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Murine Antibody Responses to the Verotoxin 1 B Subunit: Demonstration of Major Histocompatibility Complex Dependence and an Immunodominant Epitope Involving Phenylalanine 30

D. J. BAST,[1,2] J. SANDHU,[1,3] N. HOZUMI,[1,4,5] B. BARBER,[2,5] AND J. BRUNTON[1,2,6]*

Samuel Lunenfeld Research Institute, Mount Sinai Hospital,[1] The Toronto Hospital,[6] and Departments of Microbiology,[2] Molecular and Medical Genetics,[4] Immunology,[5] and Surgery,[3] University of Toronto, Toronto, Ontario, Canada

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Structurally conserved verotoxin 1 (VT1) mutant derivatives, showing reduced receptor binding and cytotoxicity, may serve as natural toxoids to protect against VT-mediated disease. In this study, the antibody responses to the wild-type VT1 B subunit, a B-subunit mutant (Phe30Ala B), and the corresponding holotoxin (Phe30Ala HT) were examined in three inbred mouse strains. BALB/c (*H-2*^{d}) and CBA (*H-2*^{k}) mice produced strong antibody responses to both wild-type and mutant B subunits. VT1 B-raised sera reacted more strongly with VT1 B than with Phe30Ala B in enzyme-linked immunosorbent assays, while Phe30Ala B-raised sera reacted equally with VT1 B and Phe30Ala B. C57BL/6 (*H-2*^{b}) and congenic BALB/c (BALB · B [*H-2*^{b}]) mice produced no detectable antibody response to either VT1 B or Phe30Ala B. However, an anti-VT1 B antibody response was detected in *H-2*^{b} mice immunized with biologically active Phe30Ala HT. Based on these observations, we conclude that the VT1 B subunit possesses a B-cell immunodominant epitope formed partly by phenylalanine 30 and that the B-subunit antibody response is dependent on the *H-2* haplotype of the mouse strain. Our results also support a potential role for the A subunit in providing the T-cell help necessary to overcome a deficient B-subunit antibody response in *H-2*^{b} mice.

Infection with verotoxin (VT)-producing *Escherichia coli*, in particular serotype O157:H7, is associated with gastrointestinal diseases including bloody and nonbloody diarrhea and hemorrhagic colitis (17, 25). Of greater significance is the association of VT-producing-*E. coli* infections with hemolytic uremic syndrome (HUS), a leading cause of acute renal failure in children (10, 12). There is strong evidence, both direct and circumstantial, that VTs play a critical role in the pathogenesis of HUS (22–24). It has therefore been suggested that immunization against VTs may prevent HUS (4). Although there is no evidence that antibodies to VTs are protective in humans, animal studies (4, 15) have shown that immunity to VTs protects against VT-mediated disease.

VT1, VT2, and VT2c, which each consist of an enzymatically active A subunit and a pentameric receptor binding B subunit, belong to a group of related toxins that enter the cytosol and inhibit eukaryotic protein synthesis (7, 27). Because of the cytotoxicity of the holotoxin (HT), vaccine development studies have concentrated on the nontoxic B-subunit pentamer (26, 30, 31). Specific serum antibody responses towards the B subunit have been shown to be protective against the cytotoxic action of the toxins both in vitro (4) and in vivo (3, 15). More recently, however, it has been reported that the B subunit may induce programmed cell death upon binding to its receptor, globotriaosylceramide, on human germinal-center B lymphocytes (14). In turn, this may prevent an effective antitoxin humoral response and raises important questions about the efficacy of the wild-type B subunit as a natural toxoid. As an alternative to the use of chemical modification to eliminate all biological activity of VTs for use in vaccine preparations, we have used site-directed mutagenesis to construct a nontoxic, structurally conserved VT1 mutant derivative for use as a human vaccine.

We have recently described a VT1 B-subunit mutant (Phe30Ala B) which is conserved structurally but has significantly reduced receptor binding (5). The decrease in receptor binding was associated with a 10^5-fold reduction in cytotoxicity of the HT (Phe30Ala HT) on Vero cell monolayers (5). As this VT1 mutant derivative may be useful as a natural toxoid to protect against VT-mediated diseases, we examined the antibody response to both the wild-type and mutant B subunits and to the corresponding mutant HT in three inbred mouse strains (BALB/c [*H-2*^{d}], CBA [*H-2*^{k}], and C57BL/6 [*H-2*^{b}]). We also investigated the relationship between the antibody responses to the three antigens tested and the *H-2* haplotype of the mouse strain used.

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Wild-type and mutant B subunits and HTs used in this study were purified from periplasmic extracts of *E. coli* JM101 transformed with the following plasmids: pJLB120 (21) and pJLB120A30 (5), encoding the wild-type and mutant B subunits, respectively, and pJLB128 (32) and pJLB128A30 (5), encoding the wild-type and mutant HTs, respectively. Purification procedures used have been described in detail elsewhere (19, 21). Six- to eight-week-old female mice of the inbred BALB/c (*H-2*^{d}), CBA (*H-2*^{k}), and C57BL/6 (*H-2*^{b}) strains were purchased from Charles River Laboratories, Wilmington, Mass. Groups of 5 to 15 mice of each strain were injected intraperitoneally with 50 μg of either wild-type or Phe30Ala B

* Corresponding author. Mailing address: Department of Microbiology, The Toronto Hospital General Division, 200 Elizabeth St., Toronto, Ontario, Canada M5G 2C4. Phone: (416) 340-3183. Fax: (416) 340-5047.

subunits emulsified 1:1 in Freund's complete adjuvant (Sigma Chemical Co.). Mice were given boosters 14 days later with 50 μg of the original antigen emulsified 1:1 in Freund's incomplete adjuvant (Sigma Chemical Co.). Control mice were injected with Freund's complete and incomplete adjuvants without antigen by the same protocol. Mice were bled by cardiac puncture 14 days after the first booster. The experimental procedures performed on the mice were conducted according to the principles of the Animal Care Committee of Mount Sinai Hospital, Toronto, Canada.

Anti-VT1 and anti-Phe30Ala B-subunit- and HT-specific antibodies were detected by using solid-phase enzyme-linked immunosorbent assays (ELISAs). Four hundred nanograms of purified wild-type or mutant B subunit or HT, dissolved in 100 μl of carbonate-bicarbonate buffer (pH 9.6), was applied to each well of an Immulon 1 microtiter plate (Dynatech Laboratories, Chantilly, Va.), and the plate was incubated overnight at 4°C. Coated wells were sequentially incubated with 2% (wt/vol) bovine serum albumin (Sigma Chemical Co.), specified dilutions of murine immune sera, peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (both heavy- and light-chain reactivity) (Bio-Rad Laboratories, Hercules, Calif.), and finally *o*-phenylenediamine dihydrochloride (Sigma Chemical Co.). The chromogen produced was measured by determining the absorbance at 490 nm on an MR600 automated microtiter plate reader (Dynatech Laboratories). Inhibition ELISAs were performed as follows. Sera, at a dilution of 1:200, were preincubated with serial twofold dilutions of VT1 and Phe30Ala B subunits (1,000 to 0.24 ng) with shaking for 90 min at 37°C. Preincubated serum–B-subunit mixtures were then applied to VT1 B-coated microtiter wells. Secondary antibody and developing stages were as described above. For both ELISAs, the wells were washed three times with phosphate-buffered saline containing 0.05% (vol/vol) Tween 20 (BDH Inc., Toronto, Canada) following each step. All incubations were for 1 h at 37°C unless otherwise specified.

The mean optical density at 490 nm (OD_{490}) for the negative-control sera at a dilution of 1:100 was subtracted from the test serum sample reading to give the reported ELISA reading (OD_{490}). The antibody titer was defined as the highest serial dilution of serum at which the OD_{490} was 2 standard deviations above the mean OD_{490} of the negative-control sera at a 1:100 dilution. Antibody titers were converted to logarithmic values [$\log_2(x)$, where x equals the reciprocal of the serum dilution] for calculation of geometric means and standard deviations. Differences in antibody reactivity and titers between groups of mice were compared by using the two-tailed unpaired Student *t* test, while those differences within groups of mice were compared by using the two-tailed paired Student *t* test. Microsoft Excel software (Microsoft Corporation) was used for statistical analysis.

Phenylalanine 30 forms part of an immunodominant B-cell epitope. Sera (at a dilution of 1:200) from VT1 B-immunized BALB/c (*H-2*^{d}) mice reacted more strongly with the VT1 B subunit than with the Phe30Ala B subunit in ELISAs (OD_{490}s, 1.271 ± 0.404 and 0.437 ± 0.218, respectively [$n = 15$] [$P < 0.0001$]). In contrast, sera from Phe30Ala B-immunized BALB/c (*H-2*^{d}) mice showed no significant difference in reactivity with either the VT1 or Phe30Ala B subunits (OD_{490}s, 1.476 ± 0.406 and 1.456 ± 0.424, respectively [$n = 15$] [$P = 0.369$]) (Fig. 1). When analyzed by serum titration, sera from a second set of VT1 B-immunized BALB/c (*H-2*^{d}) mice showed a similar reduction in antibody reactivity with Phe30Ala B compared to VT1 B. Reciprocal geometric mean titers of antibodies to the VT1 and Phe30Ala B subunits from VT1 B-raised antisera were 13.78 ± 1.06 (1:14,060) and 11.06 ± 1.27 (1:2,133), respectively ($n = 7$) ($P < 0.0001$). In contrast, Phe30Ala B immune sera showed no significant difference in reactivity with either the wild-type or mutant B subunits. Reciprocal geometric mean titers of antibodies to the VT1 and Phe30Ala B subunits from Phe30Ala B-raised antisera were 13.50 ± 1.06 (1:11,588) and 13.21 ± 1.27 (1:9,484), respectively ($n = 7$) ($P = 0.172$). Similar antibody responses were observed in VT1 B- and Phe30Ala B-immunized CBA (*H-2*^{k}) mice, suggesting that the observations made were not dependent on the strain used (data not shown).

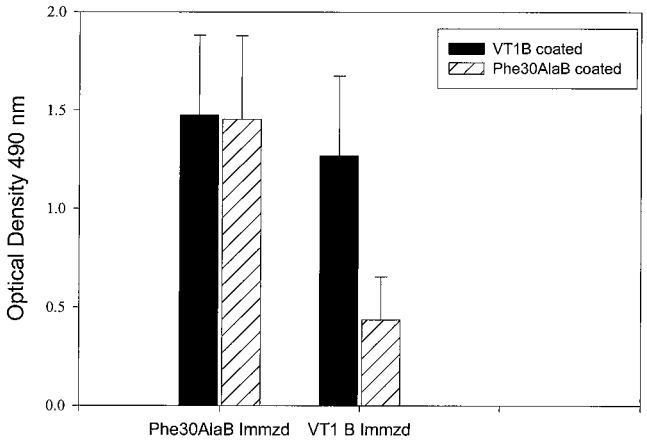

FIG. 1. Differing ELISA reactivity patterns of VT1 B and Phe30Ala B immune sera. Antisera from VT1 B-immunized (VT1 B Immzd) and Phe30Ala B-immunized (Phe30AlaB Immzd) BALB/c (*H-2*^{d}) mice were reacted with ELISA plates coated with VT1 B and Phe30Ala B. Sera were diluted 1:200. Bars represent the mean OD_{490} determined for 15 mice. Standard deviations of the means are shown. For the Phe30Ala B-immunized group of mice, there was no significant difference between the OD_{490} readings of the VT1 B-coated and Phe30Ala B-coated wells as determined by the two-tailed paired Student *t* test ($P = 0.369$). For the VT1 B-immunized group of mice, the mean OD_{490} for the VT1 B-coated wells was significantly greater than that of the Phe30Ala B-coated wells as determined by the two-tailed paired Student *t* test ($P < 0.0001$).

The application of VT1 B and Phe30Ala B to plastic microtiter plates (for solid-phase ELISA) might alter their structural conformation or might result in the preferential association of a certain epitope with the plastic surface. Therefore, the reactivity of VT1 B- and Phe30Ala B-raised antisera with the VT1 and Phe30Ala B subunits in solution was tested by inhibition ELISA. The reactivity of anti-Phe30Ala B antisera to solid-phase-bound VT1 B was inhibited equally by the soluble wild-type and mutant B subunits (Fig. 2a). The reactivity of anti-VT1 B antisera to solid-phase-bound VT1 B, however, was inhibited by wild-type VT1 B but was much less effectively inhibited by Phe30Ala B in solution (Fig. 2b). These results correlate well with the solid-phase ELISA reactivity patterns shown in Fig. 1. Furthermore, both VT1 B and Phe30Ala B immune sera neutralized the cytotoxic activity of VT1 on a Vero cell monolayer in the in vitro neutralization assay described in detail elsewhere (12). Serial twofold dilutions of antisera were preincubated with purified VT1. One 50% cytotoxic dose of purified VT1 for Vero cells was approximately 4.5 pg; two 50% cytotoxic doses were used in this assay. The neutralization titer was defined as the highest dilution of serum at which 50% of the cells were killed by the effects of the toxin. Reciprocal geometric means of 50% neutralization titers for VT1 B- and Phe30Ala B-raised antisera in BALB/c (*H-2*^{d}) mice were 11.06 ± 0.676 (1:2,138) and 10.79 ± 0.232 (1:1,778), respectively (titers were not statistically different [$P = 0.377$]).

The observation that antisera from VT1 B immunized mice

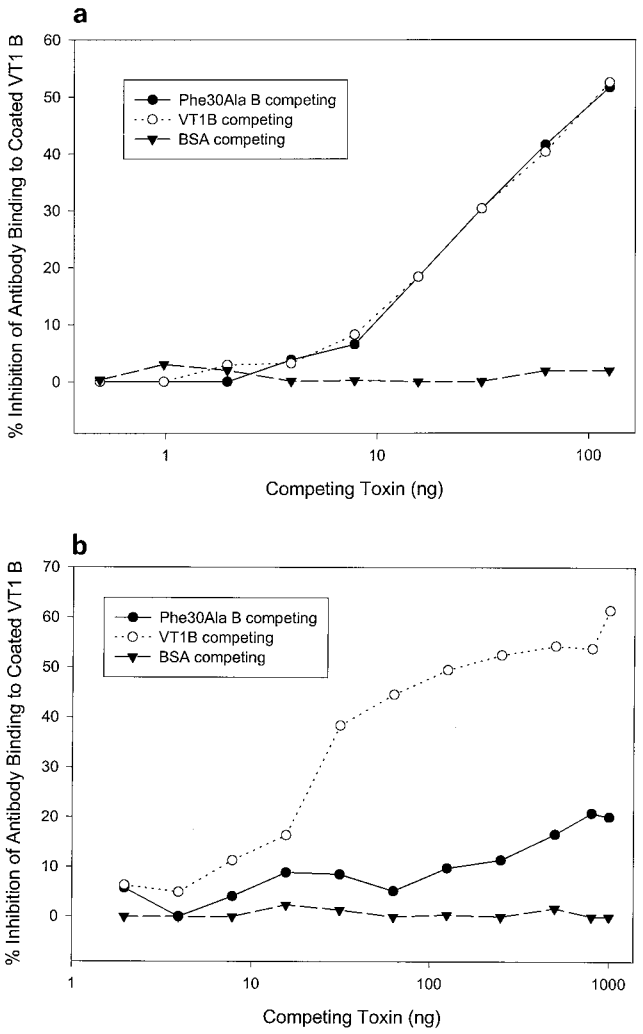

FIG. 2. Inhibition ELISAs for antisera to Phe30Ala B (a) and VT1 B (b). Antisera were diluted 1:200, incubated with the wild-type VT1 B subunit or mutant Phe30Ala B subunit for 1.5 h, and then allowed to bind to the wild-type VT1 B subunit-coated ELISA wells. Preincubation of either set of antisera with bovine serum albumin (BSA) did not inhibit antibody binding to VT1 B-coated ELISA wells. All points represent the mean of triplicate wells from a representative experiment, calculated by the following formula: percent inhibition = $100 \times [(a - b)/a]$ where a is the mean OD_{490} without inhibitor and b is the mean OD_{490} with inhibitor. Error associated with each point did not exceed 8% of the determined mean OD_{490}.

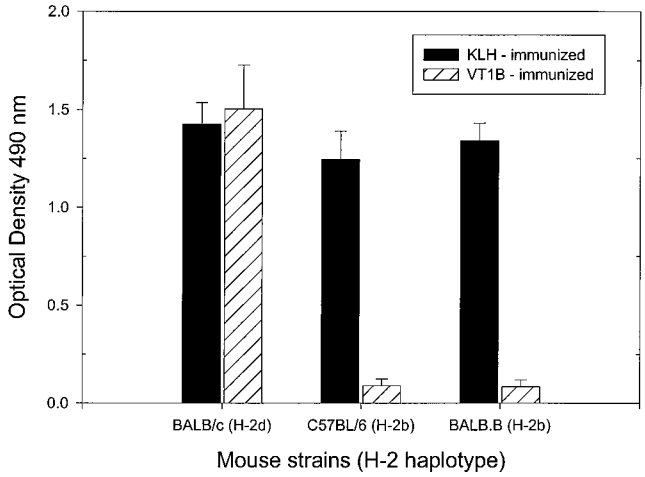

FIG. 3. *H-2* haplotype specificity and immune responsiveness of VT1 B. Anti-VT1 B-subunit-specific antibody responses were measured after intraperitoneal immunizations of BALB/c ($H\text{-}2^d$), C57BL/6 ($H\text{-}2^b$), and BALB · B ($H\text{-}2^b$) mice with purified VT1 B subunit. KLH was used as a positive control. Each bar represents the mean OD_{490} determined for five mice. Standard deviations of the means are shown.

had significantly higher titers of VT1 B- than Phe30Ala B-specific antibodies in both solid-phase and solution-based ELISAs suggests that a significant proportion of antibodies in the serum bind an epitope formed partly by the phenylalanine residue at position 30 in the VT1 B subunit. Structural data shows that phenylalanine 30 is solvent exposed at the surface of the B subunit (29), as would be expected of a B-cell epitope. Furthermore, the crystal structure of Phe30Ala B differs from that of the wild-type B subunit only in the elimination of the phenyl group of phenylalanine 30 (5). We propose that the substitution of alanine for phenylalanine 30 eliminates this immunodominant characteristic. It is of interest that the loss of this immunodominant epitope did not reduce the neutralizing antibody response.

The VT1 B subunit lacks *H-2*^{b}-restricted T-cell epitopes. In contrast to the strong antibody responses to the wild-type VT1 and mutant Phe30Ala B subunits observed in both the BALB/c ($H\text{-}2^d$) and CBA ($H\text{-}2^k$) mice, C57BL/6 ($H\text{-}2^b$) mice produced no detectable antibody response to either the VT1 or Phe30Ala B subunits (OD_{490}s [sera at 1:100 dilution], 0.088 ± 0.035 and 1.503 ± 0.224 for C57BL/6 [$H\text{-}2^b$] and BALB/c [$H\text{-}2^d$], respectively [$n = 5$] [$P < 0.0001$]) (Fig. 3). To ensure that the strains used in this study (BALB/c [$H\text{-}2^d$] and C57BL/6 [$H\text{-}2^b$]) could produce measurable antibody responses to tested immunogens, additional groups of mice were immunized with keyhole limpet hemocyanin (KLH). All mice immunized produced a detectable antibody response to KLH (OD_{490}s [sera at a dilution of 1:100], 1.245 ± 0.145 and 1.425 ± 0.110 for C57BL/6 [$H\text{-}2^b$] and BALB/c [$H\text{-}2^d$], respectively).

To determine if the absence of an antibody response towards the VT1 B subunit in C57BL/6 ($H\text{-}2^b$) mice could be under the control of genes within the *H-2* locus, a congenic mouse strain having the background of a BALB/c mouse but the major histocompatibility complex (MHC) haplotype of a C57BL/6 ($H\text{-}2^b$) mouse was used. This congenic strain is designated BALB · B ($H\text{-}2^b$) (Jackson Laboratories, Bar Harbor, Maine). Immunization of these mice with VT1 B produced no detectable anti-VT1 B response, as determined by the absence of VT1 B-specific antibodies by solid-phase ELISA (OD_{490}s [sera at a 1:100 dilution], 0.083 ± 0.036 and 1.503 ± 0.224 for congenic BALB · B [$H\text{-}2^b$] and BALB/c [$H\text{-}2^d$] mice, respectively, while both strains responded equally to KLH) (Fig. 3). The Phe30Ala B subunit also failed to produce a detectable antibody response in the congenic BALB · B ($H\text{-}2^b$) strain (data not shown). While IgG(γ)-specific anti-VT1 B- and anti-Phe30Ala B-subunit antibodies were detected only in serum samples taken from $H\text{-}2^d$ and $H\text{-}2^k$ mice, IgM(μ)-specific anti-VT1 B- and anti-Phe30Ala B-subunit antibodies were not detected in serum samples taken from any strain tested.

To investigate the possibility that the failure in the responsiveness of $H\text{-}2^b$ mice was due to the dose of antigen used, groups of BALB/c ($H\text{-}2^d$), C57BL/6 ($H\text{-}2^b$), and BALB · B ($H\text{-}2^b$) mice were immunized with various doses (0.5 to 4 μg) of B subunit. Even with the lowest dose, $H\text{-}2^b$ strains failed to induce a measurable antibody response against the B subunit.

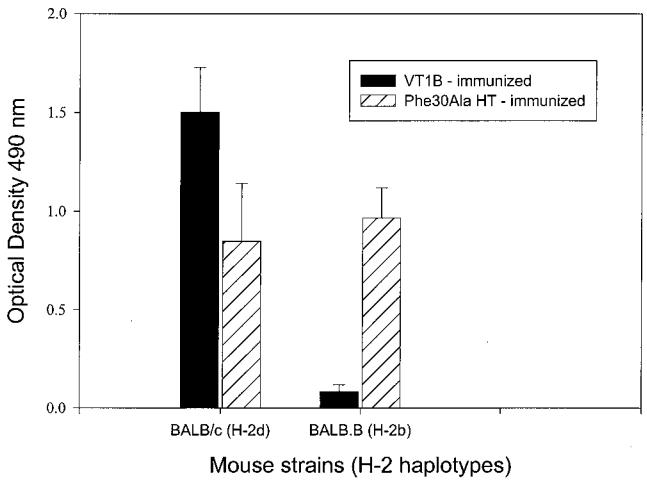

FIG. 4. Antibody responses to the VT1 B subunit in BALB/c (*H-2*^{d}) and BALB · B (*H-2*^{b}) strains following immunization with Phe30Ala HT. Each bar represents the mean OD_{490} determined for five mice. Standard deviations of the means are shown.

In contrast, the BALB/c (*H-2*^{d}) strain produced anti-VT1 B antibody responses at all doses tested (data not shown).

Although antibody responsiveness to various antigens with syngeneic and congenic mice of different *H-2* haplotypes has been previously reported (2, 6), this is the first time that such an *H-2* haplotype-dependent antibody response to the B subunit of VT1 has been demonstrated. It has been previously reported that the antibody responsiveness to cholera toxin (CT), a structurally related AB_5 subunit toxin (11), is also under *H-2* control (6). In contrast to the response observed for VT1 B, strains with the *H-2*^{b} haplotype were high responders to the B subunit of CT, while those with the *H-2*^{d} and *H-2*^{k} haplotypes were low responders (9). This genetic control of the anti-CT response was mapped to the *I-A* subregion of the *H-2* complex (6). While we believe that the lack of a detectable anti-VT1 B response is most likely due to the inability of *H-2*^{b} MHC class II proteins on the surface of antigen-presenting cells to present processed peptides of the B-subunit molecule to T lymphocytes, we cannot exclude other mechanisms. However, because *H-2*^{b} strains of mice express only an $I\text{-}A^b$ molecule, it is most likely that the B subunit lacks an epitope which can be presented by this MHC molecule.

Immunization of *H-2*^{b} mice with Phe30Ala HT overcomes the B-subunit-deficient antibody response. Intraperitoneal immunization of BALB · B (*H-2*^{b}) and C57BL/6 (*H-2*^{b}) mice with 0.5 μg of biologically active Phe30Ala HT restored anti-VT1 B responsiveness in these strains (OD_{490}s [sera at 1:100 dilution], 0.966 ± 0.153 and 0.848 ± 0.294 for BALB · B [*H-2*^{b}] and BALB/c [*H-2*^{d}] mice, respectively) (Fig. 4). Similar responses were observed in the C57BL/6 (*H-2*^{b}) strain (data not shown). The anti-VT1 B antibody responses observed in both the *H-2*^{d} and *H-2*^{b} strains were of the IgG(γ), not the IgM(μ), isotype. We hypothesize that the A-subunit portion of the toxin carries T-cell epitopes that can be presented by *H-2*^{b} MHC class II proteins to helper T lymphocytes. The dose used for the Phe30Ala HT immunizations corresponded to five times the previously reported 50% lethal dose for wild-type VT1 HT (16). Immunization at this particular dose produced no clinical symptoms typical of a VT effect. Injections with amounts equivalent to 10 times the previously reported 50% lethal dose and larger produced disease and for this reason were not used for immunization purposes. The immunization protocol was identical to that described above.

The lack of anti-VT1 B antibody responsiveness in certain mouse strains has important implications for vaccine development. To date, vaccine studies have concentrated on the B-subunit portion of the molecule as a natural toxoid (1, 26, 30, 31). If such an MHC dependency of antibody responsiveness to the VT1 B subunit exists in humans, B-subunit-derived vaccines may prove suboptimal in protecting all members of a population against VT-mediated diseases. The demonstration of a role for the A subunit in overcoming MHC restriction in mice suggests that vaccines that incorporate both the A and B subunits might be more effective. Immunization with HT would provide the immune system with a larger repertoire of T- and B-cell epitopes. Results of similar studies with both the heat-labile enterotoxin and CT have also suggested that the A subunit may be useful in overcoming any genetic restriction that may be present in the human population (8, 20). It remains to be seen whether MHC restriction will result in poor VT1 B-subunit vaccine antibody responses in outbred human populations.

While Phe30Ala HT antisera reacted with the wild-type B subunit in ELISAs (Fig. 4), the reciprocal geometric mean titer of antibody to the VT1 HT was significantly greater than the VT1 B-specific antibody titer (13.97 [1:16,032] and 9.36 ± 0.547 [1:656] for VT1 HT and VT1 B, respectively [$n = 5$] [$P < 0.001$]). Furthermore, antisera raised to Phe30Ala HT and pretreated with soluble VT1 B subunit yielded anti-VT1 HT-specific antibodies with a reciprocal geometric mean titer of 13.57 ± 0.547 (1:12,134). Anti-VT1 HT antibody titers before and after pretreatment with soluble VT1 B were not significantly different ($P = 0.177$). This result suggests that when immunized with Phe30Ala HT, mice generate higher titers of antibodies to the A subunit or conformational epitopes formed between the B and A portions of the molecule than to the B subunit alone. Therefore, the A portion of the toxin, when associated with the B subunit in a 1A:5B configuration, may be the immunodominant part of the HT. Similar A-subunit immunodominance was also observed in the Phe30Ala HT immunization of rabbits (2a), suggesting that this phenomenon is not species specific and therefore may occur in humans. Because the HT used was a mutant derivative of VT1, we cannot conclude that the same effect would be observed with the wild-type VT1 HT. It is important to note that whether the mice were immunized with HT or with the B subunit had no effect on the titer of VT1-neutralizing antibodies, despite the fact that a lower anti-VT1 B antibody response was observed in those mice immunized with Phe30Ala HT (10.96 ± 0.298 [1:1,995]). This suggests that A-subunit-specific antibodies may play an important role in VT1 neutralization. Similarly, CT-neutralizing activity of CT A-subunit-specific antibodies has also been reported (8).

Despite the lack of cross-protection of anti-VT1 antisera against the cytotoxic effects of the heterologous toxin VT2 in vitro (3, 13), Bielaszewska et al. have reported that immunity to either toxin in rabbits prevents the accumulation of both toxins in the gastrointestinal tract and central nervous system and promotes their accumulation in both the liver and spleen (3). This pattern of toxin localization in rabbits has previously been associated with immune protection against VT (23). While the significance of this observed in vivo cross-protection needs to be confirmed by formal animal protection studies, these results suggest that vaccines which include both the A and B subunits may be effective in protecting against heterologous VTs (e.g., VT2). This is an important consideration that must not be overlooked in developing vaccine strategies, as *E.*

coli strains producing heterologous verotoxins (VT2 and VT2c) are clinically as important as or more important than VT1-producing *E. coli* (18, 28). Our demonstration that immunization against Phe30Ala HT produces a higher titer of A-subunit-specific antibodies than B-subunit-specific antibodies suggests that Phe30Ala HT may be useful as a natural toxoid. However, the residual toxicity of Phe30Ala HT observed in mice at increased doses would preclude the use of such a vaccine in humans.

Conclusion. In summary, our results have implications for the design of a safe and effective vaccine to protect against VT-mediated disease. While the B subunit may serve as a natural toxoid, the A subunit should not be overlooked in vaccine development strategies. The A subunit may be a source of critical T-helper epitopes and cross-protective B-cell epitopes. Because Phe30Ala HT has proved too toxic in vivo for use as a natural toxoid, future studies will focus on the construction of less toxic yet structurally conserved mutant VT1 HT derivatives.

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REFERENCES

1. **Acheson, D. W. K., M. M. Levine, J. B. Kaper, and G. T. Keusch.** 1996. Protective immunity to Shiga-like toxin I following oral immunization with Shiga-like toxin I B-subunit-producing *Vibrio cholerae* CVD 103-HgR. Infect. Immun. **64:**355–357.
2. **Banic, D. M., P. Delplace, C. Mazingue, and D. Camus.** 1994. *H-2*^{b} restriction of the immune response to the p126 *Plasmodium falciparum* antigen. Clin. Exp. Immunol. **95:**472–478.

2a. **Bast, D. J., J. L. Brunton, M. A. Karmali, and S. E. Richardson.** 1997. Toxicity to rabbits and immunogenicity of a verotoxin 1 mutant with reduced globotriaosylceramide receptor binding. Infect. Immun. **65:**2019–2028.

3. **Bielaszewska, M., M. A. Karmali, and M. Petric.** 1994. Localization of Verocytotoxin (VT)2 and antigenic cross-reactivity of VT1 and VT2 in the rabbit model, p. 249–252. *In* M. A. Karmali and A. G. Goglio (ed.), Recent advances in Verocytotoxin-producing *Escherichia coli* infections. Elsevier, Amsterdam, The Netherlands.
4. **Boyd, B., S. Richardson, and J. Gariepy.** 1991. Serological responses to the B subunit of Shiga-like toxin 1 and its peptide fragments indicate that the B subunit is a vaccine candidate to counter the action of the toxin. Infect. Immun. **59:**750–757.
5. **Clarke, C., D. J. Bast, A. Sharp, P. M. St. Hilaire, R. Agha, P. E. Stein, E. J. Toone, R. J. Read, and J. L. Brunton.** 1995. Phenylalanine 30 plays an important role in receptor binding of Verotoxin-1. Mol. Microbiol. **19:**891–899.
6. **Elson, C. O., and W. Ealding.** 1985. Genetic control of the murine immune response to cholera toxin. J. Immunol. **135:**930–932.
7. **Endo, Y., K. Tsurugi, T. Yutsudo, Y. Takeda, T. Ogasawara, and K. Igarashi.** 1988. Site of action of a Vero toxin (VT) from *Escherichia coli* O157:H7 and of Shiga toxin on eukaryotic ribosomes. Eur. J. Biochem. **171:**45–50.
8. **Fontana, M. R., R. Manetti, V. Giannelli, C. Magagnoli, A. Marchini, R. Oliveri, M. Domenighini, R. Rappuoli, and M. Pizza.** 1995. Construction of nontoxic derivatives of cholera toxin and characterization of the immunological response against the A subunit. Infect. Immun. **63:**2356–2360.
9. **Hirabayashi, Y., S.-I. Tamura, Y. Suzuki, T. Nagamine, C. Aizawa, K. Shimada, and T. Kurata.** 1991. *H-2*-unrestricted adjuvant effect of cholera toxin B subunit on murine antibody responses to influenza virus haemagglutinin. Immunology **72:**329–335.
10. **Hofmann, S. L.** 1993. Southwestern International Medicine Conference: Shiga-like toxins in hemolytic uremic syndrome and thrombotic thrombocytopenic purpura. Am. J. Med. Sci. **306:**398–406.
11. **Holmgren, J.** 1981. Actions of cholera toxin and the prevention and treatment of cholera. Nature (London) **292:**413.
12. **Karmali, M. A., M. Petric, C. Lim, R. Cheung, and G. Arbus.** 1985. The association between idiopathic hemolytic uremic syndrome and infection by verotoxin-producing *Escherichia coli*. J. Infect. Dis. **151:**775–782.
13. **Karmali, M. A.** 1989. Infection by verocytotoxin-producing *Escherichia coli*. Clin. Microbiol. Rev. **2:**15–38.
14. **Mangeney, M., C. A. Lingwood, S. Taga, B. Caillou, T. Tursz, and J. Wiels.** 1993. Apoptosis induced in Burkitt's lymphoma cells via Gb_3/CD77, a glycolipid antigen. Cancer Res. **53:**5314–5319.
15. **Mullett, C., D. W. K. Acheson, L.-Y. Tseng, and E. Boedeker.** 1994. High titer serum antibody against the B subunit of Shiga-like toxin I protects in a model of enterohemorrhagic *Escherichia coli* (EHEC) colitis. Gastroenterology **106:**A740.
16. **O'Brien, A. D., and G. D. LaVeck.** 1983. Purification and characterization of a *Shigella dysenteriae* 1-like toxin produced by *Escherichia coli*. Infect. Immun. **40:**675–683.
17. **O'Brien, A. D., V. Tesh, A. Donohue-Rolfe, M. Jackson, S. Olsnes, K. Sandvig, A. Lindberg, and G. T. Keusch.** 1992. Shiga toxin: biochemistry, genetics, and mode of action and role in pathogenesis. Curr. Top. Microbiol. Immunol. **180:**65–94.
18. **Ostroff, S. M., P. I. Tarr, M. A. Neill, J. H. Lewis, N. Hargrett-Bean, and J. M. Kobayashi.** 1989. Toxin genotypes and plasmid profiles as determinants of systemic sequelae in *Escherichia coli* O157:H7 infections. J. Infect. Dis. **160:**994–998.
19. **Petric, M., M. A. Karmali, S. E. Richardson, and R. Cheung.** 1987. Purification and biological properties of *Escherichia coli* verocytotoxin. FEMS Microbiol. Lett. **41:**63–68.
20. **Pizza, M., M. R. Fontana, M. M. Giuliani, M. Domenighini, C. Magagnoli, V. Giannelli, D. Nucci, W. Hol, R. Manetti, and R. Rappuoli.** 1994. A genetically detoxified derivative of heat-labile *E. coli* enterotoxin induces neutralizing antibodies against the A subunit. J. Exp. Med. **6:**2147–2153.
21. **Ramotar, K., B. Boyd, G. Tyrrell, J. Gariepy, C. Lingwood, and J. L. Brunton.** 1990. Characterization of Shiga-like toxin 1 B purified from overproducing clones of the SLT-1 B cistron. J. Biochem. **272:**805–811.
22. **Remuzzi, G., and C. Joja.** 1994. Endothelial cell physiology, p. 309–316. *In* M. A. Karmali and A. G. Goglio (ed.), Recent advances in Verocytotoxin-producing *Escherichia coli* infections. Elsevier, Amsterdam, The Netherlands.
23. **Richardson, S., T. Rotman, V. Jay, C. Smith, L. Becker, M. Petric, N. Olivieri, and M. Karmali.** 1992. Experimental verocytotoxemia in rabbits. Infect. Immun. **60:**4154–4167.
24. **Richardson, S. E., M. A. Karmali, L. E. Becker, and C. R. Smith.** 1988. The histopathology of the hemolytic uremic syndrome (HUS) associated with verocytotoxin-producing *Escherichia coli* (VTEC) infections. Hum. Pathol. **19:**1102–1108.
25. **Riley, L., R. Remis, S. Helgerson, H. McGee, J. Wells, B. Davis, R. Herbert, E. Olcott, L. Johnson, N. Hagrett, P. Blake, and M. Cohen.** 1983. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. N. Engl. J. Med. **308:**681–685.
26. **Ryd, M., N. Verma, and A. A. Lindberg.** 1992. Induction of a humoral immune response to a Shiga toxin B subunit epitope expressed as a chimeric LamB protein in a Shigella flexneri live vaccine strain. Microb. Pathog. **12:**399–407.
27. **Sandvig, K., S. Olsnes, J. E. Brown, O. W. Brown, and B. van Deurs.** 1989. Endocytosis from coated pits of Shiga toxin: a glycolipid-binding protein from *Shigella dysenteriae* 1. J. Cell Biol. **108:**1331–1343.
28. **Scotland, S. M., G. A. Willshaw, H. R. Smith, and B. Rowe.** 1987. Properties of strains of *E. coli* belonging to serogroup O157 with special reference to production of Verocytotoxins VT1 and VT2. Epidemiol. Infect. **99:**613–624.
29. **Stein, P. E., A. Boodhoo, G. J. Tyrrell, J. L. Brunton, and R. J. Read.** 1992. Crystal structure of the cell binding B oligomer of Verotoxin-1 from *Escherichia coli*. Nature (London) **355:**748–750.
30. **Su, G. F., H. N. Brahmbhatt, V. de Lorenzo, J. Wehland, and K. N. Timmis.** 1992. Extracellular export of Shiga toxin B subunit/haemolysin A (C-terminus) fusion protein expressed in *Salmonella typhimurium aroA*-mutant and stimulation of B-subunit specific antibody responses in mice. Microb. Pathog. **13:**465–476.
31. **Su, G.-F., H. N. Brahmbhatt, J. Wehland, M. Rohde, and K. N. Timmis.** 1992. Construction of stable LamB-Shiga toxin B subunit hybrids: analysis of expression in *Salmonella typhimurium aroA* strains and stimulation of B subunit-specific mucosal and serum antibody responses. Infect. Immun. **60:**3345–3359.
32. **Tyrrell, G. J., K. Ramotar, B. Toye, B. Boyd, C. A. Lingwood, and J. L. Brunton.** 1992. Alteration of the carbohydrate binding specificity of verotoxins from Galα1-4Gal to GalNAcβ1-3Galα1-4Gal and vice versa by site-directed mutagenesis of the binding subunit. Proc. Natl. Acad. Sci. USA **89:**524–528.

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