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# Localization of Intravenously Administered Verocytotoxins (Shiga-Like Toxins) 1 and 2 in Rabbits Immunized with Homologous and Heterologous Toxoids and Toxin Subunits

M. BIELASZEWSKA,[1]† I. CLARKE,[1] M. A. KARMALI,[1,2] AND M. PETRIC[1,2]*

*The Research Institute and Division of Microbiology, Department of Pediatric Laboratory Medicine, The Hospital for Sick Children,[1] and The Department of Microbiology, The University of Toronto,[2] Toronto, Canada M5G 1X8*

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**Rabbits challenged intravenously with Shiga toxin or with *Escherichia coli* verocytotoxin 1 or 2 (VT1 or VT2) are known to develop diarrhea, paralysis, and death, which can be prevented by immunization with a toxoid. The pathological effects of VT1 in the central nervous system and the gastrointestinal tract of unimmunized rabbits correlate with the localization of $^{125}$I-VT1 in these tissues, whereas in immunized animals, localization of $^{125}$I-VT1 in target tissues is inhibited and labeled toxin is cleared by the liver and spleen. By using the approach described above in this study, rabbits immunized with VT1 toxoid, VT2 toxoid, or with the A or B subunit of each toxin were challenged with intravenous $^{125}$I-VT1 or $^{125}$I-VT2. After 2 h, the animals were sacrificed, and selected tissues were analyzed for uptake of labeled toxin. It was found that animals immunized with either VT1 toxoid or VT2 toxoid were protected from target tissue uptake of both $^{125}$I-VT1 and $^{125}$I-VT2. Rabbits immunized with either the VT1 A or VT2 A subunit were also protected from target tissue uptake of both the homologous and heterologous $^{125}$I-labeled holotoxins. In contrast, in animals immunized with the toxin B subunits, protection extended only against challenge by the homologous toxin. These results provide evidence of VT1 and VT2 cross-neutralization in vivo in the rabbit model and indicate that the in vivo cross-neutralization is a function, mainly, of antibodies directed to the VT A subunits. This suggests that the VT1 A or VT2 A subunit may be a suitable immunogen for immunizing humans against systemic VT-mediated disease.**

Infection by verocytotoxin (VT)-producing *Escherichia coli* (28, 29), also referred to as Shiga-like toxin (SLT)-producing *E. coli* or Shiga toxin-producing *E. coli* (36, 37), is closely associated with a spectrum of disease (17, 23) that includes nonspecific diarrhea, hemorrhagic colitis (44), and the hemolytic-uremic syndrome (HUS) (24, 27). HUS, the most serious complication of VT-producing *E. coli* infection, is characterized by acute renal failure, thrombocytopenia, and microangiopathic hemolytic anemia and occurs a few days after the onset of a bloody diarrheal illness (23, 30). It is associated with significant long-term morbidity, renal dysfunction in up to 40% of the patients, and a mortality of 3 to 5% (30, 32, 48, 52). The syndrome occurs most frequently in young children, with an annual incidence in North America of about two to three cases per 100,000 children under 5 years of age (18, 45, 50), in contrast to a roughly 10-fold-higher incidence in this age group in Argentina (33). While considerable progress has been made in the medical management of HUS, no specific treatment is currently available (30).

Human VTEC strains produce at least three serologically distinct subunit toxins, either alone or in combination (23, 38, 51). These three toxins (36) are VT1 [SLT I; type strain C600(H19J)], VT2 [SLT II; type strain C600(933w)], and VT2c [SLT IIc and SLT II vh; type strains E32511, B2F1, and 7279]. The prototype toxin VT1 is virtually identical to Shiga toxin produced by *Shigella dysenteriae* type 1. By using in vitro neutralization tests in Vero cells, VT1 has been shown to be serologically distinct from VT2 (and VT2c) in that these toxins showed no cross-neutralization by heterologous antisera. Conversely, VT2 can be completely neutralized by antiserum to VT2c, whereas VT2c is only partially neutralized by antiserum to VT2 (26, 38, 46, 47).

A large body of evidence supports the hypothesis that VTs are of direct significance in the genesis of HUS. Histologically, HUS is characterized by widespread thrombotic microangiopathy (TM) in the renal glomeruli, gastrointestinal (GI) tract, and other organs such as the brain, pancreas, and lungs (15, 30, 42, 53, 55). This is associated with a characteristic swelling of glomerular capillary endothelial cells accompanied by widening of the subendothelial space (15, 30). The occurrence of widespread systemic TM lesions in HUS with a characteristic pathology in endothelial cells is consistent with a systemic toxemic disease with endothelial cells as primary target sites for VT action. Evidence to support this includes observations that (i) VTs are cytotoxic to human renal microvascular endothelial cells in vitro (34, 39), (ii) VT1 binds to glomerular endothelial cells in human renal sections (31), and (iii) intravenous administration of VT1 in rabbits leads to dose-dependent symptoms of diarrhea and flaccid paresis (9, 22, 43) which are associated with an underlying microvascular pathology in the cecum and the central nervous system (CNS) strikingly similar to the TM observed in the kidneys, gut, CNS, and other tissues in HUS (42). Evidence supporting the hypothesis that

* Corresponding author. Mailing address: Division of Microbiology, The Hospital for Sick Children, 555 University Ave., Toronto, Canada M5G 1X8. Phone: (416) 813-6111. Fax: (416) 813-5993.

† Present address: Institute of Medical Microbiology, The 2nd Medical Faculty, Charles University, Prague, Czech Republic.

the tissue pathology in rabbits is due to the direct action of VT1 is our observation that in rabbits challenged intravenously with $^{125}$I-labeled VT1, the labeled toxin disappears rapidly from the circulation and localizes at the sites of pathology (gut and CNS), where it binds specifically to vascular endothelial cells (43).

The peak age-related incidence of HUS in young children suggests that susceptibility to it, as to other specific infectious diseases of childhood, may be related to the absence of specific immunity, possibly antitoxin (23, 25). This is supported by the findings of Howard, over 40 years ago, that the injection of Shiga toxoid into laboratory animals gives rise to neutralizing antibodies (NAbs) and protects the animals against the lethal effects of Shiga toxin (22). MacLeod and Gyles (35) have shown that active immunization with the pig edema disease toxoid, VTe, protects pigs against experimental disease induced by VTe. Furthermore, Harari et al. (20, 21) showed that active immunization with synthetic peptides from Shiga toxin B subunit protects mice against the lethal effects of Shiga toxin. Acheson et al. (1) immunized rabbits orally with a recombinant *Vibrio cholerae* vaccine strain (CVD 103-HgR) in which 94% of the cholera toxin A subunit had been deleted and which contained a plasmid encoding genes for the VT1 B subunit. Such a strain given orally to rabbits was able to induce serum NAbs to VT1.

Traditional methods for investigating immunity in experimental animal models have depended on 50% lethal dose ($LD_{50}$) experiments involving a large number of animals. However, in the experimental rabbit model of VT-induced disease, we have shown that the uptake of $^{125}$I-labeled VT1 by specific target tissues correlates directly with characteristic pathology induced in these tissues by unlabeled toxin and, furthermore, that toxin uptake by target tissues is prevented in animals immunized with VT1 toxoid (43). This inhibition of radiolabeled VT1 uptake by target tissues in animals immunized against VT1 opens up a more humane approach for testing the immunogenicity of different toxoid preparations as vaccine candidates.

The choice of a VT vaccine immunogen and route of delivery for optimal protection against human VT-associated disease remains unresolved. The lack of cross-neutralization between VT1 and VT2 in vitro (38) would suggest that immunization with multiple immunogens may be necessary to provide broad protection against exposure to all the VTs. On the other hand, we have observed, in preliminary studies (7) using the $^{125}$I-labeled VT uptake method, that immunization of rabbits with VT2 toxoid led unexpectedly to the inhibition of target tissue uptake of $^{125}$I-labeled VT1 and, conversely, that immunization of rabbits with VT1 toxoid was associated with the inhibition of target tissue uptake of $^{125}$I-labeled VT2. These findings suggest strongly that immunization with a single toxoid can provide immunity to the homologous toxin and cross-immunity to the heterologous toxin. The objective of the present study was to further characterize the basis of this cross-reactivity by studying the uptake of radiolabeled VTs in rabbits immunized with homologous and heterologous VT toxoids and with their respective A and B subunits.

## MATERIALS AND METHODS

**Toxin purification.** VT1 was purified as described previously (40) from JB28, an *E. coli* TB1 strain, provided by J. Brunton, that had been transformed by recombinant plasmid pUC19B containing VT1 genes cloned from bacteriophage H19B (12). VT2 was purified from *E. coli* R82pJES 120DH5a, kindly provided by J. E. Samuel. The purity of these toxin preparations was established by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoretic analysis. The Iodobead method (Pierce) was used to radiolabel purified VT1 and VT2 preparations with $^{125}$I. The labeled toxins were characterized for specific activity ($1.7 \times 10^5$ to $2.3 \times 10^5$ cpm/μg) and biological activity ($3.8 \times 10^5$ and $1.9 \times 10^3$ 50% cytotoxic doses [$CD_{50}$s]/μg for VT1 and VT2, respectively). Unlabeled VT1 and VT2 toxin preparations have respective specific activities of $5 \times 10^5$ and $8 \times 10^4$ $CD_{50}$s/μg. Vero cell binding activity was assessed by quantitating the proportion of the labeled VT (100,000 cpm) that bound to three sequential monolayers of Vero cells. The Vero cell binding activities, which represent the percentage of the input activity bound to the 25-cm$^2$ monolayers after 1 h of incubation and three washes, were 48% for VT2 preparations and 75% for VT1 preparations.

**Immunization of rabbits.** VT1 and VT2 toxoids were prepared by glutaraldehyde treatment (16). New Zealand White rabbits weighing approximately 2 kg were immunized subcutaneously with doses of 60 μg of toxoid mixed with equal volumes of Freund's incomplete adjuvant in four sequential weekly intervals. For subunit immunizations, VT1 and VT2 were separated into the A and B subunit fractions by SDS-polyacrylamide gel electrophoresis. Coomassie blue-stained fractions were excised from the gel slabs, dialyzed for 2 h to decrease their SDS content, macerated by passage through an 18-gauge syringe, and used to immunize the rabbits by repeat subcutaneous injection in the presence of Freund's incomplete adjuvant.

**Antibody determinations.** Determinations of antibody to the homologous and heterologous toxin were performed by the cytotoxicity neutralization assays (24) and by the indirect antibody capture enzyme-linked immunosorbent assay (ELISA) adapted from that described by Anderson et al. (2). This assay employed Immunolon 2 (Dynatech, Inc., McLean, Va.) flat-bottom plates coated with goat anti-rabbit immunoglobulin G (IgG) (heavy- plus light-chain specificity; Tago Immunochemicals, Burlingame, Calif.) diluted to 1:500 in phosphate-buffered saline (PBS) to form the antibody capture solid phase. The test sera together with the high- and low-positive controls as well as the negative control were tested at a dilution of 1:50 in PBS containing 0.15% Tween 20 and 0.5% gelatin (PBS-T-G). For titrations, sera were diluted in PBS-T-G from 1:100 to 1:6,400,000. All the washing steps were performed with PBS-Tween 20. Purified VT1 and VT2 (5 μg/ml) were then added to separate reaction wells, which were subsequently reacted with monoclonal antibodies PH1 (8) (provided by C. Lingwood) and BC5BB12 (13) (provided by N. Strockbine) at a dilution of 1:2,000 in PBS-T-G. The peroxidase-conjugated goat anti-mouse IgG (Bio-Rad Laboratories, Mississauga, Ontario, Canada) was used at a dilution of 1:16,000 in PBS-T-G with *ortho*-phenylenediamine as a chromogen, and the reactions were monitored for absorbance at 414 nm. Sera were considered positive for VT antibody if the absorbance was greater than 0.025 and the ratio of the values for the positive- to negative-control standards was greater than 2.00.

Development of antibody to the VT subunits was monitored by Western blotting (immunoblotting) (41). Briefly, preparations of purified VT1 and VT2 were subjected to SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membranes, which were then incubated in 3% gelatin–3% skim milk powder–10% goat serum for 2 h at 37°C. Preparations of the sera, diluted 1:50 in 50 mM Tris-buffered saline and 1% skim milk powder as a blocking agent, were incubated with strips of the membrane overnight at 4°C. After washing, the strips were transferred to a 1:1,000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad) and incubated for 2 h at room temperature. The strips were developed in a solution of 4-chloro-1-naphthol-hydrogen peroxide.

**Intravenous administration of VT1 and VT2 to rabbits.** In the initial study, $^{125}$I-labeled VT1 and VT2 were administered intravenously to rabbits, in groups of three, which had been immunized with VT1 or VT2 and to three nonimmune controls. In a subsequent study, $^{125}$I-labeled VT1 and VT2 were administered to rabbits which had been immunized, in groups of three, with either the A or B subunit of VT1, the A or B subunit of VT2, or with VT1 or VT2 holotoxoids and to nonimmune controls. Following administration of approximately $4 \times 10^6$ cpm of labeled VT through the ear vein, a 1- to 2-ml blood specimen was collected from the ear artery. After a 2-h period, the animals were sacrificed by lethal injection, selected tissues were removed and weighed, and representative portions of these were analyzed for radioactivity (43).

## RESULTS

The results reported below were performed as two sequential studies (studies 1 and 2). This first addressed cross-immunity in rabbits immunized with VT1 and VT2 toxoids. These findings were extended in the subsequent study of VT1 and VT2 utilizing A and B subunits as immunogens to gain additional insight into which component of the toxin is most relevant to cross-immunity.

**Antibody status of the rabbits.** Sera collected from all rabbits before immunization were negative for VT-NAb and for anti-VT IgG measured by ELISA (ELISA antibody) to VT1 and VT2. Antibody titers in the sera of immunized rabbits (collected immediately prior to the administration of the $^{125}$I-VT preparations) are shown in Table 1. Control, nonim-

TABLE 1. Immune responses to VT1 and VT2 in rabbits immunized with VT1 toxoid, VT2 toxoid, VT1 A or B subunit, or VT2 A or B subunit

| Immunogen | Antibody titer | | | | | |
|---|---|---|---|---|---|---|
| | NAb VT-1[a] | NAb VT-2[b] | ELISA VT-1[c] | ELISA VT-2[d] | WB VT-1[e] | WB VT-2[f] |
| Study 1 | | | | | | |
| VT1 | 1:3,883[g] | <1:8 | 1:1,269,921[g] | 1:158,740[g] | ND[h] | ND |
| VT2 | <1:8 | 1:3,025[g] | 1:200,000[g] | 1:1,007,937[g] | ND | ND |
| Study 2 | | | | | | |
| VT1 A (6)[i] | 1:64–>1:128 | <1:8 | Positive | Negative | Positive | Negative |
| VT1 B (6) | 1:16–1:64 | <1:8 | Positive | Negative | Positive | Trace |
| VT2 A (4) | <1:8 | 1:16–1:128 | Positive | Positive | Negative | Positive |
| VT2 B (5) | <1:8 | 1:32–>1:128 | Negative | Negative | Trace | Positive |
| VT1 (2) | >1:128 | <1:8 | Positive | Positive | Positive | Positive |
| VT2 (4) | <1:8 | >1:128 | Positive | Positive | Positive | Positive |

[a] NAb titer to VT1.
[b] NAb titer to VT2.
[c] Anti-VT1 IgG detected by ELISA.
[d] Anti-VT2 IgG detected by ELISA.
[e] Anti-VT1 IgG detected by Western blotting.
[f] Anti-VT2 IgG detected by Western blotting.
[g] Mean titer of three animals.
[h] ND, not done.
[i] Numbers in parentheses indicate numbers of animals tested.

munized rabbits remained negative for NAbs and ELISA antibodies to VT1 and VT2. Immunized rabbits developed a significant NAb response to the homologous toxin but not to the heterologous toxin (Table 1). On the other hand, they developed ELISA antibody responses to both the homologous and heterologous toxins, although the responses to the heterologous toxin were 5- to 10-fold lower. This indicates that there are cross-reactive epitopes on these toxins which are detectable by the ELISA but not by the NAb assay.

The immune responses of the rabbits immunized with toxin subunits are shown in the section of Table 1 concerned with study 2. Designed to confirm that the rabbits had developed an immune response, the NAb was measured in sera at dilutions of only 1:8 to 1:128. The ELISAs were performed at a serum dilution of 1:100. Rabbits immunized with the VT A and B subunits developed NAb to the homologous toxin but not to the heterologous toxin. Rabbits immunized with the A subunits were reactive by ELISA with the homologous toxin but were less, if at all, reactive to the heterologous toxin. Immunization with the B subunits led to the appearance of ELISA antibody

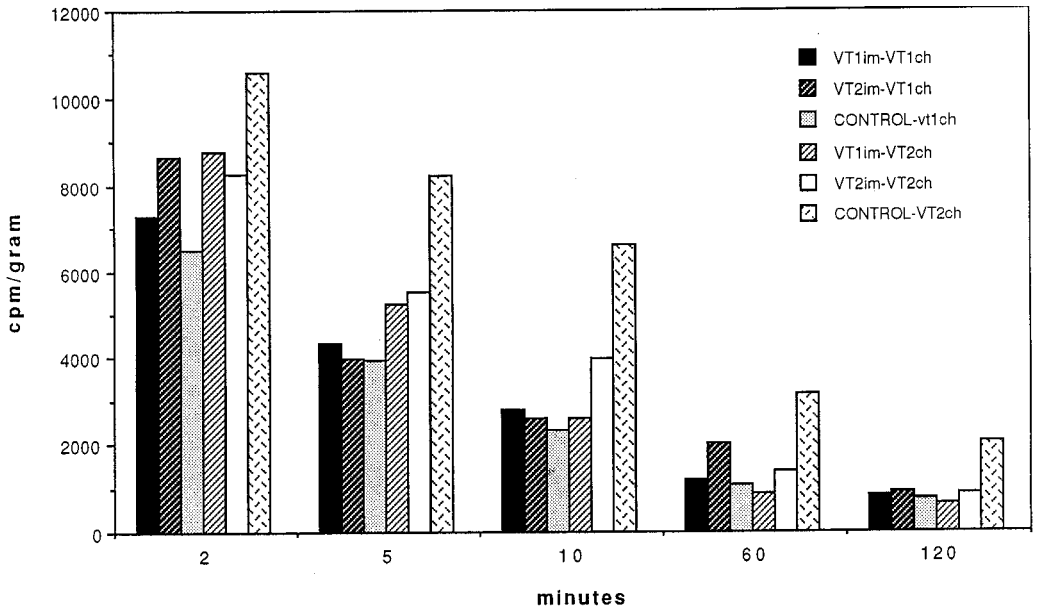


FIG. 1. Clearance of $^{125}$I-VT from blood. Blood specimens collected from the rabbits at the indicated times were weighed and counted for $^{125}$I activity. Each bar represents the average of the specimens from the designated group of three rabbits at the specific time point. The rabbit immune status is designated (e.g., VT1im refers to VT1 immune). The radiolabeled toxin is also designated (e.g., VT1ch refers to VT1 challenge).

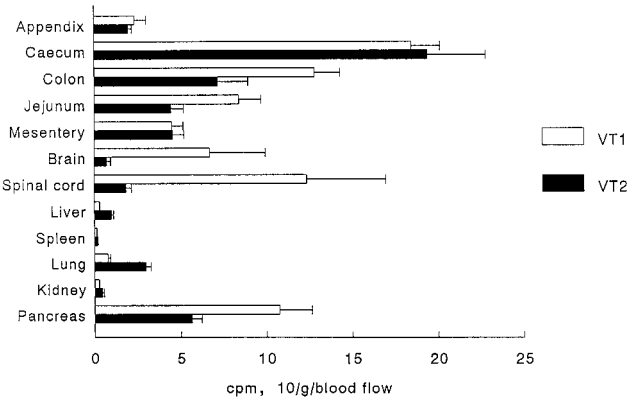


FIG. 2. Tissue binding distribution of $^{125}$I-VT2 and $^{125}$I-VT1 in nonimmune rabbits. Two hours after intravenous injection of $^{125}$I-labeled VT1 and VT2, designated organs of the rabbits were removed and a defined portion was counted for $^{125}$I activity. Toxin present in the tissue was normalized to blood flow through the organ to maintain consistency with our previously reported findings (43). Bars represent average counts from three rabbits per determination.

to the homologous toxin in the case of VT1 but did not lead to a detectable level of antibody in the case of VT2. Immunization with VT1 and VT2 toxoids resulted in strong ELISA antibody responses to both homologous and heterologous toxins. However, the toxoids in this case were prepared with glutaraldehyde, which may have led to the exposure of different epitopes. Analyses of the immune response by Western blotting showed results that were similar to those seen by ELISA, with specific responses to the toxin subunits being detected.

**Clearance of VT1 and VT2 from the blood.** The clearance of intravenously administered $^{125}$I-VTs from blood was determined by monitoring blood samples, collected at 2, 5, 15, 60, and 120 min after labeled-toxin administration, for $^{125}$I activity (Fig. 1). The half-life of $^{125}$I-VT1 was ~2 to 5 min, and the counts generally reached a steady-state background level by 1 h among nonimmune and immune animals. The rate of clearance of $^{125}$I-VT2 appeared similar to that of VT1 in animals immune to either VT1 or VT2, although it was somewhat lower in the control animals. In animals immunized with the toxin subunit fractions, only the 2-min and terminal 120-min blood specimens were analyzed for radioactivity, and these showed a consistent drop to background levels in all experiments.

**Uptake of VT1 and VT2 by tissues of nonimmune rabbits.** Figure 2 shows the localization of $^{125}$I-VT1 and $^{125}$I-VT2 in selected organs expressed as counts per gram of tissue normalized for blood flow through the tissue. Both toxins were found to bind to similar degrees to the cecum, mesentery, and, to a lesser extent, the appendix. There were substantial differences of localization of the two toxins in the brain and spinal cord compared to that in the jejunum and colon. The binding of $^{125}$I-VT1 to the brain and spinal cord was significantly higher than that for $^{125}$I-VT2. Not previously observed was the appreciable binding of both toxins, but especially of VT1, to the pancreas.

**Uptake of VT1 and VT2 by tissues of rabbits immunized with VT1 or VT2 toxoid.** The amount of $^{125}$I-VT1 bound per gram of specific organ normalized for the blood flow through the organ is shown in Fig. 3a. As expected, $^{125}$I-VT1 administered to nonimmune control animals localized in the intestinal tissues and the CNS, as reflected by the uptake in the cecum and spinal cord. In rabbits immunized with either VT1 or VT2 toxoids, virtually all the $^{125}$I-VT1 was detected in the liver and spleen while the CNS and GI tract were protected. While confirming previous studies for rabbits immunized with the homologous VT1, these results were unexpected for rabbits immunized with the heterologous toxoid, VT2, since in vitro NAb tests show no cross-neutralization between VT1 and VT2 and the animals exhibited no cross-protective antibody in their sera by this test.

The pattern of $^{125}$I-VT2 uptake (Fig. 3b) by tissues of rabbits immunized against VT1 and VT2 was similar to that observed for $^{125}$I-VT1. However, in this case, it was observed that in the immunized animals, splenic uptake of $^{125}$I-VT2 was significantly lower in animals immunized with the heterologous toxoid than in those immunized with the homologous toxoid, whereas uptake by the liver was high in both cases. While this difference was also noted in immune rabbits challenged with $^{125}$I-VT1, it was more pronounced in the case of the $^{125}$I-VT2-challenged animals.

**Uptake of $^{125}$I-labeled VT1 by tissues in rabbits immunized with the A and B subunits of VT1 and VT2.** In rabbits immunized with either the VT1 A subunit or the VT2 A subunit, the highest $^{125}$I-VT1 uptake was in the liver and spleen, with a general lack of localization in the target GI and CNS organs (Fig. 4a). Thus, immunization with either A subunit alone stimulated an immune response against the homologous toxin and cross-immunity against heterologous toxins. In rabbits immunized with the VT1 B subunit, $^{125}$I-VT1 again localized in the liver and spleen, with the GI and CNS tissues being spared.

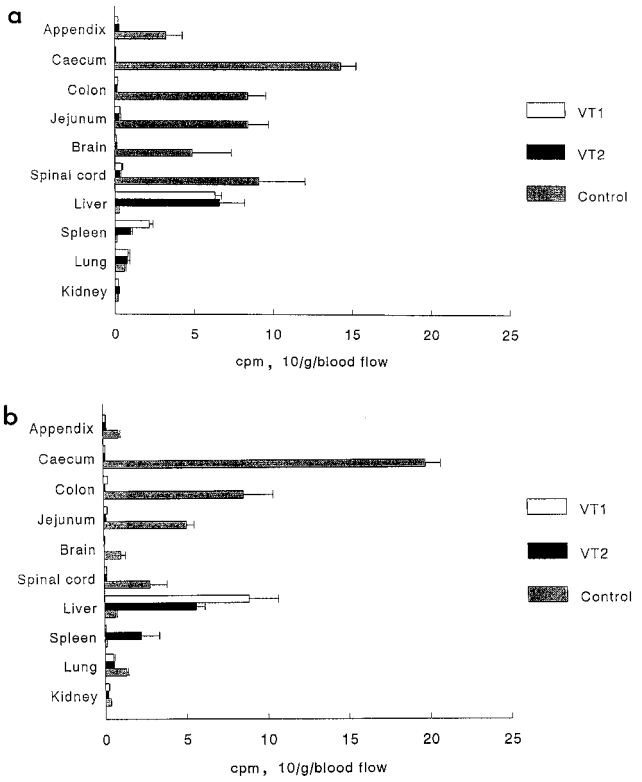


FIG. 3. Tissue binding distribution of $^{125}$I-VT1 (a) and $^{125}$I-VT2 (b) in immunized rabbits. Two hours after intravenous injection of $^{125}$I-labeled VTs to rabbits immunized with VT1 holotoxin, VT2 holotoxin, or nonimmune controls, designated organs were removed and a defined portion was counted for $^{125}$I activity. Bars represent the average counts from three rabbits per determination.

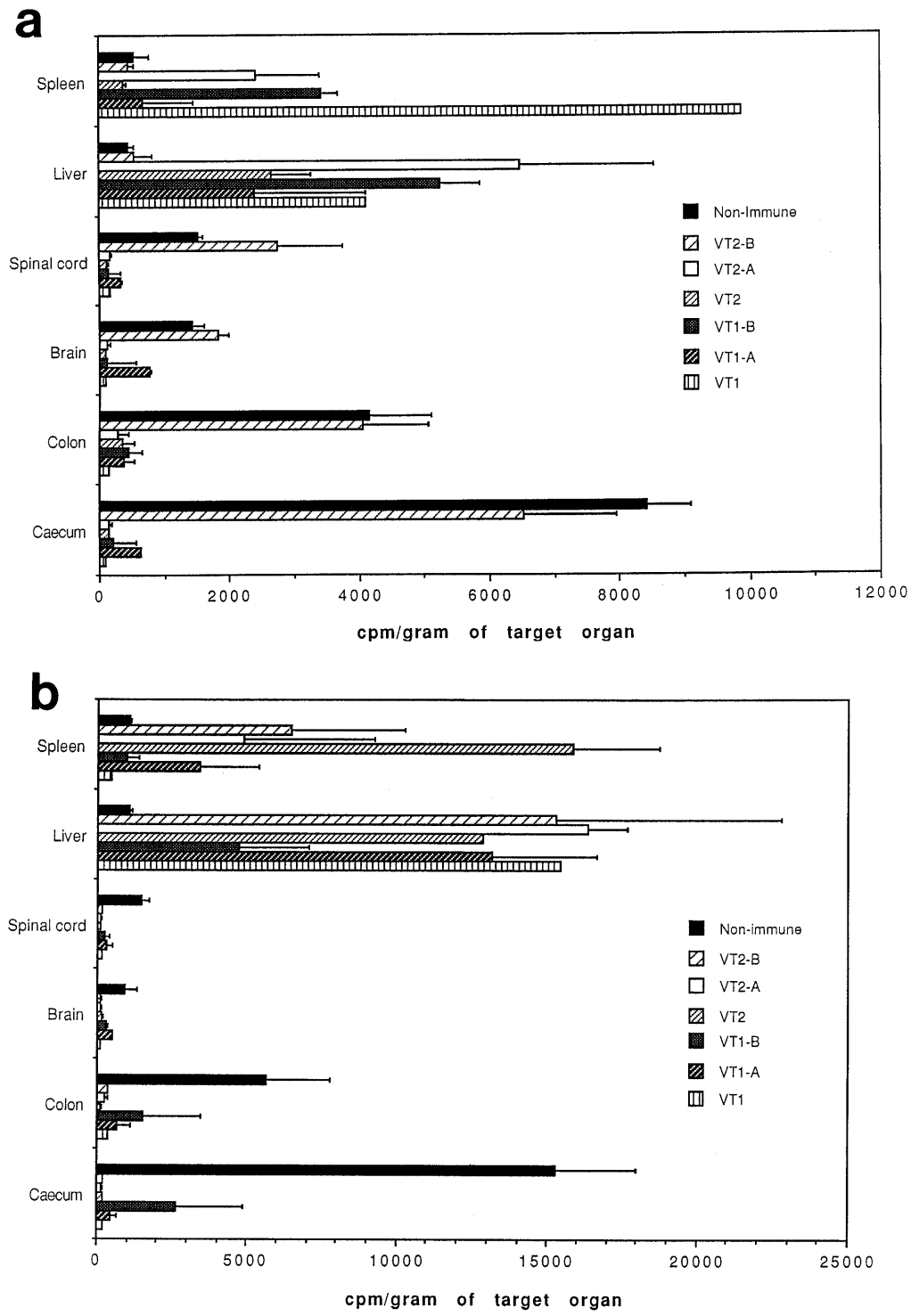


FIG. 4. Tissue binding distribution of $^{125}$I-VT1 (a) and $^{125}$I-VT2 (b) and their subunits in immunized and control rabbit organs. Two hours after intravenous injection of $^{125}$I-labeled VTs to rabbits immunized with VT1 holotoxin, VT2 holotoxin, their A and B subunits, or nonimmune controls, as indicated, designated organs were removed and a defined portion was counted for $^{125}$I activity. Bars represent the average counts from three rabbits per determination.

In contrast, no evidence of cross-protection against $^{125}$I-VT1 uptake was evident in rabbits immunized with the heterologous (VT2) B subunit.

**Uptake of $^{125}$I-labeled VT2 by tissues in rabbits immunized with the A and B subunits of VT1 and VT2.** Similar to the findings in rabbits challenged with $^{125}$I-VT1, the uptake of $^{125}$I-VT2 was inhibited in animals immunized with the homologous (VT2 A) and heterologous (VT1 A) A subunits and with the homologous (VT2) B subunit but not in animals immunized with the heterologous (VT1) B subunit (Fig. 4b).

## DISCUSSION

Shiga toxin, given intravenously, has been shown to be lethal to rabbits, with an $LD_{50}$ of about 0.2 μg/kg (40, 43). Following Shiga toxin challenge, animals develop dose-dependent symptoms of diarrhea and paralysis (9, 11). Similar pathological effects are seen when either VT1 (43) or VT2 (5) is administered to rabbits.

It has been known for over 40 years that immunization of rabbits with Shiga toxoid protects the animals against the lethal effects of injected Shiga toxin (22). In a previous study, we showed that the pathological effects of VT1 in the CNS and the GI tract of rabbits correlated with the uptake of $^{125}$I-labeled VT1 by the same tissues (43). We further showed that the uptake of $^{125}$I-VT1 by these target tissues was inhibited in animals immunized with VT1 toxoid and that labeled toxin uptake in immunized animals was highest in the liver and spleen, an observation consistent with the clearance of immune complexes by the reticuloendothelial system. The uptake of $^{125}$I-labeled VT1 in immunized and nonimmunized animals (43) thus provides a convenient approach for studying immunity against toxin challenge following immunization of animals with different antigens.

It is well established that antisera to VT1 and VT2 do not cross-neutralize the heterologous toxin in the in vitro cell culture-based neutralization assays (26, 47, 49). However, whether this lack of cross-neutralization, observed in vitro, extends to the in vivo setting had not been investigated. Knowledge of this would be very helpful in formulating the design of immunogens for vaccinating humans against VT-mediated disease. The objective of the present study was, therefore, to determine if immunity to one VT provided cross-immunity to another VT in vivo by characterizing the uptake of $^{125}$I-VT1 and $^{125}$I-VT2 in rabbits immunized with either VT1 toxoid, VT2 toxoid, or their A or B subunit.

Our findings, which were unexpected, provide clear evidence that VT1 and VT2 are antigenically cross-reactive in the experimental rabbit model. We show that immunization of animals with VT1 toxoid prevents VT2 uptake by specific target tissues. Similarly, immunization of animals with VT2 toxoid prevents VT1 uptake by the target tissues. The present understanding of the action of VT indicates that toxin neutralization at the cellular level is likely to involve the interaction of antibody with the receptor binding B subunit. However, our observations suggested that the in vivo cross-neutralization might reflect the action of antibodies directed to the larger, more conserved toxin A subunits, leading to the formation of immune complexes that are removed by the liver and/or spleen (Fig. 3). To test this hypothesis, we immunized animals with the VT1 and VT2 A and B subunits and challenged them with $^{125}$I-VT1 and $^{125}$I-VT2. We confirmed that animals immunized by either the VT1 A subunit or the VT2 A subunit were protected from target tissue uptake of both the homologous and heterologous $^{125}$I-labeled holotoxins. In contrast, in animals immunized with the toxin B subunits, protection was extended only against challenge by the homologous toxin. Our findings therefore indicate that the in vivo cross-neutralization is a predominant function of antibodies directed to the VT A subunits. This suggests that the VT1 A or VT2 A subunit may be a suitable immunogen for immunizing humans against systemic VT-mediated disease. Further studies, by $LD_{50}$, to determine cross-protection are still required.

It should be noted that the quantity of $^{125}$I-labeled challenge toxin was considerably in excess of the $LD_{50}$. However, the immunizations produced a very strong immune response in the case of the holotoxoid and a sufficient response in the case of the toxin subunits to have led to either full protection against binding to target organs or at least protection against the binding of the homologous toxin. Further studies to quantitate the antibody levels needed to confer protection from toxin binding to target organs are indicated.

The serological studies (Table 1) confirmed the lack of in vitro cross-neutralization between VT1 and VT2. Thus, the sera of animals immunized with VT1 toxoid failed to neutralize VT2 in vitro, and the sera of animals immunized with VT2 failed to neutralize VT1 in vitro. On the other hand, cross-reactivity between the toxins was clearly evident when antibodies were assayed by the ELISA, and, to some degree, by Western blotting. This is consistent with the concept that antibodies involved in in vitro neutralization are expected to be directed predominantly to the B subunit, whereas the ELISA assay presumably measured antibodies to both the B and A subunits. Thus our findings of in vivo cross-neutralization correlates with the occurrence of cross-reactive antibodies detected by ELISA.

Studies of immunity and cross-immunity to VT1 and VT2 have also been conducted with mice by Wadolkowski et al. (54). Strain 933cu-rev (which produces both VT1 and VT2), derived from the prototype *E. coli* O157:H7 strain 933, kills streptomycin-treated mice after the animals are fed this strain. Wadolkowski et al. (54) investigated whether VT1, VT2, or both contributed to the death of mice challenged orally with strain 933cu-rev. Groups of mice were pretreated by intraperitoneal injections of monoclonal antibodies to VT1, VT2, or both toxins and treated orally with 933cu-rev. Mice pretreated with monoclonal antibodies to VT2 A and VT2 B survived, whereas those challenged with VT1 B did not. It was concluded from these findings, and from other experimental data, that death in the orally infected mice was due solely to VT2. Of interest, with respect to immunity and cross-immunity, were the findings that monoclonal antibodies against both the VT2 A and VT2 B subunits were protective (against VT2-mediated disease), whereas monoclonal antibodies to the VT1 B subunit did not provide cross-immunity. These findings corroborate our own observations in rabbits regarding immunity to the homologous toxin following immunization with the homologous A or B subunit and the lack of cross-immunity to the heterologous toxin after immunization with the toxin B subunit. However, it should be noted that our studies of actively immunized rabbits are not directly comparable to the studies reported in mice (54) which employed passively administered monoclonal antibodies.

In addition to providing insights into aspects of immunity and cross-immunity between the holotoxins and their respective subunits, our studies have also revealed novel findings related to the uptake of labeled toxins in rabbits. We found that the localization of $^{125}$I-labeled VT2 to the target tissues differed from that we have previously described for $^{125}$I-VT1. Labeled VT2 was found to localize to a substantially lesser extent in the CNS tissues than VT1, although the levels of binding to the GI tissues were similar. One additional observation not heretofore noted was that VT1 and VT2 both localize in the pancreas. This observation is consistent with reports of insulin-dependent diabetes and pancreatic islet cell necrosis being associated with HUS (3, 10).

Depending on the immunogen used for the rabbit, there was a consistent difference between the uptake of $^{125}$I-labeled toxin by the liver and that by the spleen. In the case of animals immunized by the homologous holotoxin, the spleen showed greater specific radioactivity than the liver, but in the case of animals immune to the heterologous toxin or to any of the toxin subunits, the specific radioactivity localized in the spleen was less than that in the liver. These findings may reflect the

variability of the immune complexes formed under the conditions described above. It is expected that antibodies of animals immunized with the homologous holotoxin would be reactive towards a greater number of epitopes on the injected labeled toxin and form larger immune complexes than those from animals immunized with the heterologous toxin or toxin subunits. It has been shown in animal models that large immune complexes have a greater tendency to be cleared by the spleen whereas smaller ones are cleared by the liver (19). Most studies on the clearance of immune complexes have been done by injecting preformed immune complexes into animals (4, 6, 14). Our observations address the formation and clearance of the immune complexes from animals with different levels of immunity and provide added insight into the functions of the liver and spleen in this process.

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