 6×10^6 units per mg was prepared by Dr. N. Fuchsberger (Institute of Virology, Bratislava). This IFN was further purified by affinity chromatography on a NK2-Sepharose column (kindly donated by Dr. D. Secher, MRC, Cambridge, G. Britain) using monoclonal antibodies. The resulting specific activity of the IFN preparation was 10^8 units per mg of protein.

Immunization. Group of 6 BALB/c mice of 10—12 weeks age was immunized with IFN for 22 weeks. The first 10 doses were applied intraperitoneally, subcutaneously, and intravenously using $4—10 \times 10^6$ units of IFN per dose according a scheme presented on Fig. 1. After the 10th dose, the sera of mice were assayed for anti-IFN activity. The mice with the highest anti-IFN activity (1 : 200 000) received a booster dose (10^7 units) intravenously. After 72 hr, the mice were killed by cervical dislocation and their spleens were used for preparation of cells used in fusion experiments. (The titre of neutralizing anti-IFN antibodies in the sera of mice at the time of fusion was 1 : 400 000).

Myeloma cells. The immunoglobulin non-synthesising and non-secreting variant NS-1 of the myeloma cell line NSO(B) was used. The cells were cultivated in the Dulbecco minimal essential medium (D-MEM) supplemented with 15% pretested horse serum (ELAN-CLONE, Bratislava), 0.028 mmol/l 2-mercaptoethanol, 4 mg per ml glucose, 0.4 units per ml of PVR-insulin, 2 mmol/l L-glutamine and 1 mmol/l sodium pyruvate. The cells were cultivated at 37 °C in an atmosphere with 7.5% CO_2.

Cell fusion. The method of Fazekas de St. Groth and Scheidegger (1980) has been utilized. The ratio of spleen cells from immunized mice to myeloma NSO cells was 5 : 1. Polyethylenglycol (50%, Serva, GFR) was supplemented with 5% DMSO (Koch and Light, G. Britain) and 7.5% $NaHCO_3$ in 1 ml. The cells were explanted in 8 flat-bottomed microcultivation plates (768 wells) (Flow Labs., G. Britain) in amount of 2.5×10^5 spleen cells per well for 1—2 min. In the second experiment, altogether 832 wells were filled with the cell mixtures.

Selection and cultivation of hybridomas. After fusion, the cell-suspensions were transferred to selective HAT-medium (Littlefield, 1964) containing 0.1 mmol/l hypoxanthine, 0.004 mmol/l aminopterine and 0.016 mol/l thymidine in D-MEM. This medium was supplemented with 20% of aged medium obtained from the culture of NSO cells in the exponential phase of their growth. The cells were cultivated in 96-well flat bottomed plates (Flow Lab., G. Britain) in a concentration of 250×10^3 spleen cells per 200 µl medium per well. Within 10 days, the medium was changed for HAT-D-MEM and the supernates of growing clones were tested for the presence of anti-IFN antibodies. The positive (antibody-producing clones) were transferred into 24 wells plates and incubated for 48 hr in Petri-dishes with 60 mm diameter. The hybridomas were maintained in Petri-dishes in D-MEM with 15% serum (Elan-Clone) every 48 hr.

Primary screening. The hybridomas producing anti-IFN monoclonal antibodies (MoAb) were detected by employing 3 mutually complementary methods:

a) immunoenzymatic test on nitrocellulose discs (IFN-ELISAN)
b) immunoenzymatic test on polystyrene plates (IFN-ELISA)
c) virus-neutralization test (modified according Novák *et al.*, 1965) (IFN-VNT).

Cloning. The hybridomas were cloned by limiting dilution technique according Goding (1980) on feeder layers prepared 48 hr in advance. The positive (antibody-producing) clones were recloned until all positive hybridoma subclones started MoAb production.

Preservation of hybridomas. The medium used for freezing of hybridomas consisted of 45% D-MEM and 45% of sera (Elan-Clone) supplemented with 10% DMSO. The cells were freezed in concentration of 2 to 4×10^6 cells per ml according to the method of Kennett *et sl.* (1980) and kept in liquid nitrogen.

Chromosomal analysis. The method of Rothfels and Siminowitch (1958) was utilized for karyological analysis of hybridomas. At least 20 mitoses were evaluated in each analysed clone.

Production of monoclonal antibodies in vivo. The positive (anti-IFN antibody producing) hybridomas were inoculated intraperitoneally into Pristan-sensitized BALB/c mice. The ascitic fluid was exhausted during 7 to 14 days. MoAb were isolated from the ascitic fluid according to the procedure of Staehelin *et al.* (1981) and stored at —30 °C until use.

Table 1. The yield of fusion experiments

Number of microcultures		Number of hybridomas in culture				Total number of hybridomas
Total[a])	No. of hybridomas	1	2	3	4	
832[b])	353	265	71	17	–	458
768[c])	174	153	18	3		198

[a]) The microplates were filled with 300 000 cells in 200 μl per wells (the fusion ratio on spleen cells: NSO cells was 5 : 1).
[b]) Fusion experiment No. 1.
[c]) Fusion experiment No. 2.

Isotype analysis. The MoAb were assayed according to McDougal *et al.* (1983) using anti-isotype sera (Serotec, G. Britain).

Assay of IFN. The cytopathic effect inhibition method in MDBK cells using the vesicular stomatitis virus (VSV) was used for IFN assay (Havell and Vilček, 1972).

Results and Discussion

Our aim was to obtain a large number of hybridomas producing MoAb against human IFNα and with their means to analyse the structure and bological activity of IFN molecules (Borecký *et al.*, 1985 and others). Two groups of 6 BALB/c mice were immunized with IFNα (456×10^8 units per mouse in 12 doses during 22 weeks). Only spleen cells from mice with an blood antibody titre over 200,000 were used in the fusion experiments. Two fusion experiments resulted from immunization. In the fusion experiment No. 1, hybridomas were detected in 353 (42.4%) out of 832 microcultures (Table 1). In 71 cases the wells contained 2 hybridomas, while in 17 wells 3 hybridomas were found. The remaining 265 wells contained 1 hybridoma per well.

In fusion experiment No. 2 the hybridomas were formed in 174 wells from a total number of 768 microcultures (22.6%). The prevailing majority of wells (153) contained one hybridoma. These data may be important, since especially in the case of preparation of anti-IFNα MoAb the primary screening

Table 2. The distribution of antibody producing and non-producing hybridomas

Number of hybridomas		
producing		non-producing Ig
specific antibodies	non-specific Ig	
32[b]/9%	317/89.8%	4/1.1%
19[c]/10.9%	147/84.4%	8/4.5%

For legend see Table 1.

Table 3. Isotype analysis of hybridomas producing MoAb against human alpha interferon

MoAb type	No. analyzed	Isotype analysis							
		IgM	IgG1	IgG2a	IgG2b	IgG3	IgA		
Neutralizing[a])	17	10	7	—	—	—	—	—	17
Non-neutralizing[b])	34	13	13	—	4	4	—	—	34

[a]) MoAb which neutralized the antiviral activity of IFN (2 units).
[b]) MoAb which were positive in ELISA.

is time consuming and the selection of producing clones as well as securing of their monoclonality is necessarily delayed. As follows from Table 2, in accordance with Westerwoundt *et al.* (1984) and Bíleková *et al.* (1985) the number of 300×10^3 cells per well seems to be optimal for fusion.

The tests used so far for the primary screening of hybridomas producing anti-IFNα MoAb had several disadvantages: a) They were time consuming (biological tests based on inhibition of the antiviral effect of IFN (Stewart, 1979). b) They detected only hybridomas producing neutralizing MoAbs (Secher and Burke, 1980). c) They required relatively large amount of IFNα

Table 4. Chromosome and growth analysis of hybridomas producing monoclonal antibodies against human interferon alpha[a])

Hybridomas	Average No. of chromosomes[b]) per cell	Standard deviation	Doubling time of hybridoma
8E12/21	61.35	3.07	18.25
7C1/J	74.25	3.27	22.22
2B7/1	74.85	2.64	15.31
3C2/25	74.55	2.60	21.08
3C2/7	76.00	2.03	19.59
4E1/20	72.65	2.39	20.48
2H5/J	78.55	3.25	19.12
1H4	73.85	2.37	21.11
6B3/9	76.85	2.20	18.02
6B3/28	74.80	2.01	17.43
6B3/4	73.55	2.01	18.72
5G10/32	68.10	2.07	19.97
5C12/36	71.80	2.25	13.95
1A1/E1J	72.20	2.52	23.30
1C8/22	73.20	2.06	20.45
1H1	72.30	2.28	20.60
2D10/29	76.20	2.64	21.95
5G10/31	71.50	2.92	22.85
2D6/1	68.80	2.99	25.76

[a]) The hybridomas produced non-neutralizing antibodies against IFN α
[b]) Twenty mitoses evaluated

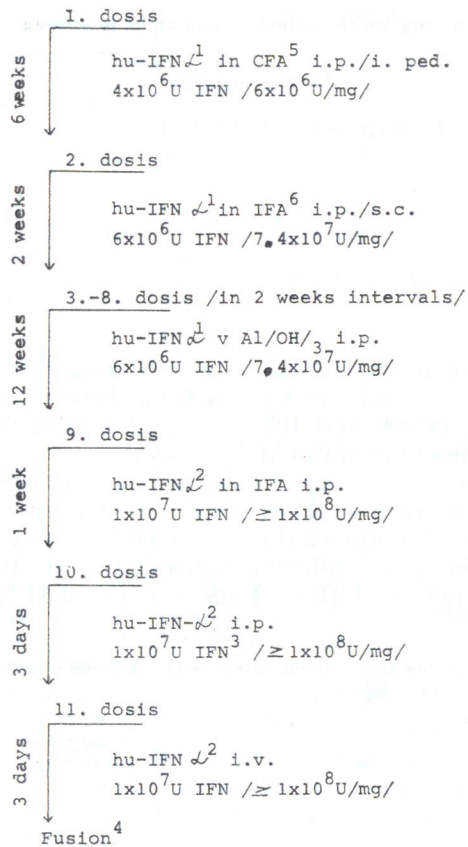

Fig. 1.

Immunization scheme of BALB/c mice with human alpha interferon

[1] Lymphoblastoid IFN (Namalva); [2] Leukocyte IFN purified on NK-2 Sepharose; [3] IFN stabilized in BALB/c mouse serum; [4] Titer of neutralizing antibodies 1 : 400,000; [5] Complete Freund adjuvans; [6] Incomplete Freund adjuvant.

(ELISA) and/or special instrumentation (RIA). These problems were partially solved using a modified assay system named: IFN-ELISAN (Novák *et al.*, 1986). It is based on the known ability of nitrocellulose filters to bind proteins quickly and firmly the adhering amount being related to the amount of applied protein. The sensitivity of such test follows from the fact that in both fusion experiments the positive (i.e. antibody producing) hybridomas were detected in a time interval of 8 to 20 hr as soon as on the 7th day. In that time, in 1 ml of supernate from the hybridoma culture, in average, 10 ng of MoAb can be present (Loevborg, 1984).

From a total of 527 hybridomas in 2 experiments, monoclonal Abs were produced by 515 hybridomas, while 12 hybridomas produced no immunoglobulin. In the further analysis it was shown that 51 (out of 515) hybridomas produced antibodies with anti-IFNα activity (10.09%) Seventeen hybridomas out of 51 produced antibodies capable of neutralizing the biological activity of IFN, while the remaining 34 hybridomas reacted with the IFN molecule without influencing its biological activity. Using anti-isotype sera (Serotec,

G. Britain), it was found that 23 hybridomas produced antibodies of IgG1 subclass, 4 hybridomas — of IgG2b and 4 hybridomas — of IgG3 subclass. All the tested MoAbs had the light chain of "K" type (Table 3).

Nineteen hybridomas from experiment No. 2 were analysed for chromosome number and growth properties. As shown on Table 4, the average number of chromosomes was 61.35 to 78.55. A higher variability was found in studying the population doubling time of hybridomas: 13.95 to 25.76 hr.

In conclusion, the characterization of hybridoma products may contribute to a better evaluation of their usefulness in biologic and antigenic analysis.

References

Bíleková, E., Novák, M., Borecký, L., Leššo, J., and Kontsek, P. (1985): Effect of concentration of spleen cells in microculture after fusion on the number and activity of hybridomas (in Slovak) fusion on the number and activity of hybridomas (in Slovak). *Congress of Czechoslovak Immunologists* (Abstracts) Nitra, 9—.13. Sept. 1985.

Borecký, L., Novák, M., Krchňák, V., and Říha, J. (1985): Attempt to characterize synthetic interferon fragments, pp. 15—22. In F. Dianzane and G. B. Rossi (Eds.): *The Interferon System. System.* Raven Press, N. York.

McDougal, J. S., Browing, S., Kennedy S., and Moore, D. (1983): Immunodot assay for determining the isotype and light chain type of murine monoclonal antibodies in unconcentrated hybridoma culture supernates. *J. immunol. Meth.* **63**, 281—290.

Fazekas de St. Groth, S., and Scheidegger, D. (1980): Production of monoclonal antibodies: strategy and tactics. *J. immunol. Meth.* **35**, 1—21

Goding, J. W. (1980): Antibody production by hybridomas. *J. immunol. Meth.* 39, 285—308.

Havell, E. A., and Vilcek, J. (1972): Production of hight-titered interferon in cultures of human diploid cells. *Antimicrob. Agents Chemother.* **2**, 476—484.

Kennett, R. G., McKearn, T. J., and Bechtold, K. B. (1980): *Monoclonal Antibodies* (Hybridomas: A new dimension in biological analyses). Plenum Press, Orlando.

Littlefield, J. W. (1964): Selection of hybrids from matings of fibroblasts in vitro and their presumed recombinants. *Science* **145**, 709—710.

Loevborg, U. (1984): Early screening for hybrids antibodies. *Develop. biol. Standard.* **55**, 173—179.

Nannel, D., De Maeyer, E., Kemler, R., Cachard, A., and De Mayer-Guignard, J. (1982): A rat monoclonal antibody against mouse and interferon of all molecular species. *Nature (Lond.)* **196**, 664—665.

Rothfels, K. H., and Siminovitch, L. (1958): An air-drying technique for flattering chromosome in mammalian cells grown in vitro. *Stain Technol.* **33**, 73—77.

Novák, M., Borecký, L., Kontsek, P., Pospíšil, M., and Bíleková, E. (1986): IFN-ELISAN. *J. immunol. Meth.*, in press.

Secher, D. S., and Burke, D. C. (1980): A monoclonal antibody for large scale purification of human leukocyte interferon. *Nature (Lond.)* **285**, 446—450.

Staehelin, T., Hobbs, D., Hsiand-fu-Kung, Chu-Yen Lai, and Pestka, S. (1981): Purification and characterization of recombinant human leukocyte interferon (IFN r A) with monoclonal antibodies. *J. biol. Chem.* **256**, 9750—9754.

Stewart, W. E. II (1979) :The Interferon System, Springer-Verlag, New York.

Westerwoudt, R. J., Naipal, A. M., and Harrison, C. M. H. (1984): Improved fusion technique. II. Stability and purity of hybrid clones. *J. immunol. Meth.* **68**, 89—101.