Immunogenicity of Tick-borne Encephalitis Virus Glycoprotein Fragments: Epitope-specific Analysis of the Antibody Response

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SUMMARY

After digestion with trypsin, α-chymotrypsin, or chemical cleavage using CNBr, fragments of the tick-borne encephalitis (TBE) virus glycoprotein were isolated which retained their reactivity with neutralizing monoclonal antibodies defining a denaturation-resistant antigenic domain. Upon immunization of mice, these fragments induced antibodies reactive with the immunizing peptide, the denatured glycoprotein and the native glycoprotein as a constituent of the whole virus. The immune sera revealed the same properties as the monoclonal antibodies that were used to select the fragments for immunization: neutralizing activity; haemagglutination-inhibiting activity; blocking of the binding of antibodies used for selection; enhancement of the binding of other monoclonal antibodies defining a denaturation-sensitive antigenic domain. It was shown that the natural immune response against certain functionally important, denaturation-resistant immunogenic domains on the native protein can be closely mimicked by immunization with defined protein fragments. Antigenic sites present on these fragments may therefore represent essential constituents of a synthetic vaccine. The fine specificities of antibody populations in anti-peptide or anti-protein immune sera were analysed on the basis of single antigenic determinants by blocking assays using radiolabelled monoclonal antibodies that define eight distinct epitopes on the TBE virus glycoprotein. Quantitative differences in the blocking of certain monoclonal antibodies were also observed between human convalescent sera. The establishment of such blocking profiles using a panel of well-characterized monoclonal antibodies may represent a general method for dissecting the specificities of antibody populations present in polyclonal immune sera and could allow investigations on determinant-restricted differences of immune responses and its possible implications for the course of the disease.

INTRODUCTION

As a member of the flavivirus genus of the family Togaviridae (for review, see Porterfield, 1980), tick-borne encephalitis (TBE) virus contains three structural proteins (E, C, and M). The glycoprotein E (mol. wt. 53 000) has been shown to induce haemagglutination (HA)-inhibiting, neutralizing, and protective antibodies and confers immunity to TBE virus after active immunization (Heinz et al., 1981).

In an attempt to characterize immunochemical properties of antigenic determinants on the TBE virus glycoprotein we have prepared monoclonal antibodies and established a model showing the topological relationships, serological specificities and functions of eight distinct epitopes on the TBE virus glycoprotein (Heinz et al., 1983 a). The properties of these epitopes are compatible with results obtained with polyclonal immune sera and we can assume therefore that the monoclonal antibodies define those sites which are immunologically active also during a natural immune response. Although each epitope reveals distinct specificities, seven of the eight

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epitopes cluster to form two major non-overlapping antigenic domains, which are both involved in HA inhibition, neutralization, and protection. These two domains however not only are spatially separated but also reveal great differences with respect to their sensitivity to conformational changes and fragmentation (Heinz et al., 1983b). The antigenic reactivity of domain A (defined by monoclonal antibodies 6E2, 2B6, 4D9) was destroyed by incubation at pH 5.0 or treatment with guanidine hydrochloride or SDS, whereas domain B (defined by 1B3, 5D6, 2E7, 1G2) was resistant. For clarity, these epitopes have recently been redesignated as A1, A2, A3 and B1, B2, B3, B4 corresponding to the domains they specify (Heinz et al., 1984). As both domains bind HA-inhibiting and neutralizing antibodies and the presence of two different receptor binding sites is unlikely, different mechanisms for the function of these antibodies have been proposed: direct or closely adjacent binding to the receptor binding site on the one hand (domain A) and inactivation of the receptor binding site by the induction of a conformational change on the other (domain B). There is indeed evidence that binding of antibodies to domain B may alter the structural properties of domain A (Heinz et al., 1984).

Immunochemical analysis of proteolytic and CNBr cleavage products revealed the presence of fragments that were reactive with polyclonal immune sera and also with certain monoclonal antibodies (Heinz et al., 1983b). After trypsin and α-chymotrypsin digestion fragments of mol. wt. 9000 were shown to contain the epitopes of the denaturation-resistant domain B. A fragment with an estimated mol. wt. of 19000 was obtained by CNBr cleavage which reacted only slightly with one monoclonal antibody against domain B but revealed strong reactivity with the non-neutralizing monoclonal antibody 1C4 (redesignated C1). The epitope defined by this antibody is also denaturation-resistant and is located outside domains A and B. These analyses have shown that glycoprotein fragments can be prepared by SDS–PAGE which retain their reactivities with certain neutralizing monoclonal antibodies and with immune sera obtained by immunization with the native protein.

In the present study we describe the immunogenicities of these fragments in mice and characterize the antibody response with respect to specificity and function. Furthermore, we demonstrate that blocking assays using defined 125I-labelled monoclonal antibodies may provide a means of dissecting the specificities of antibody populations present in polyclonal immune sera on the basis of single antigenic determinants.

METHODS

Growth and purification of virus. TBE virus (strain Neudörfl) was grown in primary chick embryo cells, concentrated by ultracentrifugation, and purified by two cycles of sucrose density gradient centrifugation as described previously (Heinz & Kunz, 1981).

Preparation of glycoprotein 'rosettes'. The preparation of defined glycoprotein complexes containing both membrane proteins (E and M) was performed according to published procedures (Heinz et al., 1981). Briefly, purified virus was treated with Triton X-100 at a detergent to protein ratio of 10 : 1 and then subjected to density gradient centrifugation into detergent-free sucrose gradients to allow reassociation of the solubilized membrane proteins to form polymeric complexes. These 'rosettes' were located in the gradient by scanning at 280 nm and then dialysed against TAN (0.05 M-triethanolamine, 0.1 M-NaCl), pH 8.0.

Isolation of trypsic, α-chymotryptic, and CNBr glycoprotein fragments for immunization. In a previous publication (Heinz et al., 1983b) trypsinic and α-chymotryptic fragments of 9000 mol. wt. and a 19000 mol. wt. CNBr fragment were shown to be immunoreactive with neutralizing and protective monoclonal antibodies. For the preparative isolation of these fragments by SDS–PAGE, 600 μg of 'rosettes' were used as starting material for each preparation. Trypsin and α-chymotrypsin were added at an enzyme to protein ratio of 1 : 50 and incubated for 24 h at 37 °C. Then the samples were precipitated with TCA and resuspended in sample buffer for SDS–PAGE. For CNBr cleavage the TCA-precipitated rosettes were resuspended in 70% formic acid before the addition of CNBr (CNBr to protein ratio 2 : 1) and incubation for 24 h at room temperature in the dark. Formic acid and residual CNBr were removed by lyophilization.

The samples were then subjected to preparative SDS–PAGE in the system described by Laemmli & Favre (1973). By coelectrophoresis of prestained molecular weight markers (Bethesda Research Laboratories) in separate wells on the same gel the positions of the desired fragments could be located. The corresponding gel strips were removed from the gel, cut into four equal pieces (four immunizations), and stored at −80 °C. For immunization each piece was homogenized in 1 ml of phosphate-buffered saline (PBS), pH 7.4, and emulsified with 1 ml of complete or incomplete Freund's adjuvant.
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**Immunization of mice.** For each preparation, groups of nine adult mice were used. These were immunized intraperitoneally with 0.2 ml per dose. For the first immunization complete Freund's adjuvant was used and three further booster doses, each 4 weeks apart, were given in incomplete Freund's adjuvant. Four weeks after the last immunization blood was collected by eye vein puncture and identical serum volumes from individual mice out of each group were pooled.

**Blocking assays using 125I-labelled monoclonal antibodies.** For these tests, polystyrene beads (6.4 mm diam., Precision Plastic Ball Co., Chicago, Ill., U.S.A.) coated with purified TBE virus were used as a solid phase and the test procedure was performed as described previously for the topological mapping of monoclonal antibody-defined epitopes (Heinz et al., 1983a). Briefly, TBE virus-coated beads were first incubated with a fixed dilution (1:20) of immune serum and then with each of the eight radiolabelled antibodies to analyse the influence of the specific antibodies present in immune sera on the binding of each monoclonal antibody. To correct for non-specific binding, uncoated balls were tested in parallel and results were expressed as the percentage of counts bound in the absence of immune serum.

**Immunoblotting.** This was performed essentially according to Towbin et al. (1979) as described in detail previously (Heinz et al., 1983b). Mouse immune sera to be tested for their reactivity with tryptic, α-chymotryptic and CNBr fragments were assayed at a dilution of 1:100.

**Enzyme immunoassay (EIA).** Microtitre plates (Nunc) coated with a standard concentration (2 µg/ml) of purified TBE virus, the Far Eastern subtype of TBE virus (strain Sofyn), louping ill virus, or West Nile virus were used as a solid phase to analyse the reactivities and serological specificities of mouse immune sera. The exact performance of this test has been described (Heinz et al., 1983a).

**Haemagglutination inhibition (HI) and neutralization tests.** HI assays were performed as described by Clarke & Casals (1958) using goose erythrocytes and a final pH of 6.4. Neutralization tests were performed in PK-15 cells grown in microtitre plates. Serial twofold serum dilutions were incubated with 100 TCID<sub>50</sub> of TBE virus and incubated at 37 °C for 90 min. PK-15 cells were added to the incubation mixtures and after incubation for 3 days at 37 °C the cells were checked for the presence or absence of c.p.e. to determine the neutralization titre. Negative mouse immune serum was included as a control.

**RESULTS**

Proteolytic and CNBr fragments of the TBE virus glycoprotein, which have previously been shown to be immunoreactive with polyclonal immune sera and certain neutralizing monoclonal antibodies (Heinz et al., 1983b), were isolated by preparative SDS-PAGE as described in Methods. Mice were immunized four times with approximately equal molar amounts of (i) native glycoprotein rosettes, (ii) a fragment of 9000 mol. wt. obtained after trypsin digestion (tryp-9000), (iii) a fragment of 9000 mol. wt. after α-chymotrypsin digestion (α-chym-9000), and (iv) a fragment of 19000 mol. wt. after CNBr cleavage (CNBr-19000). The corresponding amounts applied per immunization and per mouse were therefore 16.6 µg, 2.7 µg, 2.7 µg, and 5.7 µg, respectively. Fig. 1 shows the analysis of the resulting immune sera in an immunoblotting experiment.

The immune serum against glycoprotein rosettes (Fig. 1a) reacted with the denatured E protein, with tryp-9000 and α-chym-9000 as well as with several CNBr fragments, again confirming that antibodies against denaturation-resistant antigenic determinants are induced after immunization with the native glycoprotein (compare also Heinz et al., 1983b). After immunization with tryp-9000 and α-chym-9000, antibodies reactive with these fragments as well as with the whole glycoprotein were detected (Fig. 1b, c). However, no reactivity with CNBr-19000 could be observed.

The immune serum against CNBr-19000 also recognized the whole E protein, but not the 9000 mol. wt. fragments obtained after trypsin and α-chymotrypsin treatment (Fig. 1d). Apart from the reaction with the fragment used for immunization (19000 mol. wt.), this serum also reacted with higher mol. wt. bands apparently derived from incomplete cleavage. The same bands were also detected by the immune serum against the native glycoprotein (compare Fig. 1a).

To analyse whether these anti-peptide immune sera also reacted with a more native conformation of the glycoprotein, an enzyme immunoassay was performed using whole purified TBE virus as the solid phase (Fig. 2, filled circles). As can be seen from the figure, these fragments induced antibodies reactive also with the native glycoprotein present on whole virus (filled circles in Fig. 2b, c, d), although the titre was approximately tenfold lower compared to
the immune serum obtained after immunization with equimolar amounts of native glycoprotein rosettes (filled circles in Fig. 2a).

In this assay we also analysed the serum from each mouse used for immunization to find the degree of variation in the individual immune responses (data not shown). Series of titration curves were obtained for the sera from each group of mice with the titres spreading over a 10- to 100-fold range. Seroconversion was 100%.

The serological specificities of these sera were determined in the same assay by analysing their reactivities with TBE strain Sofyn (Fig. 2, empty circles), louping ill virus as a member of the same flavivirus serocomplex (Fig. 2, filled triangles), and West Nile virus as a more distantly related flavivirus (Fig. 2, filled squares). Anti-CNBr-19000 revealed somewhat reduced reactivity with louping ill virus and Sofyn, whereas anti-tryp-9000 and anti-α-chym-9000 did not differentiate between TBE virus Western subtype, Far Eastern subtype, and louping ill virus. These serological specificities correspond to those of the monoclonal antibodies which define epitopes on these fragments.

The functional activities with respect to HI and neutralization are summarized in Table 1. The fragments analysed induced HI and neutralizing antibodies at a significant level compared to negative controls; the titres however were considerably lower than those obtained with the glycoprotein rosettes.

To analyse whether the anti-peptide sera revealed similar binding specificities on the native glycoprotein as the monoclonal antibodies that define epitopes on these peptides, competitive blocking experiments were performed. These tests assayed the influence of each immune serum on the binding of the eight monoclonal antibodies previously described (Heinz et al., 1983a), which define eight distinguishable epitopes on the TBE virus glycoprotein. The results are presented in Fig. 3. All eight monoclonal antibodies were blocked (with the possible exception of A1) by the immune serum against the native glycoprotein. The blocking profile obtained with the anti-peptide sera however was significantly different. Anti-tryp-9000 and anti-α-chym-9000 both efficiently blocked the binding of B1, B2, B3, and B4. These antibodies define epitopes that constitute the denaturation-resistant antigenic domain B on the native glycoprotein, and we
Fig. 2. Titration of mouse immune sera in EIA using microtitre plates coated with purified TBE virus (●), the Far Eastern subtype (strain Sofyn) (○), louping ill virus (▲), and West Nile virus (■). Immune sera were obtained after immunization with (a) glycoprotein rosettes, (b) tryp-9000, (c) α-chym-9000 or (d) CNBr-19000.

Table 1. Functional activities of mouse immune sera to TBE virus obtained after immunization with glycoprotein fragments

<table>
<thead>
<tr>
<th>Antibody titre</th>
<th>HI test</th>
<th>NT</th>
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<tbody>
<tr>
<td>Anti-E-rosettes</td>
<td>640/1280</td>
<td>256</td>
</tr>
<tr>
<td>Anti-tryp-9000</td>
<td>40</td>
<td>16</td>
</tr>
<tr>
<td>Anti-α-chym-9000</td>
<td>40</td>
<td>16</td>
</tr>
<tr>
<td>Anti-CNBr-19000</td>
<td>20</td>
<td>8</td>
</tr>
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have shown recently (Heinz et al., 1984) that the same monoclonal antibodies also enhance the binding of other monoclonal antibodies (A1, A2, A3) which define a separate, conformation-dependent antigenic domain. The same phenomenon was also revealed by the anti-peptide sera (Fig. 3b, c), which further indicates that similar antigenic determinants are involved in the polyclonal immune response against these fragments. Yet another blocking profile was obtained with anti-CNBr-19000: strong blocking of C1, enhancement of A1 and little influence on the binding of the other monoclonal antibodies except B4. In summary, the fragments used for immunization induced antibodies which resembled the immune serum against the native glycoprotein in their ability to block monoclonal antibodies B1, B2, B3, B4, and C1. Antibodies blocking A3, A2, and probably A1 (i.e. antibodies against the denaturation-sensitive domain) were induced only by the native protein.

These results obtained with immune sera against glycoprotein fragments revealed that blocking assays using defined monoclonal antibodies may represent a valuable test for assaying the determinant specificity of antibodies present in polyclonal sera. We therefore further...
analysed whether differences exist in the blocking profiles obtained with different animal and human sera (Fig. 4). The rabbit hyperimmune serum obtained by immunization with whole purified virus blocked all eight monoclonal antibodies (Fig. 4a). A mouse immune serum obtained after immunization with a suspension of formalin-inactivated infected mouse brain revealed a similar pattern, with the exception of A1 which was not only not blocked but enhanced. Two human TBE convalescent sera were also included in this study (Fig. 4c, d). Although all monoclonal antibodies were blocked to a certain extent, quantitative differences in the relative blocking efficiencies were observed. For instance, both sera revealed approximately equal blocking of A3, which defines the neutralizing epitope of the denaturation-sensitive antigenic domain A (Heinz et al., 1983a). Antibodies B1 and B2, however, which define two neutralizing epitopes within the denaturation-resistant domain B, were blocked much more
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efficiently by serum 2 than by serum 1. The methodology applied therefore allows, at least on an operational basis, elucidation of differences in the polyclonal immune response against a given set of monoclonal antibody-defined antigenic determinants.

DISCUSSION

It has been shown repeatedly for different viruses (Breschkin et al., 1981; Burstin et al., 1982; Chanas et al., 1982; Kimura-Kuroda & Yasui, 1983; Massey & Schochetman, 1981; Mathews & Roehrig, 1982; Nowinski et al., 1981; Volk et al., 1982; Yewdell & Gerhard, 1982) including TBE virus (Heinz et al., 1983a) that only antibodies directed against critical sites on viral proteins are capable of inactivating certain viral functions, like HA activity or infectivity. This is also important for the design of synthetic vaccines. It has been shown that almost any peptide sequence that is exposed on the surface of a given protein may induce antibodies which not only react with the immunizing peptide but also with the native protein (Green et al., 1982). However, mere binding of antibodies is not sufficient for functional activity and only those which are directed against certain critical sites will neutralize the virus.

We have approached this problem by using functionally exactly characterized monoclonal antibodies as probes for searching such critical sites on the TBE virus glycoprotein and fragments derived from it. These studies revealed that at least two independent and structurally different antigenic domains are involved in the neutralization of TBE virus. The antigenic reactivity of one of the two domains (domain A) is strongly dependent on the native conformation of the protein (Heinz et al., 1983b) and therefore contains so-called 'conformational' antigenic determinants, which may even represent 'discontinuous' antigenic sites composed of amino acid sequences which are distantly located on the polypeptide chain (Atassi, 1980; Berzofsky et al., 1982; Crumpton, 1974). The construction of synthetic immunogens having a structure resembling such topological determinants represents a difficult immunochimical problem but has been approached by 'surface simulation synthesis' of certain discontinuous antigenic sites (Atassi, 1980). The second antigenic domain (domain B) on the TBE virus glycoprotein involved in neutralization, however, is resistant to conformational change and denaturation. Using monoclonal antibodies as probes, epitopes within this domain could be located on fragments obtained by proteolytic and chemical cleavage (Heinz et al., 1983b). Thus, tryptic and \( \alpha \)-chymotryptic peptides of 9000 mol. wt. and a CNBr fragment of 19000 mol. wt. were identified as carrying functionally important antigenic sites.

As shown in the present study, these fragments in mice induced antibodies which were reactive with the immunizing peptide, the denatured glycoprotein, the native glycoprotein, and which also inhibited HA and viral infectivity. The properties of the antibodies induced, including their serological specificity, corresponded to the properties of the monoclonal antibodies used to select these fragments for immunization. This is further substantiated by blocking experiments performed with the eight \( {\text{I}}^{125} \)-labelled monoclonal antibodies previously described. Immune sera against the tryptic and \( \alpha \)-chymotryptic 9000 mol. wt. fragments blocked the binding of all the monoclonal antibodies used to select these fragments (domain B). These antibodies have also been shown to induce a conformational change within domain A, resulting in enhanced binding of other monoclonal antibodies to this domain (Heinz et al., 1984) and this property is also mimicked by the polyclonal antibodies induced by these fragments. CNBr-19000 on the other hand has been selected by antibody C1 and again, anti-CNBr-19000 closely resembles the properties of C1: blocking of \( {\text{I}}^{125} \)-labelled C1 and enhancement of \( {\text{I}}^{125} \)-labelled A1. These denaturation-resistant domains are immunogenic on the native glycoprotein, but the antibody response to these sites can be closely mimicked by immunization with defined fragments obtained by proteolytic and chemical cleavage. These sites induce HI and neutralizing antibodies and therefore may represent essential constituents of a synthetic vaccine.

We have recently described cooperative effects between antibodies to distant antigenic sites including neutralizing sites (Heinz et al., 1984) which result in higher binding avidities of antibodies to their corresponding epitopes. This seems to be a frequent phenomenon for an antibody response to protein antigens including viral proteins (Lefrancois & Lyles, 1982;
Lubeck & Gerhard, 1982; Tosi et al., 1981) and may considerably enhance the effectiveness of a neutralizing immune response. With respect to synthetic vaccines, it may therefore be important to include more than one peptide carrying a critical determinant in order to induce a sufficiently protective immune response.

It is known that the antibody response to single antigenic determinants is under separate genetic control (Maron et al., 1973; Mozes et al., 1971) and genetically restricted antibody responses have recently also been shown for determinants on hepatitis B surface antigen (Milich & Chisari, 1982; Milich et al., 1983). Since the effect of antibody binding depends on the exact location of its epitope, it cannot be excluded that the fine specificities of the antibodies induced may have an influence on the outcome of the disease. The use of blocking assays as described in this paper employing a panel of well-characterized monoclonal antibodies allows us to obtain characteristic blocking profiles which can yield information about individual variations in the fine specificities of polyclonal immune sera. Further analysis will be necessary to assay whether, apart from other factors of virus-host interactions, variations in the immune-responsiveness to certain antigenic determinants may influence the course of infection.

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