A NEW APPROACH TO HYBRID HYBRIDOMA CONSTRUCTION BASED ON THE USE OF AN ACTINOMYCIN D-RESISTANT MYELOMA CELL LINE

Yu. S. Massino, E. A. Kizim, and A. D. Dmitriev

UDC 616-006.441-092.9-07

KEY WORDS: tetradoma; quadroma; resistance to actinomycin D; bispecific antibodies; α-endorphin

One of the most commonly used methods of obtaining bispecific monoclonal antibodies (McAb) is by the fusion of two hybridomas, secreting antibodies to different antigens. Under these circumstances hybrid (tetradoma) clones secreting recombinant immunoglobulins may be formed [14]. As a rule, selective markers are introduced beforehand into one of the parental hybridomas: recessive – sensitivity to HAT medium (HAT^s) and dominant – resistance to antibiotics, making possible tetradoma selection [11]. We have simplified this stage and have obtained a mouse myeloma cell line resistant to actinomycin D (AD^r). It has been shown that the use of this line when obtaining parental hybridomas leads to automatic inheritance of the dominant AD^r marker. The suggested approach has been used to select tetradomas producing McAb with dual specificity: to α-endorphin (α-END) and to horseradish peroxidase (HRP). Under these circumstances, AD^r and HAT^s cells (hybridoma to HRP) were fused with wild-type cells (hybridoma to α-END).

EXPERIMENTAL METHOD

Cells resistant to AD in a concentration of 30 ng/ml were isolated from the HAT^s cell line of X63-Ag8.653 mouse myeloma cells [9] by multistage selection. The ADR cells were fused by the method in [10] with splenocytes of a Balb/c mouse, repeatedly immunized with HRP, in the ratio of $4 \cdot 10^7$ splenocytes to 10^7 myeloma cells. The hybrids were isolated with the aid of HAT medium. HAT^s clones were isolated from the hybridoma lines producing McAb to HRP by one-step selection on medium containing 20 μg/ml of 8-azaguanine (8-Ag), by the method in [11]. To obtain tetradomas, 10^6 cells of hybrid clone $36F_9$, producing antibodies to HRP and possessing two selective markers (AD^r and HAT^s) were fused [10] with an equal number of hybridoma F_8 cells [3, 4], producing McAb to α-END. The cells were seeded into a 96-well planchet ($2 \cdot 10^4$ per well) containing macrophages and grown on selective HAT medium containing 3 ng/ml of AD. The clones were tested for the presence of McAb to α-END by radioimmunoassay [4, 5]. To test McAb to HRP the planchets were saturated with affinity-purified antibodies to mouse IgG — 1 μg per well in 100 μl of 0.01 M Na-phosphate buffer, pH 7.5 (PB), overnight at 0°C. After a series of washings with PB containing 0.05% Tween-90 the planchets were incubated successively at 37°C with 1% bovine serum albumin (BSA), with the test culture fluid (50 μl per well, 90 min), and with a solution of HRP (1 μg in 100 μl PB). The enzyme reaction was carried out with the aid of *o*-phenylenediamine. To test the bispecific antibodies the planchets were saturated with a conjugate of α-endorphin with BSA [4, 5] (0.5 μg conjugate in 100 μl PB, overnight at 0°C). All the stages of determination then were identical with those during testing for antiperoxidase McAb. HRP ("Sigma") and α-endorphin ("Serva") were conjugated with BrCN-Sepharose ("Pharmacia") by the method recommended by the firm. Ascites fluid with bispecific McAb was obtained as described previously [3]. To obtain bispecific McAb the ascites fluid (0.5 ml) was diluted tenfold with PB containing 0.15 M NaCl and applied to a column with protein A-Sepharose ("Pharmacia"). IgG were eluted with 0.1 M acetic acid. Fractions containing IgG were pooled, neutralized with concentrated ammonia, and applied to a column with HRP, conjugated with BrCN-Sepharose. The unbound fraction was washed off with PB containing 0.05% Tween-20. Elution from the affinity column was carried out with 0.05 M acetic acid,

Institute of Higher Nervous Activity and Neurophysiology, Academy of Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR E. A. Zotikov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 112, No. 11, pp. 511-514, November, 1991. Original article submitted March 19, 1991.

 0007-4888/91/0011-1618$12.50

TABLE 1. Distribution of Antibodies Producing Tetradoma by Specificity

Name	Concentration		Antiperoxidase activity	Antiendorphin activity
	mg	%		
IgG fraction of ascites fluid	7,68	100	+	+
Bispecific antibodies	2,39	31,2	+	+
Antiperoxidase antibodies	1,6	20,8	+	—
Anti-α-endorphin antibodies	1,54	20	—	+
Inactive antibodies	2,37	30,8	—	—

Legend. Concentration of antibodies shown per ml ascites fluid. Concentration of antibodies determined from their absorption at 280 nm, assuming that $A_{280} = 1.3$ corresponds to a concentration of 1 mg/ml.

pH 2.0 (adjusted with HCl). Fractions of affinity antibodies were pooled, neutralized with concentrated ammonia, and purified on an affinity column with α-endorphin, conjugated with BrCN-Sepharose. Rat pituitary glands for immunohistochemical staining were obtained after perfusion of the rats through the aorta with a 4% solution of paraformaldehyde in PB. The immunohistochemical reaction was carried out on freely floating vibrotome sections 40 μ thick. The pituitary gland sections were incubated with a solution of bifunctional McAb (15 μg/ml) and of HRP (30 μg/ml) in PB containing 2% neutral goat serum and 0.2% Triton X-100 overnight at 0°C. The peroxidase reaction was carried out with diaminobenzidine [3].

EXPERIMENTAL RESULTS

A subline resistant to 30 ng/ml of AD was isolated by multistage selection over a period of 4 months from line HAT^s of mouse myeloma cells X63-Ag8.653. This concentration is 50-100 times greater than the 50% lethal dose (0.3 ng/ml) for the original culture. As a result of fusion of the AD^r HAT^s myeloma cells with splenocytes of mice immunized with HRP, 312 hybrid clones were formed (fusion efficiency about 10^{-5}), of which 28 actively secreted McAb to HRP. All the hybrid clones were resistant to the antibiotic in a dose of 10 ng/ml, which was lethal for the wild-type cells, although they were selected on HAT medium in the absence of AD. Meanwhile, with an increase in the dose of AD to 20-30 ng/ml growth of the hybridomas was suppressed, in agreement with the codominant inheritance of this trait in hybrid cells [6, 8, 13]. By contrast with certain other AD^r lines [2], the resistant phenotype was relatively stable: resistance to AD in a concentration of 10 ng/ml lasted at least after the second month of culture of the hybridomas in the absence of the antibiotic. Thus the use of AD^r myeloma cells to obtain hybridomas enables a dominant selective marker (AD^r) to be introduced into a large number of hybridomas, without the need to resort to such measures as retrovirus transfection or long-term selection for resistance of one of the parental hybridomas to the preparation [7, 11]. This approach in many cases may make it much easier to construct parental hybridomas carrying selective markers necessary for tetradoma selection.

To obtain double (AD^r HAT^s) mutants the antiperoxidase clones were subjected to back-selection to the HAT^s phenotype, which can easily be done by one-step selection on medium with 8-Ag [11]. Tetradoma clones were obtained by fusion of HAT^s AD^r cells of hybridoma $36F_9$ secreting antibodies of the IgG1 class to HRP and cells of clone $F_8/40E_9$ [3, 4], secreting class IgG_{2a} antibodies to α-END. Both parental lines died completely on HAT medium containing 3 ng/ml AD, used as a selective system for screening tetradoma cells. About 300 clones grew 10 days after fusion in the selective medium – two to four clones in each well (frequency about 10^{-4}). In all wells both antiendorphin and antiperoxidase activity was found. Cultures grown in five arbitrarily chosen wells were pooled and the resulting population was cloned by the limiting dilutions method. Of 36 clones tested 22 (61.1%) secreted antibodies to α-END only, three clones (8.3%) antibodies to HRP only, in nine clones (25%) no activity was found, and only in two clones (5.6%) was both antiendorphin and antiperoxidase activity found. These two clones were distinguished by their high stability. Of the subclones obtained by

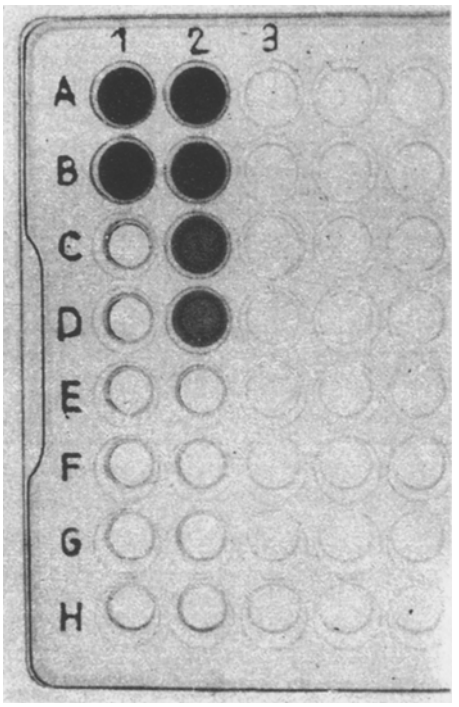

Fig. 1. Immunoenzyme testing of bispecific antibodies carrying antigenic determinants to α-endorphin and HRP. Immune planchets saturated with conjugate of α-endorphin with BSA and incubated with affinity-purified bispecific antibodies in a concentration of 5 μg/100 μl (A, B), with anti-α-endorphin antibodies in culture fluid of clone $F_8/40F_9$ (C, D), with antiperoxidase antibodies in culture fluid of clone $36F_9$ (E, F), and with washing buffer (G, H). Row 1 was further incubated with HRP solution (1 μg/100 μl), row 2 with conjugate of affinity-purified antibodies to mouse IgG with HRP. Peroxidase reaction carried out with *o*-phenylenediamine. Absorption at 472 nm was (averaged for two wells) over two in row 1 for wells A, B, and under 0.06 for wells C, D, E, F, G, and H. Corresponding values for wells A and B in row 2 were over 2, for wells C and D 1.196, and for wells E, F, G, and H under 0.13. For more details, see the section "Experimental Method".

their repeated cloning, 90% also exhibited both types of activity and were selected to detect bispecific antibodies. Bispecific antibodies were determined with the aid of immune planchets saturated with a conjugate of α-END with BSA, and subsequent incubations with the culture fluid and HRP. This testing confirmed that two clones and subclones obtained as a result of their cloning produce bispecific antibodies carrying antigenic determinants for α-END and HRP.

To purify the bispecific antibodies we used ascites fluid obtained from clone $56C_2$. Four types of antibodies were present in the ascites fluid: bispecific antibodies, antibodies to α-END, antibodies to HRP, and inactive antibodies. Light and heavy chains of immunoglobulins carrying antigenic determinants to different antigens (for example, the light chain from antibodies to α-END and the heavy chain from antibodies to HRP) recombine in all combinations in inactive antibodies. Since both parental hybridomas produce McAb belonging to the IgG class the antibodies were purified by chromatography on protein A-Sepharose. The yield of antibodies was about 8 mg/ml ascites fluid, which was taken to be 100% (Table 1). The IgG fraction was then purified by chromatography on BrCN-Sepharose, conjugated with HRP. Both the bispecific antibodies and the antibodies to HRP bind with peroxidase. Bispecific antibodies were isolated from the antibodies eluted from the HRP column by chromatography on BrCN-Sepharose, conjugated with α-END. MCA to HRP were antibodies not bound with α-END. The fraction not bound with HRP and which included McAb to α-END and inactive antibodies, was purified on an α-END column and, after chromatography, the fraction of antibodies to α-END and

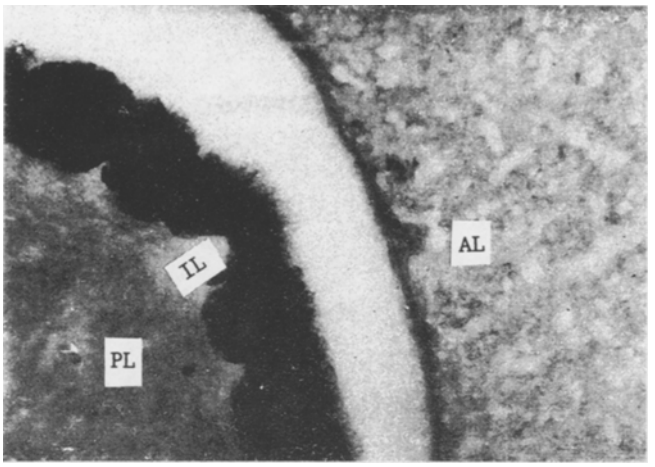

Fig. 2. Immunohistochemical localization of α-endorphin in rat pituitary gland with the aid of bispecific antibodies carrying antigenic determinants to α-endorphin and HRP. Pituitary sections incubated with a solution of purified bispecific antibodies and HRP overnight at 0°C. Peroxidase reaction carried out with diaminobenzidine. AL) Anterior lobe of pituitary, IL) intermediate lobe, PL) posterior lobe. For further details see the section "Experimental Method."

of inactive antibodies was estimated. Thus, by a combination of affinity chromatographies it is possible to estimate the fraction of bispecific antibodies, antibodies which bind only with HRP, antibodies which bind with α-END, and inactive antibodies. In addition, in each of the fractions thus obtained, ability to bind ^{125}I-α-END and HRP was determined. The results of fractionations are given in Table 1. Clearly the fraction of bispecific McAb was about 30% of the total IgG fraction, the fraction of inactive antibodies also was 30%, whereas fractions of McAb to α-END and to HRP were equal, each being 20%.

The results of immunoenzyme testing of bispecific antibodies are illustrated in Fig. 1. They show that only bispecific antibodies had the ability to bind α-END and HRP simultaneously (row 1, A, B).

In mammals endorphins are synthesized mainly in the intermediate lobe (about 90% of the endorphin-producing cells) and the anterior lobe (10-15% of endorphin-producing cells) of the pituitary [1]. Purified bispecific antibodies were tested for their ability to detect α-END immunochemically in the rat pituitary gland. It will be clear from Fig. 2 that a strong peroxidase reaction was observed in cells of the intermediate lobe of the pituitary. Single cells and groups of cells with a moderately strong peroxidase reaction were discovered in the anterior lobe of the pituitary. No peroxidase reaction could be detected in the posterior lobe of the pituitary.

Thus a tetradoma producing bispecific McAb, carrying antigenic determinants for α-END and HRP, was obtained.

LITERATURE CITED

1. A. D. Dmitriev, Progress in Science and Technology. Series: Pharmacology. Chemotherapeutic Agents. Vol. 13. Opioid Peptides and Their Receptors [in Russian], Moscow (n.d.), pp. 7-49.
2. Yu. S. Massino, E. S. Kakpakova, B. P. Kopnin, et al., Genetika, **17**, 1253 (1981).
3. Yu. S. Massino, V. V. Tsibezov, A. D. Dmitriev, et al., Byull. Éksp. Biol. Med., **103**, No. 11, 578 (1988).
4. Yu. S. Massino, V. V. Tsibezov, A. D. Dmitriev, et al., Biotekhnologiya, **3**, 730 (1987).
5. S. F. Pshenichkin, A. A. Zozulya, E. A. Kizim, et al., Biokhimiya, **53**, 309 (1988).
6. M. Berebbi and G. Meyer, Int. J. Cancer, **10**, 418 (1972).
7. A. V. Chervonsky, A. I. Faerman, L. V. Evdonina, et al., Molec. Immunol., **25**, 913 (1988).
8. I. Imbert, Y. Barra, and M. Berebbi, J. Cell Sci., **18**, 67 (1975).
9. I. F. Kearney, A. Radbruch, and B. Liesegang, J. Immunol., **123**, 1548 (1979).
10. R. D. Lane, J. Immunol. Meth., **81**, 223 (1985).

11. W. B. M. De Lau, V. A. E. Loon, K. Heije, et al., J. Immunol. Meth., **117**, 1 (1989).
12. N. Ling, R. Burgus, and R. Guillemin, Proc. Nat. Acad. Sci. USA, **73**, 3492 (1986).
13. J. S. Sobell, A. M. Albrecht, H. Reim, et al., Cancer Res., **31**, 297 (1971).
14. U. D. Staerz and M. J. Bevan, Proc. Nat. Acad. Sci. USA, **83**, 1463 (1986).

MECHANISM OF RECEPTION OF INFLUENZA VIRUSES BY SOMATIC TISSUE CELLS

V. M. Pleskov, V. P. Sukhinin, and S. S. Galitarov

UDC 578.832.1:578.23

KEY WORDS: influenza viruses; low-density lipoproteins; receptor-induced endocytosis.

Reception of virus particles by tissue cells takes place through high-affinity sites (receptors) located on the cell membrane [10].

A theory of receptor-mediated uptake of low-density lipoproteins (LDL) by tissue cells has recently been developed in detail [5, 6]. Analysis of the mechanisms or receptor uptake of virus particles and LDL indicates a large number of identical components of these processes. In both cases reception of virus and lipoprotein particles takes place through high-affinity sites, in three consecutive stages: binding of particles by receptors (internalization), endocytosis in the composition of coated vesicles, and LDL metabolism proper, or reproduction if the subject is virus.

LDL, whose composition includes large quantities of cholesterol (Chs) provide the tissue cells with this metabolite, which they need in order to construct their outer and inner membranes. The supply of Chs to the cell by the mechanism described above is therefore essential for the normal physiological activity of the tissue cells. It can accordingly be postulated that virus particles can penetrate into the cell through the same high-affinity sites as for LDL on the surface of the outer cell membranes. This hypothesis is based on the improbability that nature would create special receptors to transport virus particles inside the cell, which would ultimately lead to death of the cell.

The aim of the present investigation was to compare the structure of cellular receptors responsible for transport of LDL and virus particles, by comparing their immunological properties.

EXPERIMENTAL METHOD

Experiments were carried out on a tissue culture of human embryonic lung fibroblasts (HELF) at the 10th passage. Experiments on the cells were carried out in plastic Petri dishes 16 mm in diameter ("Falcon," USA), using the GPI-01 CO_2 inducer. The fibroblasts were used in the experiments after formation of a monolayer in 10% bovine serum in Eagle's medium; cells also were grown on Eagle's medium containing penicillin (100 U/ml), streptomycin (100 μg/ml), and nonlipoprotein serum proteins (bottom proteins) (d > 1.250 g/ml NaBr), isolated by ultracentrifugation by the method in [7], in a concentration of 5 mg/ml. Influenza virus strain A/PR8/34 was added to the cell culture in equal amounts and incubated in a CO_2 incubator at 37°C, and in an atmosphere containing 5% CO_2 for 5 h. The culture medium was then poured off, the cell culture was carefully washed with Eagle's medium containing antibiotics and bottom proteins in order to remove virus particles not bound with the cells, media of the above-mentioned composition were again added, and incubation in the

Laboratory of Pathomorphology and Pathogenesis of Influenza, All-Union Influenza Research Institute, Ministry of Health of the USSR, Leningrad. (Presented by Academician of the Academy of Medical Sciences of the USSR I. P. Ashmarin.) Translated by Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 112, No. 11, pp. 514-516, November, 1991. Original article submitted January 16, 1991.

 0007-4888/91/0011-1622$12.50