Acta virol. **30** : 199—205, 1986

PREPARATION AND CHARACTERIZATION OF HYBRIDOMAS SECRETING MONOCLONAL ANTIBODIES TO TICK-BORNE ENCEPHALITIS VIRUS

A. A. KUSHCH, *M. NOVÁK, YE. E. MELNIKOVA, S. Y. GAIDAMOVICH, *M. GREŠÍKOVÁ ,*M. SEKEYOVÁ, A. S. NOVOKHATSKY, T. G. MIKHEEVA, N. A. SVESHNIKOVA, *L. BORECKÝ, V. M. ZHDANOV

D. I. Ivanovsky Institute of Virology, U.S.S.R. Academy of Medical Sciences, 123098 Moscow, U.S.S.R.,
and *Institute of Virology, Slovak Academy of Sciences, 817 03 Bratislava, Czechoslovakia

Received August 5, 1985

Summary. — Monoclonal antibodies (MA) were prepared to two strains of tick-borne encephalitis (TBE) virus: strain 4072 isolated from a patient in the U.S.S.R. and low-pathogenic for mice strain Skalica, isolated from a bank vole *(Clethrionomys glareolus)* in Slovakia. MA specific to the 4072 and Skalica strains were produced by hybridomas of the KEN (60 clones) and NEK (65 clones) series, respectively. Chromosomal analysis of MA producing 114 hybridoma clones of both series revealed a great variability in the number of chromosomes either in the range of given clones or between individual clones. The hybridoma cells under study possessed a high degree of transformation manifested by good growth in the mouse peritoneal cavity and marked accumulation in ascitic fluid (AF).

Key words: tick-borne encephalitis virus; monoclonal antibodies; hybridomas

Introduction

Antigenic structure of TBE viruses is complex with several group- and type-specific determinants which are difficult, and sometimes impossible, to be differentiated in serological tests using polyclonal sera. MA to TBE virus were firstly prepared by Heinz *et al.* (1982, 1983, 1984). Novák *et al.* (1983), Grešíková and Sekeyová (1984) and Novokhatsky *et al.* (1984) provided further data concerning the appearance of biological activity and topographic connections of virus-specific proteins and determined subtler relationships between the viruses of the TBE complex and other flaviviruses.

This study was aimed at the preparing MA to two strains of TBE virus: strain 4072 isolated from a patient in the Ural region in the U.S.S.R. and strain Skalica isolated from a bank vole trapped in West Slovakia. The latter in contrast with the former virus strain was non-pathogenic for adult mice after subcutaneous inoculation. Our experiments resulted in obtaining

of more than 100 hybridomas secreting MA to TBE virus. This paper presents the results on production of MA and characterization of the hybridomas obtained.

Materials and Methods

Immunization of animals. BALB/c mice inoculated intraperitoneally were used throughout. In the case of strain 4072, crude 10% brain suspension of TBE-virus infected suckling mice was given 5 times in weekly intervals in 0.2 ml volumes. In the first three immunization doses, the virus was inactivated by beta-propiolactone and inoculated with an adjuvant (the mixture of arlacel and draceol), the last two doses consisted of the live virus without any adjuvant. Booster-dose of the virus in the form of 10% brain suspension thoroughly clarified by centrifugation was administered in 0.1 ml volume into the tail vein 4 days before cell fusion. Immunization with the Skalica strain was conducted using the live virus propagated in chick embryo fibroblasts and concentrated by ultracentrifugation.

Myeloma cells. The mouse myeloma cell line NS-O was used for hybridization. This cell line was chosen based on the comparative ability for cell fusion and production of hybridomas of three myeloma cell lines which synthesized neither heavy nor light Ig chains, namely P3-X63-Ag 8.653 (Kearney *et al.*, 1979), Sp2/O-Ag 14 (Schulman *et al.*, 1978) and NS-O (Thorpe *et al.*, 1983). With the use of high sensitive autoradiography (Tsoi *et al.*, 1984) there was found in three separated experiments that the myeloma cell line NS-O provided the highest yield of synkaryont hybridomas 2 hr after fusion with spleen cells.

Cell fusion. Spleen cells (2.2×10^8 and 1.5×10^8 from mice immunized with TBE virus strains 4072 and Skalica, respectively) were fused with myeloma NS-O cells in a 5 : 1 ratio, which was found according to our previous data (Tsoi *et al.*, 1984) more convenient than usually employed 2 : 1 and 10 : 1 ratios. The fusion was carried out with the use of 50% polyethylene glycol (PEG 4000) solution (Löba, Austria) according to the standard scheme (Köhler and Milstein, 1976). The resulting cells were transferred in 0.2 ml of the HAT medium to 96-well plates (Linbro) in a 1.5×10^5/well concentration.

Cultivation medium. The hybridomas were cultivated and cloned in Eagle s medium modified by Dulbecco (Gibco, U.S.A.) supplemented by 15% foetal calf serum, glucose (4.5 g/l), glutamine (2 mmol/l), gentamycin (50 μg/ml), bovine insulin (0.2 units/ml), and HEPES (10 mmol/l). The selection of hybrid cells was carried out in selective HAT medium (Littlefield, 1964) supplemented by hypoxanthine (0.1 mmol/l), aminopterine (0.004 mmol/l), and thymidine (0.016 mmol/l). Insulin and HEPES were added into the selective HAT medium as well as to the cloning medium. Suitability of the addition of these components was demonstrated in the series of special experiments, the results of which are given below.

Selection of antibody-secreting hybridomas was conducted by means of indirect immunofluorescence (IF) technique. Subconfluent monolayers of SPEV cells grown in the medium 199 without serum on the slides in the penicillin bottles were infected with 4—5 log LD_{50} units/0.1 ml of the TBE virus. After 24—48 hr depending on the degree of accumulation of virus antigens in the cells as evaluated by probatory staining, the slides with virus-infected cells were removed, washed with phosphate buffered saline solution, pH 7.2, fixed with cold acetone for 15 min and stored at —70 °C until used. The IF test was performed as described (Sveshnikova and Melnikova, 1984), using rabbit fluorescein-labelled anti-mouse globulin (N. F. Gamaleya Institute, Academy of Medical Sciences of the U.S.S.R.). To increase the contrast of staining, rhodamine-labelled bovine albumin was employed.

Karyological analysis. Chromosomal preparations were made according to the method of Moorhead *et al.* (1960). The number of chromosomes was calculated in 30 cells of each hybridoma line.

Results

Improvement of cloning conditions

To improve conditions of hybridoma cloning the series of 4 experiments aimed at determining the effects of insulin and HEPES on the cloning capability of hybrid cells were performed. In preliminary experiments there

was found that out of the HEPES buffer concentrations tested (5, 10, 15, 20, and 25 mmol/l), the best results were achieved with those of 10—20 mmol in l, which increased the rate of cell division and clone formation ensuring, at the same time, the necessary maintenance of pH in the medium.

The effects of insulin and HEPES were estimated based on the ability of clone formation and secretion of MA to influenza virus by 31-2-2, 31-2-3, 31-7, and 31-8 hybrid cells (Kushch *et al.*, 1985). To compare the number of clones formed in 96-well plates over the period of 6 days after introduction of hybrid cells (1 cell/well), one panel was used for cell cloning of each hybridoma in the presence and absence of insulin and HEPES. Calculation of mean values and their statistical analysis showed that significantly higher number of clones was produced in the medium containing insulin and HEPES ($61.4 \pm 13.5\%$) than in the absence of these components ($14 \pm 4.3\%$).

Fusion and cloning of hybrid cells

The abovementioned preliminary experiments allowed to proceed the hybridization studies. Two experiments were carried out: a) in the first experiment, the myeloma cell line NS-0 was fused with the spleen cells from mice immune to the 4072 strain of TBE virus, b) in the second one, the same myeloma cell line was fused with the Skalica virus immune spleen cells. In the former experiment 18 out of 77 samples were positive as to the production of MA to the 4072 strain at the first testing. Of them, 7 lost MA secreting ability during 1—2 weeks of cultivation and in 3 further wells the cells stopped growing 3 weeks after fusion. Remaining 8 positive cell cultures were cloned using the method of limiting dilutions. Spleen cells from non-immune mice in a 10^5/well concentration were used as feeders. The cell cloning resulted in obtaining of 115 clones, 60 of them secreting MA to the 4072 strain (Table 1). The clones of this series were designated KEN.

As a result of the latter experiment, 35 out of 88 samples were found to produce MA to the Skalica strain at the first testing on day 14 after fusion. However, at repeated testing on days 17—21 after fusion only 8 cell cultures retained their virus-specific MA secreting ability. When cloning the cells of these cultures by limiting dilution technique of 105 obtained clones 65 positives were selected. The clones of this series were designated NEK.

As follows from the data in Table 1, the cloning efficiency of hybrid cells secreting MA differed and varied from 12.5% (KEN-4) to 39.5% (KEN-2), being 30% on the average for 8 cultures tested. The proportion of positive clones also varied from 8 to 81% for the cells of KEN-7 and KEN-6 series, respectively.

Karyological analysis of hybridomas

Karyological analysis was performed with 56 hybridoma clones of the KEN series secreting MA to the 4072 strain and with 58 hybridoma clones of the NEK series producing MA to the Skalica strain. The number of metaphase chromosomes was counted. As can be seen in Table 2, the number

Table 1. Cloning of hybridomas secreting MA antibodies to the 4072 strain of TBE virus

	Hybridoma clone (designation)							
	KEN-1	KEN-2	KEN-3	KEN-4	KEN-5	KEN-6	KEN-7	KEN-8
Number of wells with clones	17/48	19/48	16/48	6/48	17/48	16/48	12/48	12/48
Cloning efficiency (%)	35	39,5	33	12,5	35	33	25	25
Number of clones secreting MA	8/17	5/19	11/16	4/6	9/17	13/16	1/12	9/12
Proportion (%) of productive clones	47	26	69	67	53	81	8	75

of chromosomes in the cells of the hybridoma clones of NEK series varied from 56 to 93 and in the cells of KEN series from 57 to 84, respectively. Statistical distribution of the mean values of the chromosome numbers in the cells of 114 investigated clones of either series also varied considerably (Table 2). No correlation was found, however, between the karyological characteristics and the stability of antibody synthesis or its intensity at the stage of cultivation under study.

Hybridomas of both KEN and NEK series were investigated as to their ability to induce tumour formation following administration into syngeneic animals. Adaptability of cells to mice and their capability to grow after intraperitoneal inoculation were compared. For this reason BALB/c or Pristane-presensitized outbred mice were inoculated intraperitoneally with the KEN 46-8, KEN 6-4, NEK 9-4 and NEK 12-4 cells. Data of the experiments are summarized in Table 3. They show that hybridomas of selected clones induced ascitic tumour formation in 50—70% inoculated mice and the number of cells in AF surpassed that of the inoculated cells. It is worth mentioning that ascitic tumours were also obtained after inoculation of hybrid cells into outbred mice, adaptation of the cells being similar (60%) to that in BALB/c mice. The results obtained testify to the good adaptability of the hybrid cells and their high proliferating activity during in vivo cultivation.

Discussion

Two experiments on the hybridization of the myeloma cell line NS-0 with the spleen cells of mice immunized with two strains (4072 and Skalica) of TBE virus resulted in obtaining of more than 100 clones secreting MA to the TBE virus strains in question. This was promoted by prevention of marked variation in pH values of the cultivation medium due to optimal HEPES buffer concentration and by stimulation of cell division due to insulin. As shown in our experiments, the addition of these components to the cloning medium significantly increased the cloning efficiency of the hybridomas.

Table 2. Chromosomal analysis of hybridomas secreting MA to TBE virus

Series (clones)	No. of	Distribution of means of chromosome numbers in hybridoma clones											
KEN	chromosomes	57–60	61–63	64–66	67–69	70–72	73–75	76–78	79–81	82–84			
	clones	3	4	5	12	14	12	4	1	1			
NEK	chromosomes	56–58	59–61	62–64	65–67	68–70	71–73	74–76	77–79	80–82	83–85	86–88	91–93
	clones	1	3	4	13	13	10	9	1	0	1	2	1

Table 3. Induction of ascitic tumour formation by hybridomas of the KEN and NEK series

TBE virus strain	Clone designation	No. of mice inoculated	No. of ascites obtained	Adaptability (%)	Mean No. of inoculated cells ($\times 10^7$)	Mean No. of cells in ascitic fluid ($\times 10^7$)	Titres of antibodies in IF test
4072	KEN series						
	6–4	17	9	53	not tested	not tested	not tested
	46–8	153	113	74	1–3	22.4	10 240
Skalica	NEK series						
	9–4	128	75	59	1–3	17.2	5 120
	12–4	166	97	58	not tested	not tested	not tested

Chromosomal analysis of the hybrid clone cells revealed a great variability in the chromosome number found between the individual clones. This indicates a high chromosomal instability of hybrid cells, in which, regardless of their intraspecies and/or intraline properties, considerable changes in the chromosome number occur within a comparatively short period (6—8 weeks) after clone formation.

The current experimental data allow to evaluate the stability of MA production by hybridomas. In our experiments out of 18 cultures secreting MA to the 4072 strain, 15 continued to grow rapidly, but only 8 of them (about 50%) retained MA production ability upon further passaging. Even less stable were hybridomas secreting MA to the Skalica strain: of 35 positive cultures only 8 (23%) remained productive during further passaging.

The loss of MA synthetizing and secreting ability of hybridomas was frequently encountered in the literature. Thus, out 28 clones producing MA to St. Louis encephalitis virus a stable MA production after further cultivation was preserved by 21 clones only (Roehrig *et al.*, 1983). Of 800 well-growing hybridomas, 50 secreted antibody to hepatitis B virus antigen (HBeAg) which they were prepared to (Ferns and Tedder, 1984), but only 18 clones (36%) maintained the ability to produce specific MA during further cultivation. Männel *et al.* (1982) obtained 250 hybridomas, 17 of them produced MA to interferon, but only one clone remained a stable MA producer. In the experiments on the preparation of MA to alpha-foetoprotein, 22 of 130 clones (17% of all wells) gave positive reaction for the presence of MA at 3 week interval after fusion, which was an efficiency by 10% lower than found at first testing on day 17 after fusion (Yazova *et al.*, 1984).

Therefore, hybridomas producing antibodies to various antigens displayed a great instability as to the preservation of the differentiated function of parent immunocytes. One can assume that this effect is most probably caused by the loss of structural or regulatory genes of heavy and/or light Ig chains of hybrid cells during their passaging. The great chromosomal instability observed in the KEN and NEK series of hybrid cells testifies this suggestion.

Of great practical importance is the retention in hybridomas of transformation properties of parent myeloma cells, namely the capability to induce tumours in inoculated animals. Our results demonstrated the high malignancy of investigated hybridomas producing MA to TBE virus strains manifested by both good adaptability of the hybrid cells to mice and high proliferative activity ensuring their accumulation in the ascitic fluid. The obtained hybrid cells of the KEN and NEK series secreting MA to TBE virus may be used for further investigations of the hybrid cell functions as well as with the analysis of secreted MA.

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