

# Variation of the Specificity of the Human Antibody Responses after Tick-Borne Encephalitis Virus Infection and Vaccination

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## ABSTRACT

Tick-borne encephalitis (TBE) virus is an important human-pathogenic flavivirus endemic in large parts of Europe and Central and Eastern Asia. Neutralizing antibodies specific for the viral envelope protein E are believed to mediate long-lasting protection after natural infection and vaccination. To study the specificity and individual variation of human antibody responses, we developed immunoassays with recombinant antigens representing viral surface protein domains and domain combinations. These allowed us to dissect and quantify antibody populations of different fine specificities in sera of TBE patients and vaccinees. Postinfection and postvaccination sera both displayed strong individual variation of antibody titers as well as the relative proportions of antibodies to different domains of E, indicating that the immunodominance patterns observed were strongly influenced by individual-specific factors. The contributions of these antibody populations to virus neutralization were quantified by serum depletion analyses and revealed a significantly biased pattern. Antibodies to domain III, in contrast to what was found in mouse immunization studies with TBE and other flaviviruses, did not play any role in the human neutralizing antibody response, which was dominated by antibodies to domains I and II. Importantly, most of the neutralizing activity could be depleted from sera by a dimeric soluble form of the E protein, which is the building block of the icosahedral herringbone-like shell of flaviviruses, suggesting that antibodies to more complex quaternary epitopes involving residues from adjacent dimers play only a minor role in the total response to natural infection and vaccination in humans.

## IMPORTANCE

Tick-borne encephalitis (TBE) virus is a close relative of yellow fever, dengue, Japanese encephalitis, and West Nile viruses and distributed in large parts of Europe and Central and Eastern Asia. Antibodies to the viral envelope protein E prevent viral attachment and entry into cells and thus mediate virus neutralization and protection from disease. However, the fine specificity and individual variation of neutralizing antibody responses are currently not known. We have therefore developed new *in vitro* assays for dissecting the antibody populations present in blood serum and determining their contribution to virus neutralization. In our analysis of human postinfection and postvaccination sera, we found an extensive variation of the antibody populations present in sera, indicating substantial influences of individual-specific factors that control the specificity of the antibody response. Our study provides new insights into the immune response to an important human pathogen that is of relevance for the design of novel vaccines.

The genus *Flavivirus* of the family *Flaviviridae* comprises several important mosquito- and tick-transmitted human pathogens, including yellow fever (YF), dengue, West Nile (WN), Japanese encephalitis (JE), and tick-borne encephalitis (TBE) viruses (1). For humans, live-attenuated vaccines are available against YF (2) as well as JE (3), and inactivated vaccines are available against JE (3) and TBE (4), but no dengue vaccine has reached the market so far (5). The induction of neutralizing antibodies is generally believed to be essential for long-lived flavivirus immunity (6, 7). Studies with polyclonal and monoclonal antibodies (MAbs) have provided detailed insights into the mechanism of virus neutralization through the inhibition of viral entry functions (7–9). In contrast, less is known about antibody populations with different specificities in polyclonal sera, their relative concentrations, and their contributions to virus neutralization. Nevertheless, deconstructing the antibody specificities in sera is essential for investigating the antibody repertoire produced by long-lived plasma cells (LLPCs) in the bone marrow, which are the main suppliers of antibodies circulating in the blood (10–12). The as-yet-undefined mechanisms of selecting only a subset of cells to generate the LLPC

repertoire in the bone marrow are certainly an important factor contributing to the poorly understood phenomenon of antibody immunodominance (13).

Flaviviruses have a relatively simple molecular organization, with a nucleocapsid (composed of the positive-stranded RNA and the capsid protein C) that is surrounded by a lipid envelope with two membrane-associated proteins, M and E (14). Virus assembly

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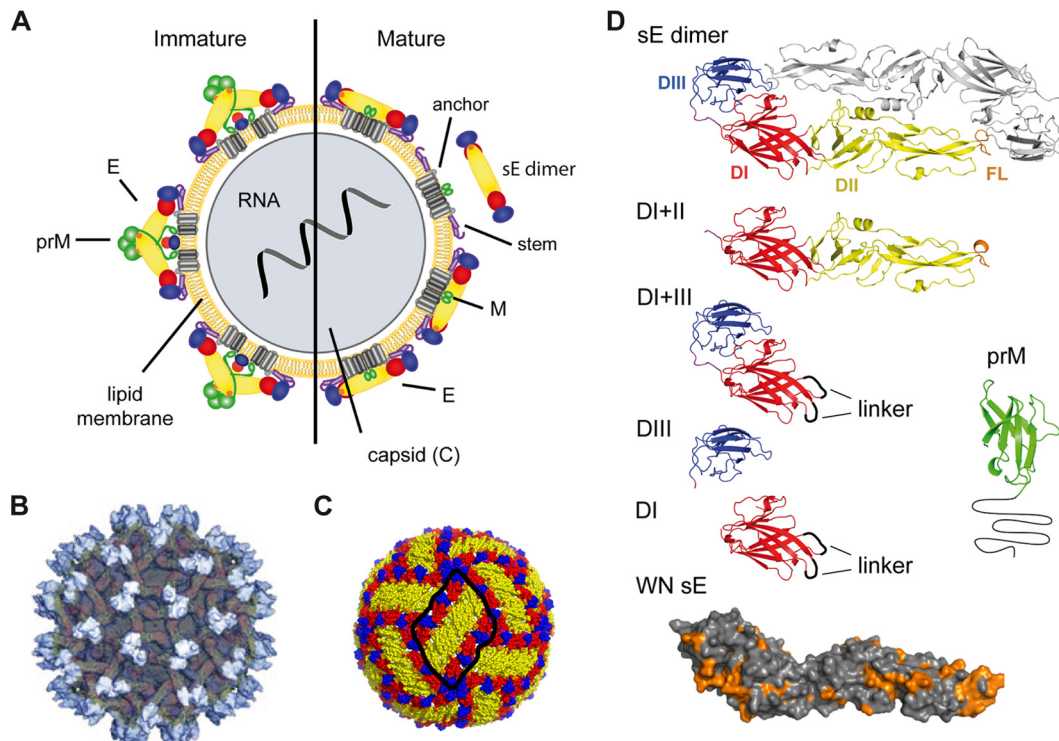
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**FIG 1** Schematic models and structures of immature and mature flavivirus particles and recombinant antigens employed. (A) Schematic representation of a virion with the immature form on the left and the mature form after prM cleavage on the right side. The nucleocapsid, surrounded by a lipid bilayer, is composed of C proteins and contains the viral RNA. In immature virions, prM and E form heterodimers. During virus secretion from host cells, prM is cleaved and E proteins are rearranged into homodimers. sE dimer, soluble form of the E dimer, lacking the stem and anchor regions. Reproduced from reference 27 under the CC BY license. (B) Surface representation of an immature dengue 2 virus as determined by cryoelectron microscopy. A total of 60 trimers of E-prM heterodimers protrude from the surface of immature virions. prM proteins at the tip of the spikes are colored in light gray. E proteins are shown in darker gray. Reprinted from reference 79 with permission of the publisher. (C) E protein arrangement on the surface of mature dengue 2 virus particles. Three E dimers form a raft (encircled in black) of the herringbone-like lattice covering the virion surface. Reproduced from reference 27 under the CC BY license. (D) Ribbon diagrams of the TBE sE dimer and other recombinant antigens used in the study (DI+II, DI+III, DIII, DI, and prM). In the surface representation of WN sE, amino acids conserved between TBE and WN viruses are highlighted in orange. Color code: DI, red; DII, yellow; DIII, blue; the fusion loop (FL) is highlighted in orange. prM (displayed is the structure of the dengue 2 prM [80], assuming that, like E, the overall structure of the TBE prM protein will be similar) is shown in green and black. Gly linkers introduced in constructs of DI and DI+III are shown as black loops.

occurs in the endoplasmic reticulum and first leads to the formation of immature, noninfectious forms of the virion containing the precursor of M (prM), which is proteolytically cleaved by furin during exocytosis to generate infectious, M-containing virions (Fig. 1A). Structural details have been elucidated by X-ray crystallography of isolated E proteins from several flaviviruses, a prM-E complex of dengue 2 virus, as well as cryoelectron microscopic studies of both mature and immature dengue and WN virus particles (reviewed in reference 15). Immature virions are studded with 60 spikes of trimers of prM-E heterodimers, whereas mature virions display 90 antiparallel E dimers that form a closed herringbone-like shell (Fig. 1B and C). The externally accessible part of E is composed of three domains (DI, DII, DIII) (Fig. 1D) that are the major targets of neutralizing antibodies, generally believed to mediate long-term protection against flavivirus disease (6, 7).

Studies with both mouse and human MAbs revealed that any site accessible at the virion surface can function as a target for virus neutralization. At least in the mouse system, the most strongly neutralizing MAbs were directed against DIII (reviewed in reference 15), whereas broadly flavivirus cross-reactive antibodies recognizing the highly conserved fusion loop at the tip of DII had no or only low neutralizing activity both in mice and humans

(16–19). Consistent with the relatively large size of antibody binding sites in protein antigens (around  $1,000 \text{ \AA}^2$  [20, 21]), MAbs were found to bind not only to individual domains and junctions between domains of the same E protein molecule but in some instances also to more complex quaternary antigenic sites, comprising amino acid residues from both molecules of the E dimer (22, 23) or even from adjacent dimers that are found only at the virion surface (24, 25). Evidence for the involvement of such complex antigenic sites has also been obtained from the analysis of human sera, primarily from dengue patients (26) and YF vaccinees (27).

It was the major objective of our work to study the specificities of antibodies in peripheral blood induced by natural TBE virus infection as well as vaccination with the inactivated TBE vaccine, which has proven to be highly effective in the field (28–30). As a specific goal, we wanted to gain insights into the extent of individual variation of antibody reactivities and immunodominance patterns and their possible consequences for virus neutralization through the analysis of relatively large panels of serum samples from both cohorts. For this purpose, we established a platform of immunoassays with recombinant antigens of E and prM, making use of the modular domain organization of E that allows not only the production of the whole protein E but also of its individual

**TABLE 1** Characteristics of the study cohorts of TBE patients and vaccinees

Characteristic	Value	
	Patients	Vaccinees
No.	38	43
Gender	11 female/27 male	23 female/20 male
Median age (range) in yrs at sample collection	56 (19–77)	61 (19–78)
Median no. of yrs (range) after onset of symptoms	1.9 (1.6–3.9)	
Median no. of days (range) after booster vaccination		24 (16–43)

domains and combinations thereof. These analyses yielded quantitative information on the proportions of antibody populations directed to different domains of the viral envelope proteins and thus a serological fingerprint for each individual serum sample. By depletion analyses, we were able to assign most of the virus neutralizing activity, both in postinfection and postvaccination sera, to antibodies reacting with DI and DII and/or the junction between the two domains. We also found evidence for neutralization epitopes dependent on the E dimer structure, and the involvement of even more complex quaternary epitopes cannot be excluded. Postinfection sera had a higher frequency of antibodies to prM, whereas a higher degree of flavivirus cross-reactivity was found in postvaccination sera. Independent of these overall characteristics, both groups displayed a substantial degree of individual variability with respect to the antibody specificities found in different sera and their contribution to virus neutralization.

## MATERIALS AND METHODS

**Study groups/cohorts.** TBE postinfection sera were collected from 38 patients >1.5 years (median of 1.9 years) after admission to the Hospital Ceske Budejovice. A total of 43 healthy persons previously vaccinated against TBE received a booster vaccination (FSME-IMMUN, 0.5 ml; Baxter) at the Department of Virology of the Medical University of Vienna, and blood was withdrawn 2.5 to 5 weeks (median of 24 days) after vaccination (Table 1).

**Ethics statement.** The studies were approved by the ethics committees of the Hospital Ceske Budejovice, Czech Republic (approval number 8/2008), and the Medical University of Vienna, Austria (approval number 590/2007), and all patients gave their written informed consent.

**Virus production.** Production and purification of formalin-inactivated TBE virus was performed as described in reference 31. Briefly, TBE virus strain Neudörfel (GenBank accession number U27495) was used to infect primary chicken embryo cells. The supernatant was harvested 48 h postinoculation, and the virus was concentrated by ultracentrifugation. Purification was carried out by rate zonal and equilibrium sucrose density gradient centrifugation. For inactivation, the purified virus was treated with 37% formalin at a final dilution of 1:2,000 at 37°C for 24 h.

**Production of recombinant proteins.** All recombinant proteins of TBE virus were derived from strain Neudörfel (GenBank accession number U27495), and the WN virus soluble E (sE) protein was derived from strain NY99 (GenBank accession number AF196835). The recombinant antigens (displayed in Fig. 1D) contained the following amino acids for TBE virus: sE, 1 to 400; DI+II, 1 to 302; DI+III, 1 to 52 + 8 Gly linker + 137 to 192 + 8 Gly linker + 285 to 400; DI, 1 to 52 + 8 Gly linker + 137 to 192 + 8 Gly linker + 285 to 302; DIII, 302 to 398; prM, 1 to 129 (containing a deletion at the furin cleavage site as described in reference 32). For the WN virus, the sE protein contained amino acids 1 to 400.

With the exception of DIII, all antigens were produced with a tandem

Strep-tag in the *Drosophila* expression system (Invitrogen) as described previously (27). The expression vector pT389 (kindly provided by Thomas Krey and Felix Rey, Institut Pasteur, France) encodes the export signal sequence Bip, an enterokinase cleavage site, and the tandem Strep-tag. *Drosophila* Schneider 2 cells were stably transfected using blasticidin for selection. Protein expression was induced by the addition of CuSO<sub>4</sub>, and supernatants were harvested 7 to 10 days after induction. Antigens were purified via affinity chromatography with Strep-Tactin columns (IBA) according to the manufacturer's instructions.

DIII was expressed as a fusion protein with thioredoxin and a C-terminal His tag in *Escherichia coli* strain BL21 with the pET 32a Xa/LIC vector (Novagen) as described previously (33). Cell lysates were clarified, and the DIII-Trx-His protein was purified via Ni<sup>2+</sup> affinity chromatography (GE Healthcare Life Sciences) from the soluble fraction.

**Blocking ELISA.** The reactivity of recombinant proteins was analyzed with conformation-sensitive and/or neutralizing MABs (the specificities are indicated in the legend to Fig. 2) in blocking enzyme-linked immunosorbent assay (ELISA) as described in reference 34. In brief, 3-fold serial dilutions of 212 nM blocking antigen were incubated with a predetermined concentration of MABs for 1.5 h at 37°C. The mixture was added for 1 h at 37°C to microtiter plates coated with formalin-inactivated TBE or WN virus or TBE virus with a high prM content, depending on the MAB specificity. Antibodies not blocked by their reaction with the antigen in solution were detected using peroxidase-labeled rabbit anti-mouse IgG (Pierce). Results are expressed as percent MAB reactivity (absorbance) in the absence of blocking antigen.

**Chemical cross-linking.** Proteins were cross-linked by dimethyl-suberimidate (DMS) treatment as described previously (35). Briefly, purified protein in triethanolamine buffer (pH 8.0) was incubated with 10 mM DMS for 30 min at room temperature (5 µg/ml). Ethanolamine was added to a final concentration of 10 mM and incubated for an additional 15 min to stop the reaction. Proteins were precipitated by treatment with trichloroacetic acid and analyzed before and after cross-linking by SDS-PAGE in a continuous phosphate-buffered system (36).

**Sedimentation analysis.** Proteins were analyzed by rate zonal sedimentation in sucrose gradients as described previously (37). Monomer controls were prepared for each protein by incubation with 1% SDS for 30 min at 65°C. A total of 3 µg of SDS-treated and 3 µg of untreated proteins were applied to the gradients and centrifuged for 20 h at 38,000 rpm at 15°C in a Beckman SW40 rotor. The gradients were fractionated, and the amount of antigen in each fraction was quantified by ELISA (38).

**IgG ELISA.** ELISAs with human sera were carried out as previously described (16, 27) by using nontreated microtiter plates coated with 50 ng/well of recombinant antigens or 25 ng/well of TBE virions and 3-fold serial dilutions of human sera, starting at 1:100. For detection, biotin-labeled goat anti-human IgG (Pierce) and streptavidin-conjugated peroxidase (Sigma) were used.

ELISA cutoffs were determined as the mean of 8 negative sera plus three standard deviations, as recommended in reference 39. Specific IgG was quantified by the use of a standard serum assigned 1,000 arbitrary units in each assay. Four dilutions of all samples were analyzed, and ELISA units were determined after curve fitting using a 4-parameter logistic regression with GraphPad Prism 5 (GraphPad Software Inc.).

In order to harmonize the results obtained in all ELISAs, we adjusted the units obtained by the factor required to shift the standard curves of the recombinant protein ELISAs in such a way that their linear ranges coincided with that of the virion ELISA. Significances of differences in the ELISA results observed between the two groups were calculated by unpaired two-tailed *t* tests.

**TBE virus neutralization assay.** Virus neutralization tests with human sera were carried out in baby hamster kidney cells (ATCC BHK-21) as described in reference 40. Two-fold serial dilutions of sera, starting at 1:10, were preincubated with 25 50% tissue culture infective doses (TCID<sub>50</sub>) of TBE virus (strain Neudörfel) for 1 h at 37°C before inoculation of cells and incubation for 3 days. To avoid interference by the depletion

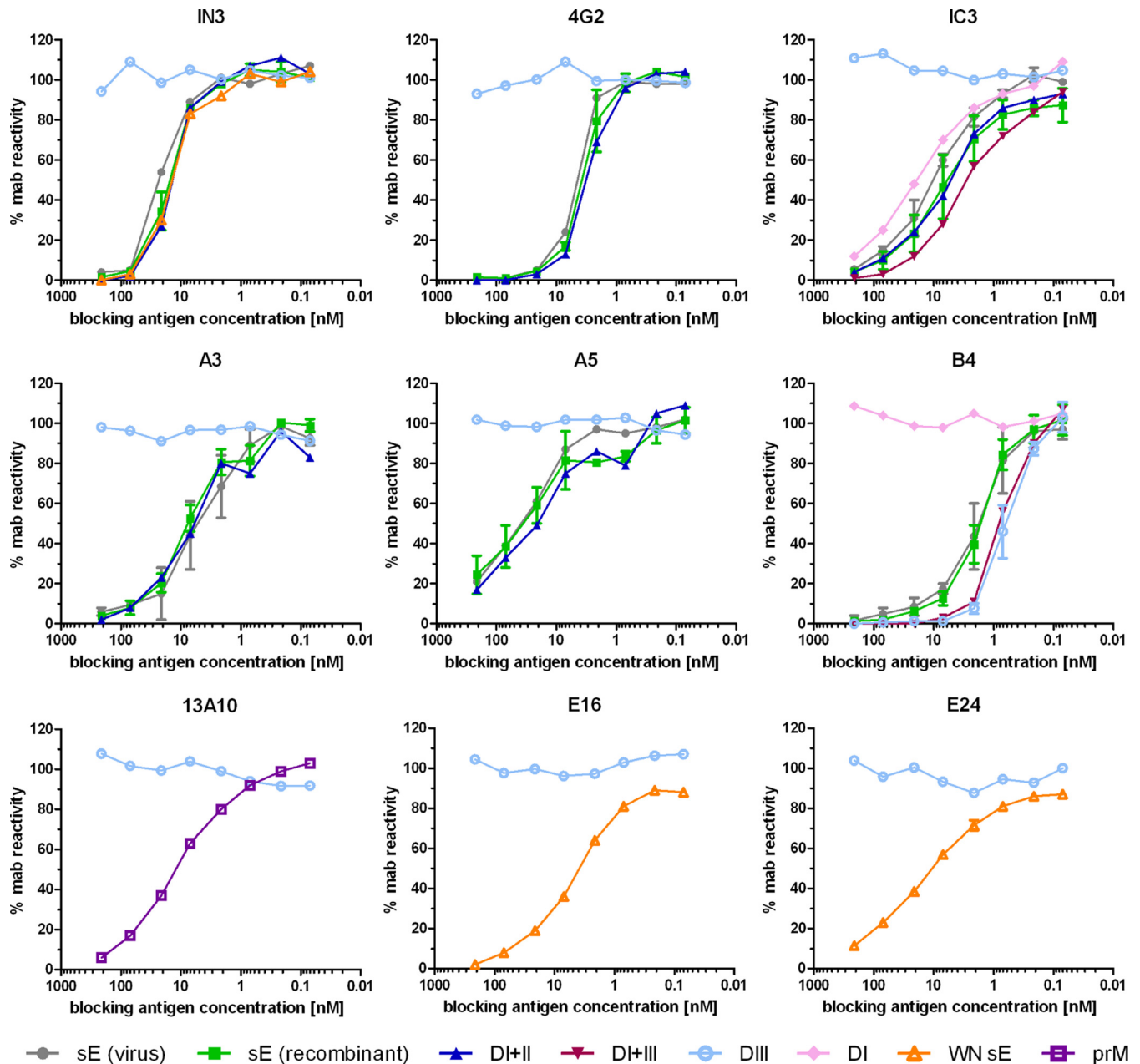


FIG 2 Blocking ELISA of recombinant antigens with MAbs. The MAbs recognize the following antigenic sites: IN3 and 4G2, flavivirus fusion loop (16); IC3, TBE DI; A3 and A5, TBE DII; B4, TBE DIII (34); 13A10, TBE prM (81); E16 and E24, WN DIII (82). The antigens analyzed are indicated in different colors, as shown at the bottom of the panel.

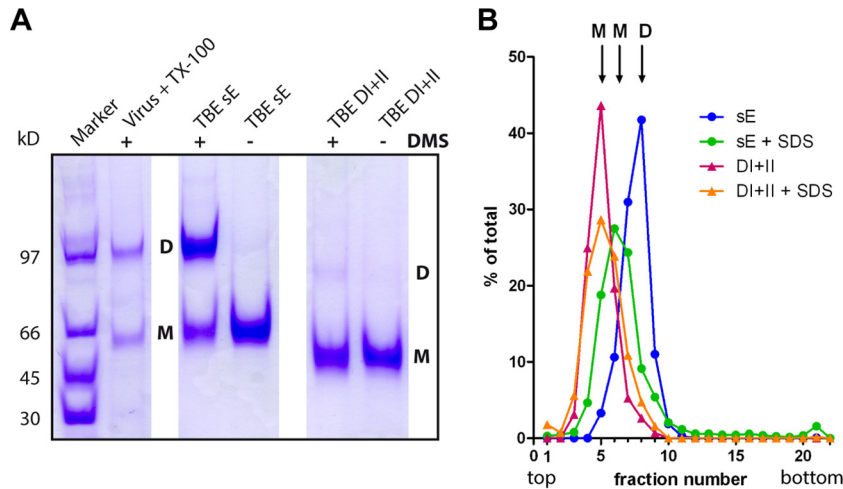
antigen, the cell culture supernatant was replaced by fresh medium 1 day after infection.

The presence of virus in the cell culture supernatant was detected by four-layer ELISA as described previously (41). Titers were calculated after curve fitting with a four-parameter logistic regression (Graph Pad Prism 5; GraphPad Software Inc.) using 50% of the absorbance in the absence of antibody as a cutoff (NT<sub>50</sub>). Titers of ≥10 were considered positive. Significances of differences in the NT<sub>50</sub> titers observed between the two groups were calculated by unpaired two-tailed *t* tests.

**Antibody depletion.** Depletion of specific antibodies from human sera was performed essentially as described in reference 33, using Dynabeads (Life Technologies) for His-tagged proteins and Strep-Tactin beads

(Qiagen) for Strep-tagged antigens. For this purpose, 10 μg antigen was bound to 33 μl paramagnetic beads for 1 h at room temperature, and after a washing step, 500 μl of 1:5 prediluted serum was added and incubated for 1 h at 37°C. Beads were pelleted by magnetic force, and the supernatant was subjected to two further rounds of depletion. The absence of nonspecific serum binding was confirmed with uncharged beads and the success of depletion by ELISA with the depletion antigen. The depleted serum samples were analyzed in virion ELISA and NT, and the data obtained were expressed as the percentage of the mean values of the nondepleted and mock-depleted sera.

**Statistical analyses of depletion results.** NT titers and ELISA units were log transformed and analyzed by a general linear model with normal



**FIG 3** Determination of the oligomeric state of TBE DI+II. (A) SDS-PAGE of recombinant TBE sE and DI+II as well as full-length E obtained from virus particles solubilized with Triton X-100 without (–) and with (+) cross-linking by DMS. Positions of monomers (M) and dimers (D) are indicated. Staining with Coomassie brilliant blue. (B) Sedimentation analysis of TBE antigens. The antigens are color coded as indicated in the panel. Monomeric controls were generated by SDS treatment (+SDS) of the antigens. Sedimentation is from left to right. The antigen content in each fraction was determined by ELISA. Positions of monomers (M) and dimers (D) are indicated.

deviates. Parameters, corresponding to the magnitude of depletion, and their standard errors were estimated by maximum likelihood. Confidence intervals for the percent depletion were calculated based on the standard errors of the parameter estimates by back transformation. Hypotheses about differences in the magnitude of depletion were tested by Wald chi-square tests. *P* values below 0.05 were considered significant.

## RESULTS

**Characterization of recombinant antigens for immunoassays.** A panel of recombinant antigens for ELISAs and depletion analysis (Fig. 1D) was produced and purified as described in Materials and Methods. All of these proteins reacted with neutralizing and/or conformation-specific MABs, and nonspecific binding was not observed using appropriate control antigens (Fig. 2). In the case of the TBE virus E-derived antigens (Fig. 1D), these reactivities corresponded to that of the sE protein isolated from purified virions. In contrast to sE, which is a dimer (42, 43), DI+II was monomeric, as shown by cross-linking and sedimentation analyses (Fig. 3), although it contains sequence elements contributing to dimerization (42). Using these antigens, we established a platform of immunoassays that allowed us to obtain information on relative quantities (expressed as ELISA units) of antibody subsets reacting with the whole dimeric sE protein and different domains or domain combinations of E, as well as prM.

**Reactivities of postinfection and postvaccination sera.** Antibody reactivities were determined with individual sera from 38 TBE patients (collected about 2 years after infection) as well as from 43 TBE vaccinees who had received a booster vaccination about 3 weeks before sample collection. These two cohorts are described in more detail in Materials and Methods, and their key features are listed in Table 1. In addition, serum pools containing identical aliquots of all postinfection and postvaccination sera were analyzed separately. The results of ELISAs and neutralization tests (NTs) are displayed in Fig. 4. In the serum pools, the ELISA reactivities against all antigens were significantly higher after vaccination, with the exception of prM-specific antibodies, which were significantly higher after infection ( $P < 0.0001$ ) (Fig. 4A and

B). The neutralization titer, however, was nonsignificantly lower in the postvaccination pool ( $NT_{50} = 1,497$ ) than in the postinfection pool ( $NT_{50} = 2,726$ ) ( $P = 0.0653$ ). The mean values of individual serum analyses (Fig. 4C and D) yielded a pattern similar to that obtained with the serum pools but revealed an extensive degree of individual variation with respect to the quantity of antibodies reacting with the different antigens and also with respect to neutralization titers.

Calculations of the correlation coefficients of subunit ELISA results as well as NT titers to the virion ELISA results yielded very good positive correlation coefficients (Pearson  $r = 0.74$  to  $0.98$ ) (Fig. 5). Only the correlation between prM and virion reactivity in TBE vaccinees was somewhat lower (Pearson  $r = 0.51$ ).

To obtain a standardized measure for the individual variation of the proportions of antibody subsets and to compensate for titer variations, we determined, for each serum, the ratios of subunit ELISA units as well as NT titers relative to virion ELISA units. These individual serum ratios are displayed as deviations from the mean values of all sera in Fig. 6A and B. For all parameters tested, there was substantial variation (including the ratio of NT to virion ELISA) reflecting quantitative differences in the specificities and composition of sera, both from infected individuals and vaccinees. This analysis provided a characteristic reactivity pattern for each serum, representing a fingerprint of TBE virus-specific antibody subsets and their neutralizing activities, shown as gray lines in Fig. 6C and D. The patterns are quite heterogeneous and divergent at the single serum level, consistent with individual-specific factors that shape the specificity of the antibody response. Three sera were selected from both groups and highlighted in Fig. 6C and D to show that sera with similar reactivity patterns can have quite divergent NT-to-virion ELISA ratios (compare black dashed and solid lines) and, vice versa, sera with divergent patterns can have similar NT to virion ELISA ratios (black and gray curves).

**Depletion analysis.** In order to gain quantitative information on the contribution of different antibody subsets to the total TBE virus-specific serum reactivity and to neutralizing activity, we de-

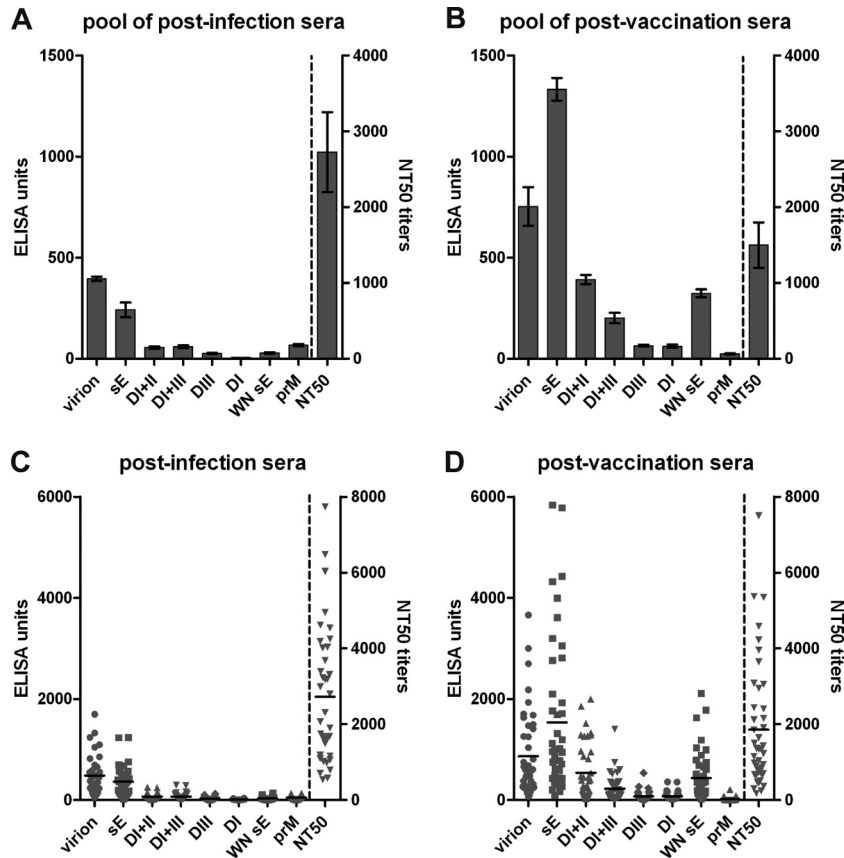


FIG 4 ELISAs and NTs of TBE postinfection and postvaccination sera. Analysis of serum pools (A, B) and individual sera (C, D) in TBE virion and subunit ELISAs as well as in neutralization tests. Error bars in panels A and B represent standard errors of the means calculated from the results from at least 3 independent experiments. Black horizontal lines in panels C and D represent the means of individual sera.

pleted the postinfection and postvaccination serum pools with the recombinant proteins shown in Fig. 1D. As controlled by ELISA with the respective depletion antigens, depletion was complete (below ELISA cutoff and/or less than 5% remaining reactivity) in all instances. The effect of the removal of distinct antibody subsets was then determined in virion ELISA as well as NTs, and the results of these analyses are shown in Fig. 7.

Depletion with sE resulted in a virtually complete removal of the total virion reactivity in the postvaccination serum pool, compared to about 75% in the postinfection pool. The majority of this

residual reactivity, however, could be removed by double depletion with sE and prM. Most importantly, in both serum pools, all of the neutralizing activity was removed by depletion with sE alone, indicating that the epitopes recognized by these antibodies are displayed completely or at least to a large extent on the soluble dimeric E protein. A substantial proportion of virion reactivity and neutralizing activity could even be removed by the monomeric DI+II protein (65% in postinfection and 85% in postvaccination pools; Fig. 7C and D), suggesting that after both vaccination and infection a considerable portion of antibodies recognized

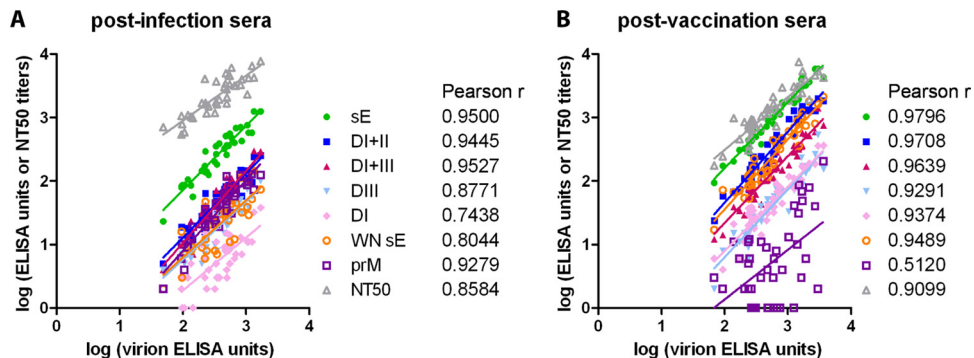
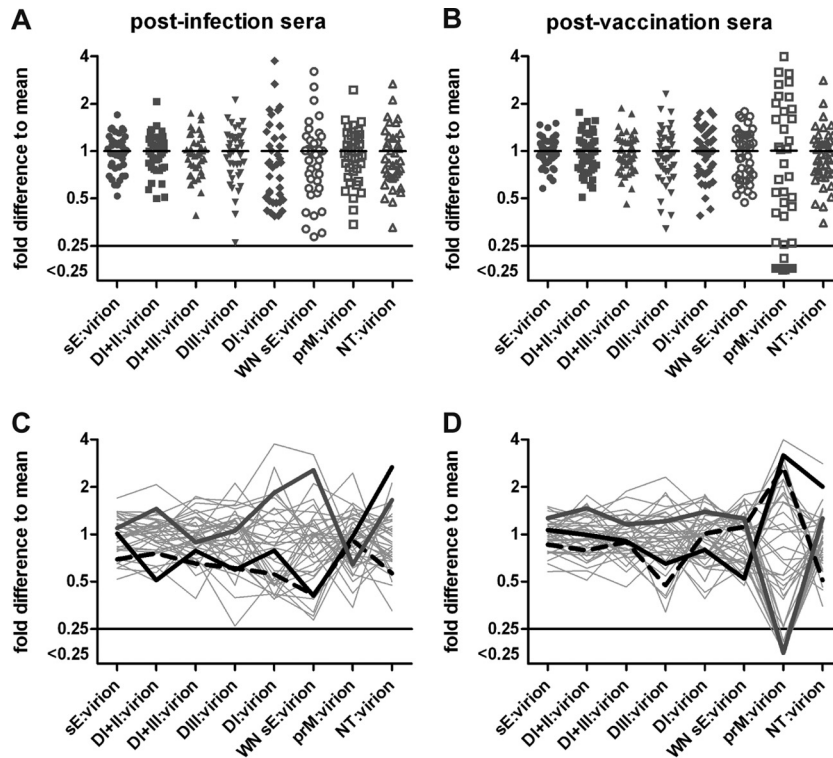


FIG 5 Correlations of individual serum reactivities in different assays. Linear regression between virion ELISA and subunit ELISA units as well as with NT titers. Pearson correlation coefficients (r) are indicated. (A) Postinfection sera; (B) postvaccination sera.



**FIG 6** Variation of reactivities of individual sera. (A, B) Ratios of subunit ELISA units and NT titers in relation to virion ELISA units, expressed as fold difference from the mean of all sera. (C, D) Ratios obtained for each individual serum are connected by gray lines. Patterns of three selected sera are highlighted. (A, C) Postinfection sera; (B, D) postvaccination sera.

antigenic sites that were largely independent of the dimer structure of E.

In none of the cases, depletion with DIII resulted in a significant reduction of virion reactivity and neutralizing activity, indicating that this domain plays a very minor role in the human antibody response to TBE virus, irrespective of its presentation as a replicating or inactivated virus. Similar results were also obtained with the isolated DI, except for a slight reduction of virion reactivity in the postvaccination pool. Significant reduction of virion reactivity and neutralizing activity, however, was observed in both serum pools after depletion with the recombinant protein comprising both DI and DIII, suggesting the involvement of epitopes at the junction between the two domains.

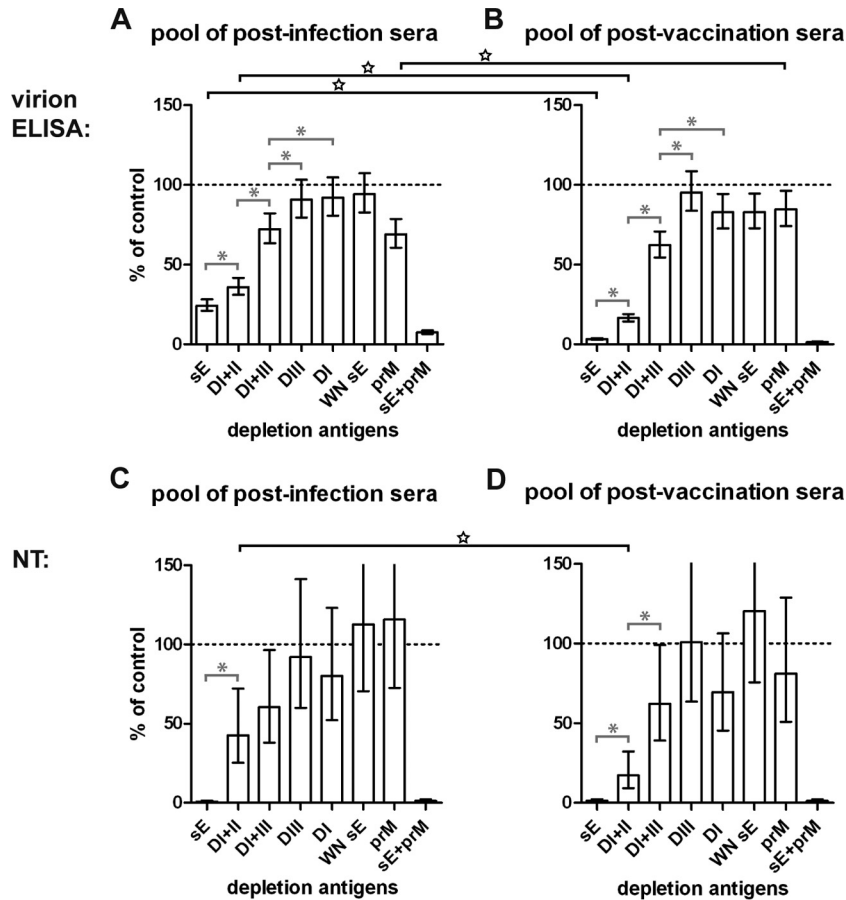
Consistent with the ELISA results shown in Fig. 4, the postinfection sera had a significantly higher proportion of prM-specific antibodies than the postvaccination sera, whereas a slight but significant degree of depletion with the cross-reactive sE from WN virus was found with the postvaccination but not the postinfection pool. In none of these cases a significant depletion effect on neutralizing activity was achieved. The small amount of cross-reactive antibodies was completely depletable with DI+II but not DI+III (data not shown), suggesting that they were directed primarily to conserved sites in DII, although amino acids conserved between TBE and WN sE are found in all domains of E (see Fig. 1D).

**Results obtained with individual sera.** To identify possible individual variations in antibody subsets contributing to the depletion patterns found with serum pools (Fig. 7), we selected three postinfection sera (I-16, I-9, I-29) and three postvaccination sera

(V-208, V-207, V-229) with relatively high neutralization titers for depletion analyses. Table 2 summarizes the characteristics of the selected sera. The results of the analyses are displayed in Fig. 8 and revealed significant deviations from the average patterns obtained with the serum pools. The most important differences relative to the pool data can be described as follows. (i) Sera I-16 and V-208 contained significantly more DIII-specific antibodies than the pools, which, however, did not contribute significantly to the neutralizing activity of these sera. (ii) In sera I-9 and V-207, the depletion of total virion reactivity with monomeric DI+II was as efficient as that with the whole sE dimer, suggesting a minor role of antibodies to dimer-specific epitopes in these cases. In serum V-207, the DI+II reactive antibodies also accounted for almost all of the neutralizing activity. (iii) In contrast to what was observed with serum V-207, most of the neutralizing activity of serum V-229 could not only be removed by DI+II but also by DI+III, suggesting that DI-specific antibodies dominated the neutralizing response in this case. (iv) Serum I-29 did not contain significant amounts of antibodies depletable with prM, different from the results obtained with the postinfection pool and sera I-9 and I-16.

## DISCUSSION

In this study, we deconstructed specificities and functional activities of antibody populations induced in the course of natural TBE virus infection and TBE vaccination through the use of immunoassays with recombinant proteins, including single domains and domain combinations of the target for neutralizing antibodies (protein E), as well as prM. The samples were collected late after infection (median of 1.9 years) and 3 to 4 weeks after booster



**FIG 7** Depletion of antibody subsets from serum pools with recombinant antigens. Postdepletion analysis of the pools of postinfection sera (A, C) and postvaccination sera (B, D) in virion ELISA (A, B) and NT (C, D). The reactivity of depleted serum pools is given as a percentage of the mean of non- and mock-depleted controls. Depletion is significant when the error bar does not reach the 100% mark (dashed line). Error bars represent confidence intervals of 2 to 4 independent experiments. Gray capped lines with asterisks within each of the panels indicate significant differences between depletions of the same serum pool with different antigens. Black capped lines with stars spanning the panels indicate significant differences between depletions of the postinfection and postvaccination pools with the same antigen. Antigens used for depletion are indicated below the panels.

vaccination, and the measured antibodies are therefore believed to be responsible for long-term protection. It was an important finding of our work that the neutralizing activity, both after natural infection and vaccination, could be almost completely removed from serum pools by depletion with the dimeric recombinant sE protein (Fig. 7C and D), suggesting that neutralizing epitopes are

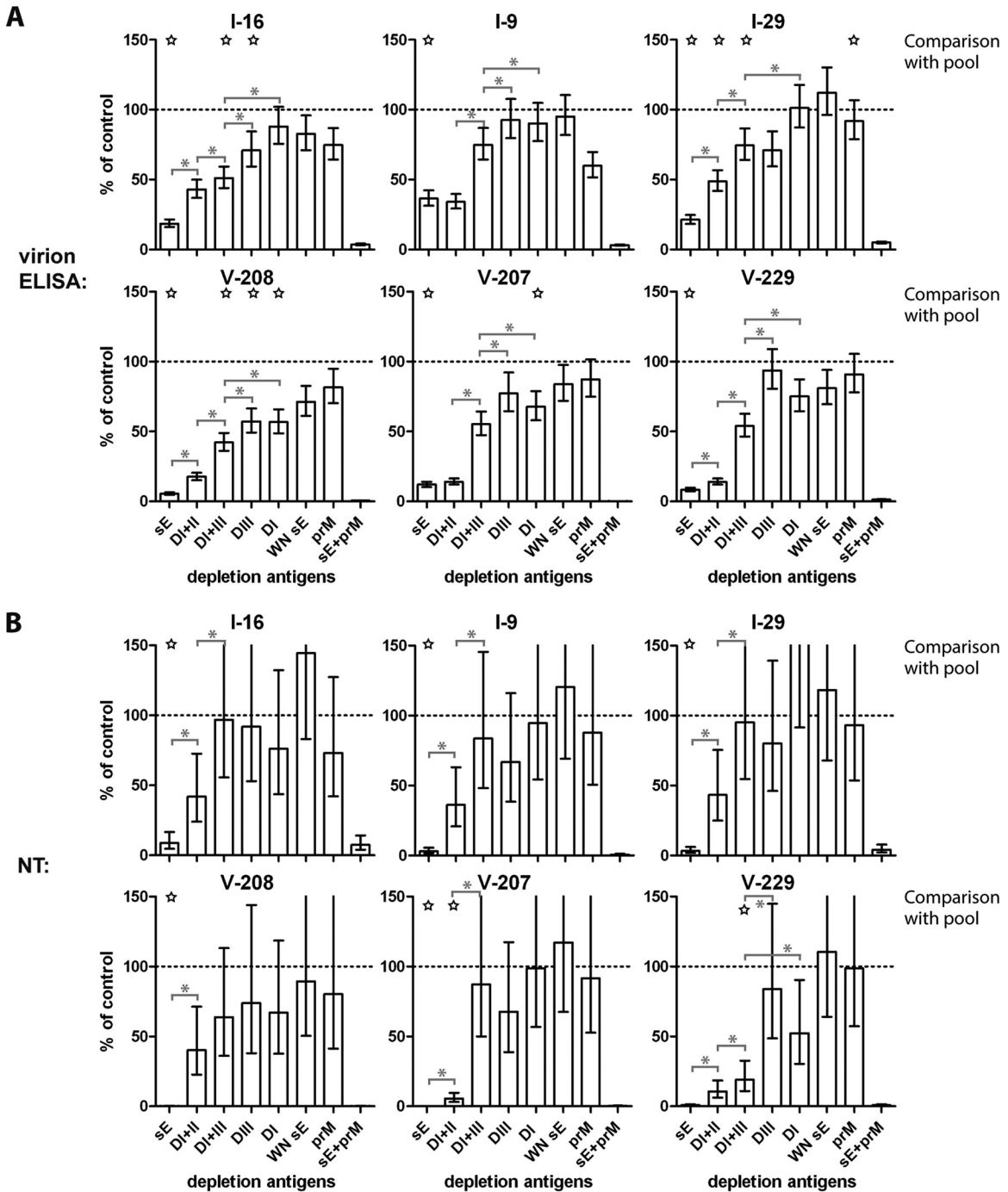
largely confined to this building block of the viral envelope. More complex quaternary epitopes (generated only by the herringbone-like arrangement of E at the virion surface) have been structurally characterized through cryoelectron microscopy analyses of certain MAb-derived Fabs complexed to WN virus (24) and dengue 1 virus (25) and were shown to have amino acid contributions from two adjacent dimers present at the virion surface. Also in these cases, however, a large proportion of the antibody contact site was confined to a single E dimer. Depletion with the sE dimer could therefore potentially also lead to the removal of antibodies to such dimer overlapping sites.

Somewhat unexpectedly, our sE depletion data did not conform to those of a related study analyzing dengue postinfection sera, which revealed that the majority of neutralizing antibodies bound only to intact virions but not to soluble E (26). Such discrepancies may be explained by structural differences between TBE and dengue viruses, especially with respect to the stability and/or the curvature of the E protein dimer in solution (44). Whereas the TBE E dimer corresponds in its curvature to that of the virion surface (42) and remains dimeric even at the relatively high dilutions employed in serological assays (Fig. 3) (45), recent

**TABLE 2** Characteristics of sera selected for depletion analyses

Serum	Time after onset of symptoms for postinfection sera (yrs) or after booster vaccination for postvaccination sera (days)	Age (yrs) of patient/vaccinee	NT <sub>50</sub> titer
<b>Postinfection</b>			
I-16	1.7	61	4,253
I-9	1.6	72	4,540
I-29	3.8	17	7,737
<b>Postvaccination</b>			
V-208	30	23	5,350
V-207	22	46	7,505
V-229	28	19	2,157





**FIG 8** Depletion of antibody subsets from individual sera with recombinant antigens. Postdepletion analysis of three postinfection sera (I-16, I-9, I-29) and three postvaccination sera (V-208, V-207, V-229) in virion ELISA (A) and NT (B). The reactivity of depleted sera is given as the percentage of the mean of non- and mock-depleted controls. Depletion is significant when the error bar does not reach the 100% mark (dashed line). Error bars represent confidence intervals of 2 to 4 independent experiments. Gray capped lines with asterisks within each of the panels indicate significant differences between depletions of the same serum with different antigens. Black stars indicate significant differences between depletions of an individual serum and the respective serum pool with the same antigen. Antigens used for depletion are indicated below the panels.

observations indicate that the dengue E protein is monomeric in solution (46). This antigen would allow only discrimination between monomer-specific and more complex quaternary epitopes but not between dimer-specific (22, 23) and herringbone-specific (24, 25) epitopes. It also has to be kept in mind that the results of

neutralization assays can be influenced by the maturation state of the virus (47, 48) as well as the cell type used in such analyses (49).

Quite remarkably, antibodies to domain III (which forms a prominent protrusion at the surface of flaviviruses [reviewed in reference 15]) played only a marginal role in the responses both to

TBE virus infection and vaccination, and depletion of DIII-specific antibodies from the serum pools had no measurable effect on virion binding and neutralization activities (Fig. 7), similar to what was observed in a study with human YF postvaccination sera (27). In a mouse immunization study with inactivated and aluminum hydroxide-adjuvanted purified TBE virions (i.e., an immunogen similar to that in the TBE vaccine), however, a substantial proportion of the total and neutralizing antibody response was directed at DIII (43). These data are consistent with previous observations made with monoclonal and polyclonal antibody responses to West Nile and dengue viruses, which had shown that the dominance of DIII responses typical of mice has no counterpart in humans (reviewed in reference 50). Considering the fact that the antigenically relevant surface of DIII differs strongly among flaviviruses (62% of the surface-exposed residues are not conserved in any pairwise comparisons between TBE, WN, JE, dengue 2, and YF viruses [data not shown]), such disparate findings are difficult to explain by differences in the antibody repertoires of humans and mice. Understanding the mechanisms underlying such a pronounced species-related divergence in antigen recognition could lead to a better understanding of the largely elusive phenomenon of antibody immunodominance in general.

A substantial proportion of virion binding and neutralizing antibodies could be depleted with a monomeric DI+II construct (Fig. 7), although the extent of these depletions was significantly lower than that of the sE depletions in serum pools. Since DIII had no effect in depletion (Fig. 7 and paragraph above), this difference provides evidence for the involvement of E dimer-specific epitopes as addressed before (22, 23) but also indicates that more than 50% of the total virion binding and neutralizing antibody response was directed to epitopes represented by the monomeric E protein. This is reminiscent of data obtained with YF postvaccination sera using a monomeric form of sE as well as YF DI+II (27). The importance of epitopes in DI- and DII-inducing neutralizing antibodies, especially involving residues at the junction between the two domains, has been shown previously in several monoclonal and polyclonal antibody studies with dengue viruses (51–53). Furthermore, the dominance of such antibodies in virus neutralization was recently demonstrated in elegant experiments using recombinant viruses with transplanted amino acid residues at the DI+II hinge region of dengue viruses (54), suggesting that this is a characteristic feature of the human neutralizing antibody response to flaviviruses in general.

Similar to what was found with DIII, antibodies depletable by the isolated DI did not contribute significantly to the neutralizing activity of serum pools after infection and vaccination. However, the construct encompassing DI and DIII removed substantial amounts of virion binding and neutralizing activity from the serum pools, suggesting that epitopes at the junction between the two domains, similar to those at the junction between DI and DII, were involved in these responses. Such an epitope has been identified previously by a dengue-specific monoclonal antibody (5H2) that was shown to recognize DI and the linker between DI and DIII (55). The importance of interdomain epitopes in the antibody response to flavivirus infections in humans has also been deduced from the application of a high-throughput dot blot assay with alanine mutant E proteins for the analysis of dengue postinfection sera (51).

The single-serum analyses of postinfection and postvaccination panels in recombinant protein ELISA platforms demon-

strated a substantial individual-specific variation with respect to the composition and specificities of antibody populations in peripheral blood (Fig. 6), similar to those reported in an analysis of the Ig responses to three distinct epitopes of E for dengue fever patients (56) and YF vaccinees (27). Functional consequences of such variations became apparent in depletion analyses of selected sera (Fig. 8) and were observed between individuals (I-16 compared to I-9 and V-208 compared to V-229) of similar age and time since infection and vaccination (Table 2). They thus apparently reflect individual-specific variations in factors controlling the stimulation and selection of specific B cells for antibody production, including germ line Ig repertoire (57, 58), competition between different B cell clones by affinity maturation in germinal centers (59), and poorly understood mechanisms leading to the selection of B cell subsets to become B memory cells or antibody-producing LLPCs in the bone marrow (12, 60).

Although postvaccination sera had significantly higher ELISA titers than postinfection sera against soluble forms of E compared to whole virion (Fig. 4), the patterns obtained in depletion analyses were remarkably similar. Specifically, the complete neutralizing activity could be depleted by the soluble E protein from serum pools of both cohorts, and also other characteristics of the antibody response, such as the immunosilence of DIII, the contribution of DI+II and DI+III junction epitopes (Fig. 7), and the extents of individual variability of antibody fine specificities (Fig. 6), were comparable in the two groups. However, prM antibodies were found more frequently and at higher titers in postinfection sera than after vaccination, and the opposite was true for broadly cross-reactive antibodies. Nevertheless, both of these antibody populations (especially the cross-reactive antibodies induced by vaccination) made up only a minor fraction of the total virion-reactive antibodies and did not contribute to neutralization, as revealed by depletion analyses. The induction of prM-specific antibodies in the course of infection suggests that immature or partially immature virus particles and possibly also soluble forms of prM circulate in the course of TBE virus infections in humans and thus stimulate a prM response, similar to what has also been observed in JE and dengue virus infections (18, 19, 61, 62).

Surprisingly, the broadly flavivirus cross-reactive response was very low after infection, in stark contrast to what has been observed in dengue and WN postinfection sera, in which fusion loop-specific antibodies were found to encompass a significant percentage of the anti-E response (18, 51, 56, 63, 64). The reasons for these discrepancies are not clear yet but could be related to virus-specific differences in the stability of E-E and E-prM complexes, as well as the dynamic motions of E at the virus surfaces (65–70), potentially affecting the transient exposure of antigenic sites that would be cryptic in the closed shell state of mature virus particles (16, 48, 71). Structural features could also contribute to the differences observed between infected and vaccinated individuals, as observed in the reactivity patterns of the recombinant protein ELISA platforms (Fig. 4), and may be a consequence of chemical modification (72) and/or stabilization of E oligomers by formalin (43, 73), adsorption to aluminum hydroxide (74, 75), and different concentrations of soluble antigen forms in the vaccine compared to those produced during infection.

In summary, our study not only highlights similarities and differences in the antibody response to TBE virus infection and vaccination in comparison to other flaviviruses but more generally points to the impact of individual-specific variations of antibody

fine specificities and their functional consequences for immune responses to virus infections and vaccines. All of these issues are related to the phenomenon of immunodominance which, in addition to structural features of the antigen, is apparently strongly modulated by mechanisms that are private to each individual. An in-depth understanding of the factors involved in these mechanisms can inform strategies for a more rational design of new vaccines (13, 76), especially against viruses with highly variable target antigens, such as influenza (77) and HIV (78).

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