MONOCLONAL ANTIBODIES DIRECTED AGAINST TICK-BORNE ENCEPHALITIS VIRUS WITH NEUTRALIZING ACIVITY *IN VIVO*

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Received December 16, 1993

Summary. – Monoclonal antibodies (MoAbs) were raised against the tick-borne encephalitis (TBE) virus, strain K23. The reactivities of 14 selected MoAbs were characterized by ELISA, Western blot analysis, haemagglutination inhibition, immunoprecipitation, *in vivo* protection and *in vitro* neutralization tests. All MoAbs reacted only with the glycoprotein E. The binding epitope of one MoAb could be delimited by a synthetic peptide to amino acids 306 – 339 representing one immunodominant loop structure of the glycoprotein E. The MoAbs exhibited individual reactivities against 13 different TBE virus isolates in ELISA and immunoblot test ranging from type-specific reactions to a broad reactivity with all isolates. Four MoAbs also showed a cross-reaction with other flaviviruses like West Nile virus and/or Yellow fever virus in immunoblot analysis. By competition ELISA the MoAbs could be divided into five different reaction patterns. Four MoAbs showed neutralizing activity with titers in the range 1:140 to 1:5 000 in an *in vitro* assay. These neutralizing activities could be confirmed by an *in vivo* mouse challenge model. The MoAbs are useful for diagnostic purposes and for differentiation of TBE virus strains and other flaviviruses.

Key words: tick-borne encephalitis virus; monoclonal antibodies

Introduction

TBE virus, a member of the flavivirus genus, is the cause of a tick-transmitted severe disease. Vaccines with inactivated TBE virus induce a protective immunity, which is mainly mediated by the glycoprotein E of TBE virus (Kunz *et al.*,1980; Klockmann *et al.*, 1989; Bock *et al.*, 1990). The virus consists of three structural proteins, core (C) protein, membrane (M) protein and envelope (E) glycoprotein. The glycoprotein E is responsible for the binding of the virus to the cellular receptor and exhibits haemagglutination activity. Several MoAbs directed against TBE virus have been described, most of them directed against glycoprotein E (Heinz *et al.*, 1982; Stephenson *et al.*, 1984; Kushch *et al.*, 1986). Most of them were analyzed for their neutralizing activity in cell culture. Here we described the characterization of 14 different MoAbs reacting with glycoprotein E in several *in vitro* tests as well as in an *in vivo* mouse challenge model.

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Materials and Methods

Cells and viruses. TBE virus strains (Klodobok, Absettarov, 2741 II, Petracova, 68/II, Dobrostan, Gbelce, Hypr, Sofyn, Tryp-sovky) as well as a TBE virus related Louping ill, causing illness in sheep, were described previously (Klockmann *et al.*, 1991). TBE virus strains were propagated in the A-549 cell line (Girad *et al.*, 1973) and subsequently inactivated by beta-propiolactone (BPL 0.05%, 4 °C, 24 hrs), followed by its degradation (37 °C, 3 hrs). Viruses were purified from cell-free culture supernatant by differential centrifugation and were pelleted through a 10% (w/v) sucrose cushion. Virus from 100 ml culture supernatant was re-suspended in 200 µ l PBS and used for coating of ELISA plates (Nunc) and immunoblot analysis. TBE virus strain K23 for the immunization of mice was obtained from Dr. Ackermann (Rhese-Kupper *et al.*, 1987). It was grown in chicken embryo fibroblasts, inactivated by 0.05% formaldehyde, and purified by continuous flow zonal ultracentrifugation (Electro Nucleonics Inc., NJ, USA). The TBE vaccine contained 3 µ g TBE virus antigen per ml and 0.2% $Al(OH)_3$ as adjuvant. Safety of this vaccine was tested in tissue cultures and laboratory animals.

Immunization, cell fusion and establishment of hybridoma cells. Four Balb/c mice were immunized intraperitoneally (ip) with 100 µg/400 µl purified TBE virus as described (Bock *et al.*, 1990); 50 µg antigen was inoculated 4 and 8 weeks later. Prior to fusion mice were boostered intravenously (iv) with 50 µg TBE virus. Three days after the last booster injection, the spleen cells were fused with SP2/0 cells according to Köhler and Milstein (1986). Ten to 14 days after fusion, supernatants of the hybridoma cell cultures growing in hypoxanthine/aminopterin/thymidine selection medium was tested for TBE virus-specific antibodies by ELISA. Hybridoma cells secreting antibodies against TBE virus were subcloned in 96-well microplate using a single cell manipulator.

Subclass determination of mouse immunoglobulins secreted by hybridomas were performed by the Ouchterlony technique.

ELISA. Microtiter plates (96-wells, Nunc) were coated with purified virus (5 – 10 µg/100 µl/well) in 0.02 mol/l Tris-HCl pH 7.6. After incubation at 4 °C for 12 hrs the plates were washed with washing buffer (0.01 mol/l Tris-HCl pH 7.2, 0.01% Tween 20), and were then incubated with 200 µl washing buffer containing 1% Lactoferin or BSA to block unspecific binding sites. After washing the plates 100 µl of hybridoma culture supernatants were added and incubated for 1 hr at 37 °C. The wells were washed and rabbit anti-mouse IgG-conjugated peroxidase (Dianova) was added and incubated for 1 hr at 37 °C. Bound enzyme detected by the reaction with tetramethyl benzidine in 0.15 mol/l citrate buffer containing 0.02% hydrogen peroxide as substrate.

Immunoblot analysis. Proteins from purified viruses were separated by 10% polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose sheets. The blots were incubated with hybridoma supernatants for 12 hrs at 4 °C. After three washes with PBS containing 0.01% Tween 20, they were incubated with goat anti-mouse IgG-alkaline phosphatase conjugate (Dianova, 1 hr, 20 °C). The blots were developed with Fast Red TR Salt (16 mg/ml in 0.2 mol/l Tris-HCl, 2 mmol/l NaCl, pH 8.0 buffer) and Naphtol As-MX phosphate (0.8 mg/ml).

Competition ELISA. Purified MoAbs were labelled with horseradish peroxidase according to Ishikawa *et al.* (1983). Unbound peroxidase was removed on Sephadex G25 column. TBE virus-coated ELISA plates were incubated with 200 µl/well cell culture supernatant (4 °C, 12 hrs) with different MoAbs. After washing the labelled MoAbs was added in concentration exhibiting A_{492} of 1.5 – 1.8 in ELISA. After 1 hr incubation the ELISA plates were washed and developed as described above. The inhibition of the binding of the second POD labelled MoAbs was considered strong reduction of the absorption above (50%), weak reduction (25 – 50%), none or only slight reduction below 25% as compared to the control.

Neutralization test. Purified MoAbs in different concentrations were incubated with 1 ml TBE strain K23 at 37 °C for 2 hrs. Two hundred µl of each mixture were seeded onto human lung carcinoma cells (line A-549) growing in microtiter plates; eight parallel cultures were used (Klockmann *et al.*, 1991). After 4 days cell cultures were examined microscopically and the neutralizing titer of the antibodies was determined as the reciprocal of the highest serum dilution inhibiting the CPE in 50% of cultures. Titers were calculated according to Reed and Muench (1938).

Immunoprecipitation assay. The immunoprecipitation assay with TBE virus antigen followed procedures described by Conraths *et al.* (1988). Anti-mouse IgG coupled to agarose (Sigma) was used for precipitating the complex. Precipitated proteins were separated by SDS-PAGE and blotted onto nitrocellulose. Immunostaining was done with TBE virus-positive human sera and anti-human IgG coupled with alkaline phosphatase (Dianova).

Haemagglutination inhibition test was performed in polystyrene microtiter plates (Greiner, FRG) essentially as described by Clarke and Casals (1958). After heating of 100 µl of purified MoAb (1 mg/ml) at 56 °C for 30 mins, 400 µl of a 20% kaolin (Behringwerke AG) suspension pH 9.0 was added and shaken for 10 mins at room temperature. After centrifugation for 10 mins at 2500 rpm (Heraeus minifuge) the supernatant was neutralized with 300 µl 0.05 mol/l phosphate/0.15 mol/l NaCl buffer pH 6.3. Fifty µl of twofold serial dilutions of pretreated MoAbs in borate buffer 0.05 mol/l borate/0.15 mol/l NaCl buffer pH 9.0 were mixed with an equal volume of TBE virus antigen. The antigen consisted of TBE virus (strain K23) suspension, concentrated and purified by continuous flow centrifugation and diluted with borate buffer to 8 haemagglutination units. After 1 hr incubation 100 µl of a 0.25% goose erythrocyte suspension in phosphate buffer (0.05 mol/l phosphate/0.15 mol/l NaCl buffer pH 6.0) were added and incubated for 1 hr at 20 °C. The mixture of equal volumes of borate buffer (pH 9.0) and phosphate buffer (pH 6.0) resulted in a final pH 6.3, the optimal pH for the test. Knob formation of erythrocytes at the bottom of the wells indicated haemagglutination inhibition. The haemagglutination inhibition titer was defined as the reciprocal of the highest serum dilution leading to knob-formation of erythrocytes.

Peptide mapping. Three linear peptides representing potential antigenic loop structures in the amino acid (aa) sequence of glycoprotein E of TBE virus were synthesizied using a modified method (Haist *et al.*, 1992), first described by Atherton *et al.* (1978) with Fmoc-(9-fluor-enylmethoxycarbonyl) protected amino acids and Tenta-Gel resin (Rapp Polymere, Tübingen, FRG) as solid support. Prior to purification by reversed phase HPLC (C2/C18 copolymer column PepS, Pharmacia/LKB) the cysteine residues in each peptide sequence were oxidized in order to form loop structures stabilized by disulfide-bridges. The peptides had the following sequence published by Pletnev *et al.* (1986) SRC-THLENRDFVTGTQGTTRVTLVLELGGCVT (aa 1-32), TVCKRDQSDRGWGNHCGLFGKGSIVACVK (aa 90-118), and MCDKTKFTWKRAPTDSGHDTVVMEVTFSGTKPCR (aa 306-339). The coating of 96-well multiplates (Nunc) with peptides was performed in carbonate buffer (0.15 mol/l pH 9.6). Then 100 µl/well hybridoma culture supernatant was added and incubated for 1 hr at 37 °C. The plates were washed (0.9% saline, 0.05% Tween 20) and 100 µl of horseradish peroxidase anti-mouse IgG (goat) conjugate (1:4000, Dianova) were added and incubated for 1 hr at 37 °C. After washing, 100 µl of the substrate (ortho-phenylenediamine, 0.1 H_2O_2 in acetate buffer pH 5.5) was added. The reaction was stopped with 100 µl 2.5 N H_2SO_4 and A_{490} read. In a competition analysis hybridoma culture superna-

Table 1. Reactivity of MoAbs in an in vivo mouse protection model

MoAb[a]	Days									Survived/infected mice	Virus isolation[b]
	0	0	2	4	6	8	10	12	14	21	21
19/75	*	+	#	#	#	#	#	#	#	2/2	0/2
19/324	*	+	#	#	#	#	#	#	#	1/2	1/2
19/694	*	+	#	#	#	#	#	#	#	2/2	0/2
19/1718	*	+	#	#	#	#	#	#	#	2/2	0/2
19/1786	*	+	#	#	#	#	#	#	#	2/2	0/2
Control	–	+	–	–	–	–	–	–	–	0/10	2/2

[a]Purified MoAbs were used.
*Mice were injected iv with 200 μg MoAb on day zero.
+Mice were challenged ip with 300 $TCID_{50}$/0.1 ml of TBE virus 4 hrs after the first dose of MoAb.
#Mice were injected ip with 500 μg MoAb on days indicated.
[b]Isolation of challenge virus was carried out in A-549 cells using homogenized mouse brain preparations. Number of successful isolations per number of animals tested is indicated.

tant was preincubated with each of the three different peptides (2 μg, 2 hrs, 37 °C) before the regular ELISA was performed.

In vivo mouse protection model. Three months-old Balb/c mice were injected iv with 200 μg of the purified MoAbs (1 mg/ml) (Table 1). After 4 hrs the mice were challenged ip with 300 $TCID_{50}$ of TBE virus. The titer of the challenge virus was evaluated in five groups of control mice with 10 animals in each group with tenfold dilutions of a standard TBE virus and calculated according to Reed and Muench (1938). On day 2,4,6,8,10,12, and 14 every mouse received additional 500 μg MoAb by ip route. The mice were checked daily for symptomes of disease and these surviving at day 21 were counted.

Results

Characterization of MoAbs

Fourteen different MoAbs directed against glycoprotein E of TBE virus (strain K23) were established. Eleven MoAbs belonged to the IgG1, two to the IgG2b and one to the IgG2a subclass (Table 2). In haemagglutination inhibition test six of the ten tested MoAbs inhibited the agglutination of erythrocytes with TBE virus showing the specificity of these MoAbs (Table 2). Using detergent-treated TBE virus in immunoprecipitation test ten MoAbs were able to precipitate the glycoprotein E.

In ELISA all MoAbs except one (19/573) showed cross-reactivity with the other virus strains (Table 3). Extremely low reactivities were observed with strains 274/II, Dobrostan and Louping ill for most of the MoAbs except 19/1493 and 19/1718, which showed relatively high cross-reactions with strains Dobrostan and Louping ill. In immunoblot analysis a more individual reaction pattern of the MoAbs against 12 different strains could be observed (Table 4). As in the ELISA, low reactivities were found with strains

Table 2. Anti-TBE virus reactivities of the MoAbs

MoAb	IgG[a]	HIT	NT	IP
19/52	IgG1	ND	ND	+
19/75	IgG1	2048	500	+
19/171	IgG1	2	<35	(+)
19/324	IgG1	–	<35	+
19/503	IgG1	ND	ND	+
19/573	IgG2a	–	<35	–
19/694	IgG1	2048	1000	+
19/1336	IgG2b	ND	ND	–
19/1367	IgG2b	512	<35	–
19/1418	IgG1	–	<35	+
19/1493	IgG1	512	<35	(+)
19/1718	IgG1	512	140	+
19/1737	IgG1	ND	ND	+
19/1786	IgG1	4096	5000	–

[a]Mouse IgG subclasses were determined used by the Ouchterlony technique.
HIT – haemagglutination inhibition test.
NT – neutralization test. MoAb titers starting from concentrated purified MoAb (1 mg/ml).
IP – immunoprecipitation test. Strong positive reaction (+), weak positive reaction ((+)), no reaction (–).
ND – not done.

Table 3. Reactivity of MoAbs with TBE virus isolates in ELISA

	Virus isolates						
MoAb	K23	Absettarov	Hypr.	274/II	Dobrostan	Klodobok	Gbelce
19/52	2.0	1.8	>2.0	0.1	0.2	1.4	1.4
19/75	0.9	1.1	1.4	0.1*	0.2*	0.7	0.9
19/171	>2.0	1.5	>2.0	0.2	0.5*	1.0	>2.0
19/324	0.5	0.2	0.3	–	–	–	–
19/503	>2.0	>2.0	>2.0	0.2*	0.4	0.6	1.3
19/573	1.3	0.2#	0.2#	–	–	–	–
19/694	1.1	0.8	1.2	0.1#	–	0.2	0.5
19/1336	1.0	0.7	0.9	0.1#	0.3	0.4	0.7
19/1367	1.7	0.8	1.3	0.4#	0.5	0.5	0.8
19/1418	1.2	0.4*	0.1	–	–	0.3*	0.3*
19/1493	>2.0	>2.0	>2.0	0.3	1.8	>2.0	>2.0
19/1718	>2.0	>2.0	>2.0	0.7#	0.7	1.1	>2.0
19/1737	>2.0	0.6	1.1	0.1*	0.5	0.3	0.5
19/1786	>2.0	>2.0	>2.0	0.2	0.2	1.2	>2.0

	Virus isolates					
MoAb	68/II	Cg1	Sofyn	Louping ill	Petracova	Trypsovsky
19/52	>2.0	>2.0	>2.0	0.3	1.5	1.6
19/72	0.8	1.0	0.7	0.2*	0.4	0.8
19/171	>2.0	>2.0	–	0.2	>2.0	>2.0
19/324	0.4*	0.4*	0.4*	0.1*	0.4*	0.4*
19/503	> 2.0	>2.0	>2.0	0.7	1.1	>2.0
19/573	–	–	–	–	–	–
19/694	0.4	0.6	0.3	0.1*	0.2	1.8
19/1336	0.7	1.0	0.9	0.5	0.4	0.7
19/1367	1.1	1.6	1.1	0.9	0.9	1.1
19/1418	0.4*	0.4*	0.3	–	0.3	0.3*
19/1493	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0
19/1718	>2.0	>2.0	1.6	1.5	1.1	0.8
19/1737	0.4	0.4	0.5	0.4	0.2	0.1
19/1786	>2.0	>2.0	>2.0	0.4	1.5	>2.0

A_{492} values were determined at different MoAb concentrations: 0.1 μg/ml (no label), 10 μg/ml (*), 100 μg/ml (#) and no reaction (–).

274/II, Dobrostan and Louping ill. Only MoAbs 19/171 and 19/1493 showed a prominent cross-reaction with these viruses (Fig. 1). A weak cross-reaction with West Nile (WN) virus was found with two MoAbs and a clear positive staining with MoAbs 19/1367 and 19/1718. Four of the MoAbs showed reactions with the vaccine strain 17D of Yellow Fever (YF) virus (19/503, 19/1367, 19/1418, 19/1493). In WN virus and YF virus antigen preparations 48 K and 62 K proteins were stained by the MoAbs (data not shown).

Epitope analysis

In competition ELISA using ten different labelled MoAbs as detection system we found five reaction patterns.

Table 4. Reactivity of MoAbs with TBE virus isolates and other flaviviruses in immunoblot analysis

	Virus isolates						
MoAb	K23	Absettarov	Hypr.	274/II	Dobrostan	Klodobok	Gbelce
19/52	++	+	+	+	(+)	+	+
19/75	++	+	+	–	–	+	–
19/171	++	++	++	++	++	++	++
19/324	++	–	–	–	–	(+)	–
19/503	++	+	+	–	(+)	++	+
19/573	++	(+)	(+)	(+)	–	(+)	(+)
19/694	++	–	–	–	–	–	–
19/1336	++	+	+	+	+	++	+
19/1367	++	+	+	–	(+)	++	+
19/1418	++	+	++	+	(+)	++	++
19/1493	++	++	++	+	++	++	++
19/1718	++	(+)	+	(+)	+	+	(+)
19/1737	++	(+)	–	–	–	(+)	–
19/1786	++	+	+	+	+	+	+

	Virus isolates						
MoAb	Cg1	Sofyn	Louping ill	Petracova	Trypsovsky	West Nile	YFV 17D
19/52	+	+	(+)	+	+	–	–
19/75	+	–	–	–	–	–	–
19/171	++	++	++	++	++	–	–
19/324	–	–	(+)	(+)	–	–	–
19/503	+	–	–	+	–	(+)	+
19/573	(+)	(+)	–	(+)	(+)	–	–
19/694	–	–	–	–	–	–	–
19/1336	+	+	++	+	+	–	–
19/1367	–	–	++	+	+	+	+
19/1418	+	+	(+)	+	+	–	+
19/1493	++	++	+	++	++	(+)	+
19/1718	–	–	(+)	(+)	–	+	–
19/1737	–	–	–	–	–	–	–
19/1786	+	+	+	+	+	–	–

Reactivity very strong (++), strong (+), weak ((+)), none (-).

To each group belonged two MoAbs with comparable reactions (19/75, 19/573; 19/171, 19/324; 19/694, 19/1786; 19/1367, 19/1418; 19/1493, 19/1718) (Table 5). Considering the blocking capacity of the unlabelled MoAbs we concluded that at least three different epitopes could be defined (Fig. 2). In the model presented the partial inhibition of MoAbs 19/725 by MoAbs 19/694, 19/1336, 19/1493, 19/1718, 19/1737 and the complete inhibition by MoAbs 19/1786 (Table 5) was not included. For the epitope 3 we did not consider the partial inhibition of MoAbs 19/1493 and 19/1718 by MoAbs 19/52 and 19/75 and the complete inhibition by MoAb 19/694.

Using ELISA plates coated with three different peptides representing three loop structures of glycoprotein E only one (19/573) of the fourteen MoAbs showed a reaction with the peptide aa 306-339. The reaction of this MoAb in ELISA could be blocked by pre-incubation with the homologous soluble peptide.

In vitro neutralization and in vivo protection assay

In neutralization test using the homologous TBE virus strain K23 only four (19/75, 19/694, 19/1718, 19/1786) of the ten tested MoAbs showed positive activity (Table 2).

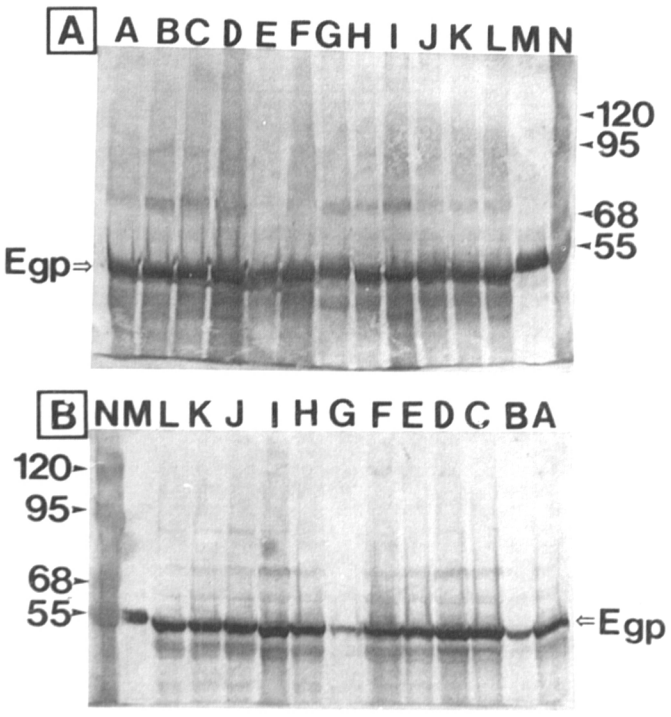

Fig. 1
Cross-reactivity of anti-TBE virus MoAbs with TBE virus strains in immunoblot
A: MoAb 19/171; B: MoAb 19/52
Strains Sofyn (lane A), Louping ill (B), Trypsovsky (C), 68/II (D), 274/II (E), Petracova (F), Dobrostan (G), Gbelce (H), Cgl (I), Hypr (J), Absettarov (K), Klodobok (L), K23 (M), molecular mass markers (N) with M_r values in thousands.

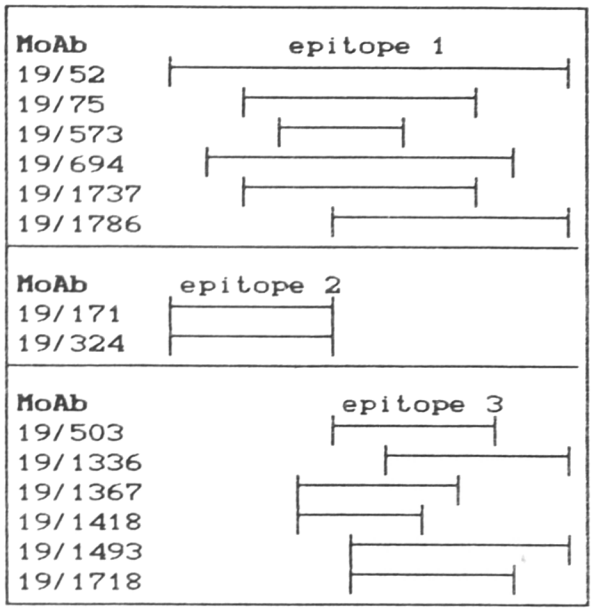

Fig. 2
Binding epitopes of MoAbs

These four MoAbs and one non-neutralizing MoAb (19/324) were used for an *in vivo* protection experiment in mice (Table 1). After challenge with 300 $TCID_{50}$ all ten untreated control mice showed symptoms of TBE virus infection beginning on day 9 and progressing until day 16 when all the mice died due to the virus infection. In contrast the MoAb-treated animals at this timepoint were still healthy except one which was treated with MoAb 19/324, lacking neutralizing activity *in vitro*. To analyze whether the mice were infected with TBE virus but without signs of infection we tried to reisolate virus from homogenized brain preparations. In none of the MoAb-treated animals we were able to isolate virus in contrast to the two control mice where the isolation was successful.

Discussion

Glycoprotein E, the major structural protein of TBE virus mediating protective immunity, has been intensively analyzed either for the formation of the polymeric complex or the antigenic and immunogenic properties (Heinz and Kunz 1979, 1980, 1981, 1982). These studies showed that the glycoprotein E is arranged as a homodimer which can be cross-linked by formaldehyde or dissociated by detergents. The antigen used for the generation of the MoAbs was inactivated by formaldehyde and purified by sucrose gradient centrifugation. Therefore we expected antibodies recognizing antigenic domains present on the surface of the virus particle as continuous or discontinuous epitopes. All fourteen MoAbs described in this investigation recognized exclusively glycoprotein E. From the immunoglobulin subtype analysis and the individual reaction patterns in different tests (ELISA, immunoblot, haemagglutination inhibition and neutralization tests) we conclude that we have selected MoAbs with different binding epitopes and avidities. Analysing the cross-reactivity with other TBE virus strains we found type-specific reactions in ELISA (MoAb 19/573) and immunoblot (MoAb 19/694) as well as broad cross-reactions in ELISA and immunoblot (MoAbs 19/171, 19/1493). Except the reduced reactivity with strains 274/II, Dobrostan and Louping ill we found a strong cross-reactivity with all other strains tested for the MoAbs 19/52, 19/1718, 19/178 in ELISA and immunoblot. This clearly demonstrates a low subtype variability of this antigenic region of glycoprotein E which is an agreement with previous results (Stephenson *et al.*, 1984; Klockmann *et al.*, 1991; Stephenson, 1985;

Table 5. Reactivity of MoAbs in competition ELISA using TBE virus strain K23 antigen

MoAb[a]	Labelled MoAbs[b]									
	19/75	19/171	19/324	19/573	19/694	19/1367	19/1418	19/1493	19/1718	19/1786
19/52	–	+	+	–	–	+	+	(+)	(+)	–
19/75	–	+	+	–	(+)	+	(+)	(+)	(+)	(+)
19/171	+	–	–	+	+	(+)	(+)	+	+	+
19/324	+	–	–	+	+	+	(+)	+	+	+
19/503	+	+	+	+	+	(+)	(+)	–	–	+
19/573	+	+	+	(+)	(+)	+	+	(+)	+	+
19/694	(+)	+	+	+	–	+	+	+	+	(+)
19/1336	(+)	+	+	+	+	(+)	+	–	–	+
19/1367	+	+	+	+	+	(+)	+	(+)	(+)	+
19/1418	+	+	+	+	+	+	–	+	+	+
19/1493	(+)	+	+	+	–	+	(+)	–	–	(+)
19/1718	(+)	+	+	+	–	+	(+)	(+)	(+)	–
19/1737	(+)	+	+	+	–	+	+	(+)	+	(+)
19/1786	–	+	+	+	–	+	+	(+)	(+)	–

[a]Purified MoAbs or cell culture supernatants were used for preincubation of TBE virus-coated plates.
[b]Labelled MoAbs were used in dilutions corresponding to A_{492} 1.5 – 1.8 determined in TBE virus ELISA.
A_{492} = (100%) – (>75%) corresponds to (+).
A_{492} = (75%) – (>50%) corresponds to ((+)).
$A_{492} \leq 50\%$ corresponds to (–).

Holzmann *et al.*, 1992). Analyzing the reactivity of the MoAbs with other members of the flavivirus family we found three MoAbs (19/503, 19/1367, 19/1493) cross-reacting with the corresponding proteins of WN and YF viruses. Two MoAbs showed only a cross-reaction either with WN virus (MoAb 19/1718) or with YF virus (MoAb 19/1418). Comparable results were published by Heinz *et al.* (1983) who also found limited cross-reactivity with WN virus for six out of sixteen MoAbs directed against TBE virus. These data demonstrated the high conservation of glycoprotein E confirmed also by serological and sequence analysis (Pletnev *et al.*, 1986; Heinz *et al.*, 1983; Mardrid and Porterfield, 1974; Mandl *et al.*, 1989).

All TBE virus-specific MoAbs described so far are directed against glycoprotein E (Klockmann *et al.*, 1989; Kushch *et al.*, 1986). Most of them were analyzed for their reactivity with a variety of TBE virus strains or related flaviviruses. A comprehensive analysis of the structural organization of glycoprotein E resulted in a two-dimensional model of this protein (Heinz and Kunz, 1982; Mandl *et al.*, 1989). On the basis of results of the peptide-ELISA we conclude that the epitope of MoAb 19/573 is represented by the region of aa 306-339 of glycoprotein E which was already described as epitope B by Mandl *et al.* (1988). The other MoAbs described here seem to react with conformational epitopes as they do not react with the continuous sequential epitopes representing loop structures of glycoprotein E. In case the other epitopes recognized by the antibodies are continuous epitopes one could localize the binding sites with sequential overlapping synthetic peptides as it was performed earlier with other MoAbs (Niedrig *et al.*, 1989).

In the competition ELISA with 10 labelled MoAbs 5 different reaction patterns could be observed. The experiments showed differences in the binding activity of some of the MoAbs depending on the antibody used for inhibiting the labelled indicator antibody. These differences can be explained by the induction of conformational changes by the unlabelled MoAb which influences binding and/or the avidity of the second labelled MoAb as demonstrated by Heinz *et al.* (1984). Grouping of the MoAbs into three different epitope reactivities neglected some minior blocking reactions which might results from conformational changes caused by the first MoAb. From the peptide- and the competition ELISA we have to assume that at least some of the MoAbs recognized discontinuous epitopes. The incompatibility binding behaviour of the MoAbs lead to an assumption of different lengths of the epitopes. The results

of the epitope model fitted also with the data of the haemagglutination inhibition test and the neutralization test. In both tests the MoAbs 19/171 and 19/324 showed no reactivity whereas the MoAbs 19/75 and 19/694 displayed good reactivity. The highest reactivity in both tests was found with MoAb 19/1786, what is an agreement with previous findings that HA-inhibiting, neutralizing and protective epitopes are topologically identical sites (Heinz and Kunz, 1982).

Four out of ten MoAbs showed *in vitro* neutralizing activity in a titer range 1:140 to 1:5 000. If we assume a molecular weight of 150,000 for the MoAb, we needed only 10 antibody molecules to neutralize 1 $TCID_{50}$ for the MoAb with the highest neutralizing activity (19/1786) and 50 molecules for MoAb 19/694. To confirm the neutralizing activity in an *in vivo* system we used a mouse protection model. In this model all mice treated with the neutralizing MoAbs (19/75, 19/694, 19/1718, 19/1786) were protected against the challenge with 300 $TCID_{50}$ of TBE virus strain K23. Treated animals did not show any sign of disease in contrast to the controls which became ill after one week and died till day 16. One of the two mice treated with MoAb 19/324, which showed no neutralizing activity *in vitro*, were infected and died on day 20. In all the mice protected we were unable to reisolate virus from brain suspensions. Stephenson *et al.* (1984) found five *in vivo* neutralizing MoAbs out of 16. However, a detailed comparison of the MoAbs described above with other neutralizing MoAbs is difficult due to differences in performing the neutralization tests.

Considering the type-specific reactivity of MoAb 19/1786 in immunoblot and its incapability to immunoprecipitate detergent-treated TBE virus, we assume that this MoAb reacts with an conformational epitope. The neutralizing MoAbs (19/75, 19/694, 19/1718) showing a low cross-reactivity in serological tests seem to recognize conformational and/or type-specific epitopes. Despite the great homology of TBE virus to other flaviviruses there is no conserved neutralizing domain within these groups (Mandl *et al.*, 1988; Grešíková and Sekeyová, 1987). This became obvious because even a single amino acid substitution in glycoprotein E resulted in a reduction of pathogenicity of TBE virus (Holzmann *et al.*, 1990). However, the homology between the TBE virus isolates seems to result in cross-protection of the vaccine against European and far Eastern subtypes (Klockmann *et al.*, 1991; Holzmann *et al.*, 1992). Furthermore, the data presented in this investigation clearly demonstrate that protection of mice by passive immunization against TBE virus infection and/or disease with the MoAbs is possible and is mediated by antibodies directed against glycoprotein E. An enhancement of TBE virus infectivity by antibodies in polyclonal sera as described by Phillpotts *et al.* (1985) was not observed in our mouse model system. Some of the neutralizing MoAbs might be useful for development of an immunoglobulin for passive immunization against the TBE virus infection. Besides that, the MoAbs are useful for diagnostic purposes and for differentiation of TBE virus strains and other flaviviruses.

Acknowledgement. The authors thank Mrs. B. Thom and Mr. G. Kaufmann for excellent technical assistance, Mr. H. Eife and Mr. H. Graf for performing the neutralization and haemagglutination inhibition tests. We also thank Prof. G. Wengler (Giessen, FRG) for providing WN virus, Prof. J. Lagé-Stehr (Berlin, FRG) for providing 17D YF virus, Dr. J.R. Stephenson (Porton Down, U.K.) for providing anti-TBE virus MoAbs and Dr. R. Johannsen for continuous interest in and support of our work.

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