Acta virol. **35**: 365-372, 1991

MONOCLONAL ANTIBODIES TO TICK-BORNE ENCEPHALITIS (TBE) VIRUS: THEIR USE FOR DIFFERENTIATION OF THE TBE COMPLEX VIRUSES

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Received May 8, 1990

Summary. - Monoclonal antibodies (MoAbs) to Central European tick-borne encephalitis virus (strain Hypr) were used for differentiation of eight viruses of the TBE complex by indirect immunofluorescence. MoAb 11/B3 (in Western blot recognizing 52 and 70 kD polypeptides) reacted with five out of the eight TBE complex viruses, MoAb 13/E5 (anti-52 kD protein) reacted with the western or eastern subtype of TBE virus only, while MoAb 12/G4 (anti-70 kD protein) distinguished the western subtype of TBE virus from the rest of the TBE complex. These three MoAbs were able to differentiate the virulent strain Hypr from attenuated strains Skalica and Hy-HK-18-„3". MoAb 2/10C (anti-56 and 70 kD proteins) which reacted with all viruses of the TBE complex, recognized both virulent and attenuated strains of TBE virus.

Key words: tick-borne encephalitis virus; monoclonal antibody; antigen differentiation

Introduction

TBE virus is a flavivirus highly pathogenic for man; TBE together with the Lyme disease is the most important disease transmitted by ticks in Europe.

Similarly to other flaviviruses, the TBE virus RNA codes for 3 structural (C pre-M, E) and 7 nonstructural (NS1, NS2a and 2b, NS3, NS4a and 4b, NS5) proteins (Mandl *et al.,* 1989).

From the biological point of view, glycoprotein E is the most important, being responsible for haemagglutination activity, binding of the virus to cellular receptors and induction of protective immune response *in vivo* (Heinz *et al.,* 1981).

Detailed mapping of glycoprotein E by MoAbs revealed 19 different epitopes localized into three non-overlapping domains (Guirakhoo *et al.,* 1989). These domains are composed of family-specific, complex-specific, type- and subtype-specific antigenic determinants. The so called TBE complex is formed by TBE

virus together with Langat, Negishi, Omsk haemorrhagic fever, Kyasanur Forest disease, and louping ill viruses (De Madrid and Porterfield, 1974). The all are very closely related and difficult to differentiate. Powassan virus was also classified into this complex (Clarke, 1964). Using antibody adsorption, TBE virus strains could be further distinguished into western and eastern subtypes (Clarke, 1964).

Several attenuated strains of TBE virus have been derived by serial passages in tissue culture (Mayer, 1964) and were shown nonpathogenic for veanling mice after peripheral infection. Low virulence characterized the Skalica strain of TBE virus isolated from the bank vole *Clethrionomys glareolus* (Grešíková *et al.*, 1976).

In the present study we describe the properties of four MoAbs reacting with structural and nonstructural protiens of TBE virus and their use for differentiation of the viruses of TBE complex.

Materials and Methods

Viruses. Following viruses from the TBE complex were used: TBE virus strains Sofjin (eastern subtype) and Hypr (western subtype), Omsk haemorrhagic fever (OHF), louping ill, Kyasanur Forest disease (KFD), Langat TP-21, Negishi and Powassan. The attenuated strain from Skalica virus was obtained by courtesy of dr. M. Grešíková, the attenuated TBE virus strain derived from Hypr virus by serial passages in hamster kidney cells was the gift of dr. V. Mayer (both from the Institute of Virology, Bratislava).

Preparation of MoAbs. Hybridomas were prepared by a modified method of Köhler and Milstein (1975). Balb/c mice were immunized with Hypr virus using a brain suspension purified by centrifugation through a sucrose gradient. The virus inactivated with β-propiolactone was administered in incomplete Freund's adjuvant intraperitoneally in four doses (the last dose without adjuvant). Fusion of spleen cells with the myeloma cells Sp2/0-Ag14 at a ratio 5:1 was performed in 42 % polyethylene glycol (molecular weight 3600–4000) with 15 % dimethylsulphoxide. Resulting cells were cultured in RPMI-1640 (Sigma) supplemented with 20 % foetal calf serum, HEPES (15 mmol/l) and hypoxanthine, aminopterine and thymidine (Sigma) in 24-well plates with peritoneal exudate feeder cells. Supernatants from individual wells were assayed for production of specific antibodies by ELISA. Producing hybridomas were cloned by limiting dilution technique in 96-well plates with feeder cells. MoAbs were obtained in the form of ascitic fluids after intraperitoneal injection of hybridoma cells into Balb/c mice primed with paraffin oil.

Indirect immunofluoresce (IIF). Monolayers of SPEV cells grown in the medium 199 without serum on coverslips were infected with 10^4-10^5 PFU of individual viruses and incubated for 24–48 hr depending on the degree of accumulation of virus antigen in the cells. The cells were fixed with cold acetone for 15 min and stored at -70 ^{0}C. IIF was performed as described (Coons and Kaplan, 1950) using rabbit fluorescein-labelled anti-mouse globulin (Gamaleya Institute, Academy of Medical Sciences of the U.S.S.R., Moscow).

Haemagglutination inhibition test (HIT) was performed according to Clarke and Casals (1958) using goose erythrocytes at pH 6.4.

Plaque-reduction neutralization test (PRNT). Dilutions of ascitic fluids in L-15 medium containing 3 % newborn calf serum were mixed with an equal volume containing 50-100 PFU of TBE virus. After incubation for 1 hr at 37 ^{0}C the mixture was titrated on PS cells by plaque method under methylcellulose overlay (De Madrid and Porterfield, 1969).

Determination of antibody class and subclass. Determination was performed by dot test according to Hořejší and Hilgert (1983) using goat specific antisera (Sigma).

Indirect ELISA. Strain Hypr virion antigen was immobilized on the surface of wells of microtitration plates KOH-I-NOOR P (Koh-i-noor Dalečín, Czechoslovakia) at protein concentration 5 μg/ml in carbonate/bicarbonate binding buffer, pH 9.6. Hybridoma supernatants were tested undiluted, titration of mouse ascitic fluids was performed in PBS containing 2 % sheep serum. The tested samples were incubated in microtitration plates for 1 hr at 37 ^{0}C in humid box. After incubation the wells were washed with PBS and incubated with horseradish peroxidase-conjugated swine anti-mouse Ig (SwAM/Px SEVAC Prague, Czechoslovakia) in PBS containing 2 % sheep serum for 1 hr at 37 ^{0}C. After washing with PBS, the substrate (o-phenylendiamine, 1 mg/ml in phosphate-citrate buffer, pH 5.0 plus 1 μl of perhydrol/ml) was added and the enzyme reaction was stopped by addition of 2N H_2SO_4. The absorbance was measured at 492 nm in Titertek Multiskan MCC/340 spectrophotometer. The content of specific antibody activity was expressed as dilution reciprocal. The titre was defined as reciprocal value of such dilution which absorbance was $A_{492} \geqq 0.2$.

Immunoblotting. PS cells uninfected and TBE-infected were analysed by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate (SDS PAGE, Laemmli, 1970). The transfer of proteins from polyacrylamide gel to nitrocellulose (NC) membrane was carried out according to Towbin *et al.* (1979). After rinsing in T-PBS (PBS, pH 7.4 containing 0.05 % of Tween 20), the NC membranes were incubated for 2 hr with mouse immune ascitic fluids diluted according to the content of specific antibodies (adjusted to 1:128 ELISA titre). NC membrane was rinsed in T-PBS and incubated for 1 hr with peroxidase conjugated swine anti-mouse Ig (SwAM/Px SEVAC Prague, Czechoslovakia) diluted in PBS. Peroxidase reaction product was coloured by incubation of the blot in substrate solution (0.1 mol/l TRIS-HCl, pH 7.6, 0.01 % H_2O_2, 0.6 mmol/l 3,3-diaminobenzidine) and inhibited with 0.5 % NaN_3.

Results

Characterization of TBE virus-specific monoclonal antibodies

The isotype determination of our MoAbs revealed that they all are of IgG_1 isotype. Using the immunoblotting technique it was shown that MoAb 2/10C reacted with polypeptides of M_r 56 and 70 kD, MoAb 11/B3 with 52 and 70 kD

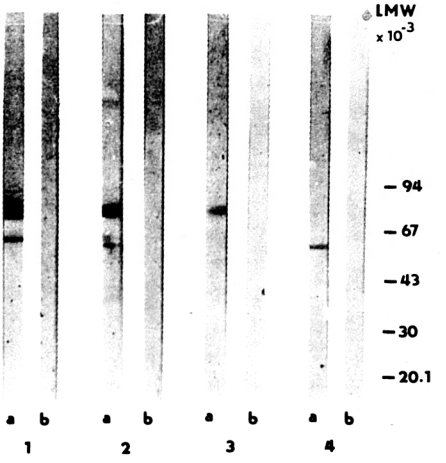

Fig. 1
Interaction of TBE virus-specific monoclonal antibodies with TBE virus antigens in immunoblot
1 - 2/10C, 2 - 11/B3, 3 - 12/G4, 4 - 13/E5
a - PS cells infected with the Hypr strain
b - uninfected PS cells
LMV - protein molecular weight standards (Pharmacia Uppsalla)

Table 1. Serological activity of TBE virus-specific monoclonal antibodies

MoAb	Isotype	ELISA	HIT	PRNT
2/10C	IgG_1	1600	< 10	<8
11/B3	IgG_1	51200	< 10	<8
12/G4	IgG_1	12800	< 10	<8
13/E5	IgG_1	51200	20	<8

Notice: titres expressed as dilution reciprocals (in all Tables)

proteins, the MoAb 12/G4 recognized a 70 kD protein and MoAb 13/E5 a protein with M_r of 52 kD (Fig. 1). On the basis of relative molecular weights of these antigens it may be deduced that our MoAbs reacted with the structural glycoprotein E (52–56 kD) and with the nonstructural protein NS3 (70 kD) (Heinz and Kunz, 1982).

The titres of MoAbs in ELISA in comparison with HIT and PRNT are shown in Table 1. The ELISA titres ranged from 1 600 to 51 200; only the MoAb 13/E5 possessed the HI activity, which confirmed its gpE specificity. No MoAb was able to neutralize the TBE virus.

Reactivity of TBE virus-specific monoclonal antibodies with the TBE complex viruses

Anti-TBE virus MoAbs differed considerably in their reactivity with individual viruses of the TBE virus complex by IIF (Table 2). MoAb 2/10C reacted with all viruses of the TBE complex, MoAb 11/B3 did not react with Langat, KFD and Powassan viruses. MoAb 13/E5 reacted with the two subtypes of TBE virus only. MoAb 12/G4 recognized a unique epitope of the western subtype of TBE virus. In most cases the differences in the reactivity with individual TBE complex viruses had a clearcut character. In a positive case MoAbs reacted in IIF until the dilution of 1:1 280 – 1:2 560, in the negative case they did not react

Table 2. Reactivity of TBE virus-specific monoclonal antibodies with the viruses of TBE complex in the indirect immunofluorescence assay

MAb	Hypr	Sofjin	Negishi	Louping ill	Langat	KFD	OHF	Powassan
2/10C	160*	80	80	20	160	160	40	80
11/B3	1280	1280	640	640	-**	-	640	-
12/G4	1280	-	-	-	-	-	-	-
13/E5	640	640	-	-	-	-	-	-

* antibody titre
** (-) denotes antibody titre < 20

Table 3. Reactivity of TBE virus-specific monoclonal antibodies with virulent and attenuated TBE virus strains in the indirect immunofluorescence assay

MoAb	Hypr	Skalica	Hy-HK-18-„3"
2/10C	160*	80	80
11/B3	1280	-**	-
12/G4	1280	-	-
13/E5	1280	-	-

* antibody titre
** (-) denotes antibody titre <20

in the dilution of 1:20.

Using TBE virus-specific MoAbs antigenic comparison of the prototype virulent strain Hypr with two attenuated TBE virus strains Skalica and Hy-HK-18-„3" was carried out in IIF (Table 3). Only MoAb 2/10C reacted with both virulent and attenuated strains. The other MoAbs clearly differentiated the virulent strain from the attenuated ones.

Discussion

Monoclonal antibodies prepared to TBE virus and recognizing the main structural glycoprotein E and nonstructural protein NS3 in immunoblotting were used for differentiation of TBE complex viruses. Individual epitopes characterized by these MoAbs seemed to represent as conserved as unique domains. While MoAb 2/10C reacted with all viruses of TBE complex and corresponding epitope could be considered group-specific, MoAb 11/B3 reacted with four viruses, and MoAb 13/E5 with the two subtypes of TBE virus only (TBE virus-specific). MoAb 12/G4 which bound to nonstructural protein NS3, was of subtype specificity, it reacted exclusively with the western European subtype of TBE virus.

For the first time MoAbs to glycoprotein E of TBE virus have been prepared by Heinz *et al.* (1982). These MoAbs were flavivirus group-reactive, TBE complex-specific, type- and subtype-specific (Heinz *et al.*, 1983). Together 19 epitopes defined by different MoAbs were (except for 3 isolated epitopes) divided into three non-overlapping domains termed A, B, and C, which differed in their serological specificity. Group-specific epitopes were only in domain A, domain B contained mostly TBE complex-specific epitopes and domain C subtype-specific determinants (Guirakhoo *et al.*, 1989). Similar more or less conserved epitopes have been demonstrated on other flaviviruses as

well (reviewed by Heinz, 1986). Sometimes such antigenic determinants have been identified by MoAbs in IIF, which presence on individual serologically defined groups did not correlate with this subgrouping (Gould *et al.*, 1985).

Attention was mostly paid to gpE, which possesses the most important biological activities. It is of interest that we found a subtype-specific epitope on the nonstructural protein NS3 which function is connected with the replication of viral RNA and which is considered for highly conserved in the *Flaviviridae* family (Chambers and Rice, 1987). Although a very close relationship between the western subtype of TBE virus and louping ill virus has been demonstrated (Heinz *et al.*, 1983; Stephenson *et al.*, 1984), MoAbs 12/G4 and 13/E5 clearly distinguished between them by IIF. MoAb 12/G4 reacted with the Hypr strain in a high titre but it did not react with other viruses of TBE complex not even in dilution 1:20. To our knowledge such MoAb unenquivocally differentiating the western subtype of TBE virus from all other viruses of this complex has not been described until now.

TBE virus-specific MoAbs were further used to differentiate the attenuated TBE virus strains Skalica and Hy-HK-18-„3" from the virulent strain Hypr. Skalica virus isolated from bank vole *Clethrionomys glareolus* (Grešíková *et al.*, 1976) was considered for TBE virus strain. It is thermosensitive, nonpathogenic for juvenile mice after extraneural infection (Rajčáni and Grešíková, 1982). The TBE virus strain Hy-HK-18-„3" derived from the virulent strain Hypr (Mayer, 1964) had similar properties. Only MoAb 2/10C reacted with both virulent and attenuated strains in IIF, while MoAbs 11/B3, 12/G4, and 13/E5 distinguished the attenuated strains from the virulent strain Hypr reacting exclusively with the latter.

Due to the lack of error correcting mechanisms in RNA replication, more frequent mutations can lead to the attenuation of a virulent RNA virus strain. With respect to relatively high percentage of changes in the nucleotide sequences coding for particular proteins of attenuated flaviviruses (Nitayaphan *et al.*, 1989), it is very likely that these changes will lead to modification of antigenic determinants typical for wild strains and that these antigenic changes would be detected by MoAbs.

Using MoAbs Gould *et al.* (1985) were able to differentiate the vaccine strains of yellow fever virus from the wild ones. They also prepared a MoAb reacting exclusively with wild type yellow fever viruses and used it to identify a wild-type antigenic variant in 17 D vaccine pools (Gould *et al.*, 1989). Three MoAbs to glycoproteins E_1 and E_2 were shown to distinguish between virulent and avirulent strains of Semliki Forest virus in ELISA and plaque reduction test (Boere *et al.*, 1985). Using the selection method in the presence of neutralizing MoAbs, Holzmann *et al.*, (1989) obtained a mutant with a single amino acid exchange in the E protein of TBE virus, which revealed a strongly reduced pathogenicity for mice after peripheral inoculation.

MoAbs to the Skalica strain have been prepared by Novák *et al.* (1983) and used for the study of antigenic relationships among viruses of the TBE

complex (Grešíková and Sekeyová, 1984). Using these MoAbs, Powassan and Langat viruses could be distinguished from other members of the TBE complex. Other collection of MoAbs prepared to TBE virus strains Skalica and 4072 (Kushch *et al.*, 1986) have been also applied to antigenic differentiation of TBE-related viruses (Gaidamovich *et al.*, 1986; Grešíková and Sekeyová, 1990). Using some of these MoAbs, it was possible to differentiate the Skalica strain from strains such as Sofjin, 4072 and Absettarov.

It is of interest that antigenic differences between highly attenuated strains Skalica and Hy-HK-18-„3" and virulent Hypr strain were found not only in the glycoprotein E, which is responsible for most biological activities of TBE virus, but also in the nonstructural protein NS3. This fact can be connected with differences in the replication rate, plaque morphology etc.

Using limited number of TBE virus-specific MoAbs it was possible to demonstrate marked antigenic differences among viruses of TBE complex. MoAb 12/G4 reacting exclusively with the western subtype of TBE virus can be used for identification of this virus using IIF test or ELISA. It was also shown, that relatively extensive antigenic changes could be connected with attenuation of flaviviruses. Greater panel of MoAbs with defined binding specificity to structural and nonstructural proteins of TBE virus together with a genome analysis are necessary for the characterization of changes responsible for attenuation of this virus.

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