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Characterization of the B Cell Epitopes Associated with a Truncated Form of *Pseudomonas* Exotoxin (PE38) Used to Make Immunotoxins for the Treatment of Cancer Patients


Recombinant immunotoxins composed of an Ab Fv fragment joined to a truncated portion of *Pseudomonas* exotoxin A (termed PE38) have been evaluated in clinical trials for the treatment of various human cancers. Immunotoxin therapy is very effective in hairy cell leukemia and has also activity in other hemological malignancies; however, a neutralizing Ab response to PE38 in patients with solid tumors prevents repeated treatments to maximize the benefit. In this study, we analyze the murine Ab response as a model to study the B cell epitopes associated with PE38. Sixty distinct mAbs to PE38 were characterized. Mutual competitive binding of the mAbs indicated the presence of 7 major epitope groups and 13 subgroups. The competition pattern indicated that the epitopes are discrete and could not be reproduced using a computer simulation program that created epitopes out of random surface residues on PE38. Using sera from immunotoxin-treated patients, the formation of human Abs to each of the topographical epitopes was demonstrated. One epitope subgroup, Ela, was identified as the principal neutralizing epitope. The location of each epitope on PE38 was determined by preparing 41 mutants of PE38 in which bulky surface residues were mutated to either alanine or glycine. All 7 major epitope groups and 9 of 13 epitope subgroups were identified by 14 different mutants and these retained high cytotoxic activity. Our results indicate that a relatively small number of discrete immunogenic sites are associated with PE38, most of which can be eliminated by point mutations. *The Journal of Immunology*, 2006, 177: 8822–8834.

Over the past 20 years, a variety of protein therapeutics has been administered to humans including mAbs, protein growth factors, and enzymes (1, 2). Compared with small m.w. compounds, proteins are usually large enough to induce an immune response in patients (3). Not surprisingly, proteins obtained from nonmammalian sources induce Abs more frequently than proteins of human origin (3–5).

Our laboratory is developing recombinant immunotoxins for the treatment of cancer (6–13). These agents are composed of a 38-kDa portion of *Pseudomonas* exotoxin A (PE38), which is of bacterial origin, and the Fv portion of a mAb genetically fused to it. The binding activity of the Fv moiety targets the immunotoxin to Ag-positive cells which are killed by the cytotoxic activity of the toxin moiety (11, 13). When immunotoxins are administered to patients, neutralizing Abs often develop within 3 wk. These Abs, which almost always react with PE38 and very infrequently with the Fv, limit the number of treatment cycles that can be given (8, 14, 15). Fortunately, patients with certain leukemias and lymphomas, including hairy cell leukemia, infrequently make Abs to the immunotoxin and can receive the benefit of many cycles (9, 12).

This nonresponsiveness probably results from damage to the immune system, either due to previous chemotherapy or because the leukemia causes immune suppression. Consequently, we have had our greatest successes when treating patients with leukemia. Over half of the patients with life-threatening drug-resistant hairy cell leukemia achieved a complete remission after receiving three or more cycles of the immunotoxin BL22 targeted to CD22 (9, 12). This result suggests that immunotoxin therapy is more likely to be successful if multiple cycles of treatment can be given.

Several approaches have been proposed to decrease the immunogenicity of foreign proteins such as PE38. One involves shielding the protein from the immune system by conjugating high m.w. polyethylene glycol (PEG) to the immunotoxin (16, 17). This approach requires attaching the PEG at locations on the toxin that do not interfere with its cytotoxic activity (18). Another approach is to identify and remove epitopes of T cells (19, 20) or of B cells (21–23) by site-directed mutagenesis. T cell epitopes recognized by Th cells are complexes of the peptide fragments derived from the Ag and the MHC molecules (24). Because of the numerous leukocyte Ag alleles present in humans (25) and because T cells stimulated by one epitope can stimulate responses to different B cell epitopes on the same Ag (26, 27), it is a formidable task to remove all possible T cell epitopes from...
a foreign protein. Pretreating patients with immunosuppressive agents has also been proposed as a way to reduce Ab responses but, to date, very little progress has been reported (15). However, patients receiving anti-B cell chemotherapy such as cladribine may exhibit long delays in responding to foreign proteins (28).

This study focuses on a strategy to identify the B cell epitopes on PE38, with the ultimate goal of removing or modifying the most prominent ones. The properties of B cell epitopes have not been as well-characterized as those of T cell epitopes. Although some investigators have proposed that any region on the surface of a protein can be an epitope (29–33), most studies have reported the existence of clusters of Ab epitopes on the Ag surface (34, 35). Examination of the interfaces of the crystal structures of Ag-Ab complexes have not yet identified a universal mode of interaction or identified specific amino acids that are uniformly important in these interactions (33, 36–40) although some prediction methods have successfully identified highly immunogenic regions (41–43). For proteins from related species, there are regions that are not particularly immunogenic because paralog proteins have “educated” the immune system (44). But for a foreign protein such as PE38, the presence of B cell epitope hot spots has not been demonstrated. To address the issue of PE38 immunogenicity, we decided it was first necessary to locate all detectable epitopes and then evaluate their distribution for evidence of hot spots. We also considered that some Ab-PE38 interactions would neutralize the toxin and some would not.

A common approach for identifying immunogenic hot spots is to use small fragments or peptides derived from a protein. We previously used this approach and identified some epitopes on PE38 (14, 45). However, this approach cannot locate discontinuous conformational B cell epitopes (46–48). Conformational epitopes can be located by evaluating loss of Ab reactivity with a series of point mutants of the Ag (49, 50). Furthermore, because each mutant should eliminate a single epitope, a series of mAbs against individual epitopes is required to distinguish each epitope from the others.

Because there is evidence that human and mouse Abs recognize the same epitopes on foreign proteins (51–54), we made a large panel of mouse anti-PE38 mAbs, and used these to determine the number and location of the epitopes. We also demonstrated that Abs to these epitopes are also present in human sera from immunotoxin-treated patients.

### Materials and Methods

#### The immunotoxins and their recombinant target proteins

Several PE38-based immunotoxins were used in this study. They are BL22 (9), LMB-2 (55), M1 (56), LMB-9 (57), SS1P (10), and T6 (58) and their inactive mutants (59, 60). We also made both domains of PE38 (domain II and III) separately (61). Table I summarizes the composition of these immunotoxins and their targets on cancer cells. All the immunotoxins and domains were made by a standard protocol established in our laboratory (63). In brief, the components of immunotoxins were expressed in *Escherichia coli* BL21 (ADE3) under a T7 promoter and harvested as inclusion bodies. The protein was solubilized in 6 M guanidine hydrochloride under reducing conditions, and then refolded by dilution into a refolding solution containing redox shuffling reagents. Active monomeric protein was purified by ion exchange and size exclusion chromatography to near homogeneity. Protein concentrations were determined by a Bradford assay (Coomassie Plus; Pierce) using BSA as the standard.

The extracellular domains of the target membrane proteins of the immunotoxins (CD22, CD25, mesothelin, and CD30) were produced as human Ig Fc fragment (HFc)-fusion proteins or rabbit Fc (RFc)-fusion proteins in 293T cells. The cells were transiently transfected with the corresponding pcDNA3-based plasmids and the Fc-fusion proteins were harvested in the culture supernatants (64, 65). The Fc-fusion proteins were purified by protein A-Sepharose (Amersham Biosciences) as described previously (64).

#### ELISA

Passive adsorption of proteins onto plastic surfaces often alters the protein conformation by destroying conformational epitopes or revealing cryptic ones (66–68). To avoid these potential problems we have devised an indirect ELISA termed an immune complex capture ELISA (ICC-ELISA). The ICC-ELISA detects Ag-Ab reactions that occur in solution. In this ELISA, microtiter plates (MaxiSorp; Nalge Nunc) were coated with 100 ng/50 μl/well CD22-HFc, CD25-RFc, or CD30-HFc in PBS overnight at 4°C. In separate tubes, the anti-PE38 mAb samples diluted in blocking buffer (25% DMEM, 5% FBS, 25 mM HEPES, 0.5% BSA, 0.1% sodium azide in PBS) were mixed with 2 μg/ml of an immunotoxin containing PE38 fused with an Fv reactive for CD22, CD25, or CD30. After washing the plates with PBS containing 0.05% Tween 20, the immunotoxin-Ab mixtures were transferred to each well (50 μl/well). The amount of immune complex captured by the Fc fusion proteins was detected by HRP-conjugated goat anti-mouse IgG (H+L; no. 115-035-146; Jackson ImmunoResearch Laboratories) or HRP-rat anti-mouse κ mAb (no. 04-0620; Zymed Laboratories) followed by tetramethylbenzidine (TMB) substrate kit (Pierce).

#### Production of mAbs

Immunization of mice with immunotoxins was conducted under various conditions to increase the chances of obtaining mAbs to all possible
epitopes. The immunogen and immunization conditions are summarized in a part of Table II and Results. All the animal protocols were approved by the Animal Care and Use Committee at the National Institutes of Health. Hybridomas were produced by a cell fusion protocol using SP2/0-neo cells (69). The ICC-ELISA was used to select 60 mAbs (57 mAbs from this study and three previous mAbs in Ref. 70) that bind to PE38 in solution. Ig isotypes were determined by mouse mAb isotyping reagents (ISO2; Sigma-Aldrich). IgG concentrations in the culture supernatants were determined by ELISA using isotype-matched IgG control mAbs (no. 90-6551, mouse Ig panel; Zymed Laboratories) as previously described (71). In some experiments, mAbs were purified using protein G Sepharose (Amersham Biosciences).

**Topographical epitope mapping**

The classification of the mAbs into epitope groups was conducted by the mutual competition of all possible pairs of the anti-PE38 mAbs (60 × 60 = 3600) in a label-free competitive ELISA (72). For example: an indicator mAb 1 was captured by goat anti-mouse IgG Fc that had been coated on the microtiter plates. In a separate tube, an excess amount of competitor mAb 2 was diluted in the blocking buffer and mixed with an appropriate concentration of T6 immunotoxin (anti-CD30) overnight at 4°C. The plates were washed twice and the mixtures in the tubes were transferred to each well. The immune complexes captured on plates were probed with CD30-HRP followed by HRP-conjugated goat anti-human IgG and TMB substrate.

The binding of mAb 1 to the mAb 2-T6 immune complex was determined as the percentage of the binding without mAb 2. As a result, each mAb 1 had a set of normalized competition indexes for each mAb 2-Ag complex. The pairwise matrix table of the competition indexes was analyzed by cluster analysis to group the mAbs based on the topographical relationship of their epitopes (72).

In some experiments, patient sera containing human anti-PE38 Abs were examined in a similar competition assay. The human serum samples were obtained before and after patients with solid tumors received treatment with the immunotoxins LMB-9 or SS1P during phase I clinical trials (National Cancer Institute (NCI) protocol IDs: NCI-431 and NCI-01-C-0011) (10, 57). These studies were approved by the Institutional Review Board of the National Cancer Institute and all the patients gave written informed consent. In the analysis, a 200-fold dilution of the human serum was applied to the mAb competition matrix (73).

**Biosensor experiments**

The binding kinetics of each mAb with PE38 was determined using an Amersham Biosensor (Amersham Biosciences) as previously described (73). The biosensor experiments were conducted by a cell fusion protocol using SP2/0-Neo cells. Hybridomas were produced by a cell fusion protocol using SP2/0-Neo cells (69). The ICC-ELISA was used to select 60 mAbs (57 mAbs from this study and three previous mAbs in Ref. 70) that bind to PE38 in solution. Ig isotypes were determined by mouse mAb isotyping reagents (ISO2; Sigma-Aldrich). IgG concentrations in the culture supernatants were determined by ELISA using isotype-matched IgG control mAbs (no. 90-6551, mouse Ig panel; Zymed Laboratories) as previously described (71). In some experiments, mAbs were purified using protein G Sepharose (Amersham Biosciences).

**Simulation analysis of the epitope location**

To determine whether the epitope clusters can be reproduced by random selection of epitopes from the surface of PE38, a computer-based simulation was conducted. A PE38 model was made by extracting residues present in PE38 (251–364 and 381–605) from the crystal structure of PE (62). Artificial epitopes were randomly created on the surface of the model and their overlap was calculated as the AB competition value in a matrix. The number and quality of the artificial clusters were compared with that from the experimental data. To create artificial epitopes on PE38, all the exposed residues were selected from the PE38 model by Lee and Richards’ method (75); artificial epitopes around the selected residues were created by collecting surface exposed atoms around the central residue incrementally until the outermost ring contained 60 or more nonhydrogen atoms. The number 60 is the average number of atoms in the periphery of the interface between four different Abs and the hen egg white lysozyme in their crystal structures. Two artificial epitopes were considered to overlap if they shared one or more atoms. Competition binding values for pairs of virtual Abs to these epitopes were defined as one if there was any overlap, zero otherwise. Ten competition-binding data sets for the evaluation were generated by selecting the exposed residues randomly.

All sets of competition values, both artificial and experimental, were clustered using the PyCluster software library developed by M. Eisen (http://bonsai.ims.u-tokyo.ac.jp/~mdehoon/software/cluster/software. htm#pycluster). For comparison, each of the experimental competition indexes (see above) was converted to either one or zero using 50% competition as the cutoff. The resulting matrix was then clustered. To evaluate the quality of each clustering, we calculated the following values (true positive rate and false positive rate) for each cluster number in the clustering and plotted as a receiver operating characteristic (ROC) curve (76).

**Neutralization assay for the mAbs**

For the neutralization assay, 6 μg/ml (40 nM) of each mAb was incubated with 5 ng/ml (80 pM) M1 immunotoxin for 30 min at 37°C and then applied to A431 cells (A431 stably transfected with CD25) (55). After 24 h, incorporation of tritium-labeled leucine into cellular protein was measured as described previously (63). The neutralizing activity of each mAb was expressed as the percent recovery of leucine incorporation (counts) relative to the specific reduction by the immunotoxin without a mAb.

**Statistics**

The association of the characteristics of the mAbs (neutralization activity, association rate constant, dissociation rate constant, and affinity constant) with the topographical epitopes was evaluated by a nonparametric statistics (Mann-Whitney U test). mAbs assigned to each epitope were tested against the other mAbs assigned to different epitopes with the null hypothesis that the two mAbs have identical distributions with the testing characteristics (p < 0.01). Ep2a was not evaluated because only one mAb was assigned to this epitope.

**Point mutants of PE38 used to locate each epitope**

To locate specific epitopes, we produced a series of single point mutants of PE38. We chose highly exposed surface residues (>70 Å2) as the first candidates. Of 347 residues, there are 98 (28%) that exhibit an average overlapping of 113.1 ± 33.3 Å2 covering 67% of the total surface area. Because an epitope usually spans 6–8 aa and occupies 400–900 Å2 (36, 37, 77), a relatively small number of mutants should be sufficient to “cover” the entire surface of PE38 if they are evenly distributed. We took advantage of our previous knowledge that mutations of either R or E, followed by D, Q, or N tended to retain toxin function (18, 78).

![FIGURE 1. ELISA to detect Abs reactive for soluble PE38. Principle of the ICC-ELISA. Immunotoxins, comprising the 38-kDa portion (domain II and III) of Pseudomonas exotoxin A fused to an Fv specific for target Ags on cancer cells, are incubated with candidate mAbs. Because the reaction takes place in solution, all epitopes on native PE38 are theoretically available. Complexes are captured to the plate with immobilized target ligands such as CD22-Fc or similar molecules. The primary Ab is then detected with an HRP-labeled secondary Ab.](http://www.jimmunol.org/doi/abs/10.4049/jimmunol.88.9.8824)
Table II.  A variety of mAbs to the surface of PE38 produced in 10 different fusions

<table>
<thead>
<tr>
<th>Fusion</th>
<th>Immunogen</th>
<th>Boost</th>
<th>Number of mAbs Obtained</th>
<th>Subgroup</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>Total</th>
<th>Average $K_A$ ($10^8 M^{-1}$)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>BALB/c</td>
<td>M1 x 4</td>
<td>E553D</td>
<td>a b</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>BALB/c</td>
<td>E553D × 7</td>
<td>E553D</td>
<td>b a</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<td>1</td>
<td>4</td>
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<tr>
<td>3</td>
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<td>M1 x 6</td>
<td>E553D</td>
<td>a b</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<td>1</td>
<td>3</td>
<td>8.1</td>
<td></td>
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<tr>
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<td>R276G × 6</td>
<td>R276G</td>
<td>c a</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>2</td>
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<td>16.8</td>
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<tr>
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<td>R276G × 4</td>
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<td>a b</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2.6</td>
<td></td>
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<tr>
<td>6</td>
<td>BALB/c</td>
<td>M1 x 5</td>
<td>M1</td>
<td>a b</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>3</td>
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<td>4</td>
<td>1</td>
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<td>M1</td>
<td>a b</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<td>4</td>
<td>1</td>
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<tr>
<td>8</td>
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<td>E553D × 4</td>
<td>E553D</td>
<td>c a</td>
<td>1</td>
<td>6</td>
<td>1</td>
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<td>3</td>
<td>1</td>
<td>2</td>
<td>18</td>
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<tr>
<td>9</td>
<td>A/J</td>
<td>M1 x 3 + E553D × 2</td>
<td>E553D</td>
<td>a b</td>
<td>2</td>
<td>1</td>
<td>1</td>
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<td>1</td>
<td>2</td>
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<td></td>
</tr>
<tr>
<td>10</td>
<td>A/J</td>
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<td>Domain III</td>
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<td>1</td>
<td>1</td>
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<td>1</td>
<td>2</td>
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<td></td>
</tr>
<tr>
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<td></td>
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<td>5</td>
<td>4</td>
<td>3</td>
<td>8</td>
<td>3</td>
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* All mAbs were IgG1(κ · γ1) except for no. 16(κ · γ2a), no. 36(κ · γ2b), no. 37(κ · γ2b), and no. 49(κ · γ2b).
* Identified by the mutual competition of mAbs to PE38 (Fig. 2).
* Immunogen used in the final boost 3 days before fusion. 10–50 μg of the mAbs were injected i.p. in PBS.
* The affinity of mAbs no. 14 (Fusion 1, Ep4b) and no. 49 (Fusion 9, Ep4a) was not measured. mAb no. 57 (Fusion4, Ep1a) was excluded because of the exceptionally high-affinity constant (5.3 x 10^11 M^-1).
* These mAbs were previously obtained (70).
FIGURE 2. Topographical epitope mapping of mAbs by mutual competition. The binding of indicator mAbs (listed in rows) to anti-CD30 T6 immunotoxin containing PE38 in the presence of 600-fold excess amounts of competitors (listed in columns). Strengths of competition are shown as percentages in each box, which are shaded according to the key at the top of the figure. The competitive patterns were analyzed by a cluster analysis (shown in Fig. 3A). The number of epitope groups and subgroups was determined based on the SI as shown in Fig. 3B. Seven epitopes and 13 epitope subgroups are separated by blue and green lines, respectively.
In addition, these residues often supply significant energy to Ag-Ab binding through polar interactions or hydrogen bonds (36, 39, 40). We included four mutants in the panel where residues had <70 Å² in area. The resulting mutant panel consists of only 41 mutants (12% of total residues) but covers 28.3% (4,693/16,560 Å²) of the PE38 surface area. The distances of the α carbons of each residue to its nearest neighbor (7.4 ± 3.0 Å) are close to the distances between all the exposed (>70 Å²) residues (5.0 ± 1.7 Å). To evaluate the side-chain contributions of Ab binding to PE38, the mutated residues were replaced with A or G except for E553D. The mutations in PE38 portion were introduced in the anti-CD22 immunotoxin, BL22, except for R276G that was in the anti-CD25 immunotoxin, LMB2. The point-mutant panel consists of P268A, R276G, E282A, E285A, P290A, A300G, R302A, Q310A, R313A, P319G, D324A, E327A, E331A, Q332A, E348A, R352A, Q353A, D403A, R412A, N416A, E420A, R427A, E430A, E431A, R432G, R458A, R467A, Q485A, R490A, R505A, R513A, L516A, R529A, R538A, E548A, R551A, E553D, R576A, K590A, L597A, and D599A. The mutant proteins were expressed and purified to homogeneity by established protocols (63). All mutant proteins were obtained with reasonable yields, usually 2–10% of starting material (data not shown).

**Competition assay to measure the reactivity of mAbs to PE38 mutants**

Each PE38 point mutant was tested for its reactivity with each mAb in solution to keep both Ag and Ab native during the reaction. We used 40 representative mAbs from the 60 mAbs in the competition assay to cover all 7 major epitope groups and 12 of the 13 subgroups (Ep2a was excluded because it contained only one mAb). To standardize the different affinity of individual mAbs assigned to each epitope, the result was evaluated as residual reactivity, which was defined as the ratio of the concentrations of each mutant and of the wild type that were required for the binding to the same amount of each mAb (79, 80). These values are close to the affinity ratios as will be described in Results. In the assay, mAbs that failed to bind mutant versions of PE38 were selectively captured by the antimesothelin immunotoxin, SS1P, which had been indirectly coated on the plates via mesothelin-Fc fusion proteins.

In brief, microtiter plates were coated with 100 ng/50 μl/well me- sothelin-RFc, followed by a 2-h incubation with 200 ng/100 μl/well SS1P. In separate tubes, a series of 4-fold dilutions of each mutant or wild-type immunotoxin (0.04–10,000 ng/ml) were mixed with an appropriate concentration of each mAb in blocking buffer at 4°C overnight. The concentration of each mAb in the mixtures had been prede- termined as a concentration that gave a half maximum signal in this ELISA. After washing the plates, the immunotoxin-mAb mixtures in the tubes were transferred to each well (50 μl/well). The uncomplexed mAb in the mixtures was captured by the SS1P coated on the plate during a 1-h incubation. The mAbs were finally detected by HRP-conjugated goat anti-mouse IgG (H+L), followed by TMB substrate. The concentrations of each mutant that reduced the signal by 50% (IC₅₀) were calculated by fitting to a four-parameter logistic curve. The resid- ual reactivity of a mutant to a mAb = IC₅₀ of wild-type PE38 to the mAb binding/IC₅₀ of the mutant to the mAb binding.

**FIGURE 3.** Characterization of the topographical epitope groups identified in the mapping experiment. A, A dendrogram generated by cluster analysis of the competition pattern shown in Fig. 2 (72). The mAbs with similar competition patterns are connected near the bottom of the tree and mAbs showing dissimilar competition patterns go up in separate branches until they approach the top of the tree. The number of epitope groups was determined based on the SI as described below. Values at each node represent percentage bootstrap support after 1000 replicates. The dotted line shows the cutoff value that gives the 13 epitope subgroups as determined by the SI (B). B. The number of topographical epitopes was determined by the SI as described in Ref. 72. SIs were defined according to the formula below and plotted against the number of epitopes:

$$SI(g) = \frac{\sum BS_i \times (H_i - CO_i)}{100 \times CO \times g}$$

where $SI(g)$ is the SI for g groups of mAbs. Each epitope group (1, 2, . . . , g ) joins to another group at each height in the dendrogram (H 1, 2 ,...,g ). CO g is the highest cutoff value of the height that makes g + 1 epitope groups of mAbs. BS 1,2 ,...,g are the bootstrap percentages of the nodes where the 1,2, . . . , g epitope groups are made. The group number for the first peak (7 epitopes) was taken as the epitope group number (blue) and the value for the second peak was taken as the epitope groups including subgroups (green, 13 subepitopes). C, Biacore sensogram for the serial injections of different mAbs. SS1P immunotoxin was covalently coupled to the sensor chip CM5. Each mAb was serially injected over the chip surface at 10 μl/min. The mAb numbers and corresponding epitopes are shown.
Results

ELISA development to detect anti-PE38 mAbs

Passive adsorption onto plastic surfaces often alters protein conformation by destroying epitopes or revealing cryptic ones (66–68). To avoid these problems, we designed an ICC-ELISA in which Ag-Ab interactions take place in solution. Once formed, the mAb-PE38 complex is captured by a target ligand tethered to the plastic surface. The mAb is then detected with an HRP-labeled second Ab (Fig. 1). A total of 60 mAbs to PE38 were characterized using this ELISA.

Production of a large panel of anti-PE38 mAbs

Because PE38 differs greatly from mammalian proteins in sequence and in structure, we hypothesized that Abs produced by humans or mice would recognize many of the same B cell epitopes (51–54) also, see below. To obtain mAbs that react with the native surface of the PE38, we immunized mice with various immunotoxin preparations and saved only those hybridomas that reacted with native PE38. Reactivity for native PE38 was detected using the ICC-ELISA—see Materials and Methods and Fig. 1 for details. To obtain a broad set of Abs, we used a variety of conditions and schedules for immunization, as summarized in Table II. In addition to the active immunotoxins, we also used inactive immunotoxin mutants for some of the immunizations. We used either E553 or R276, which are important for different events in the cytotoxicity pathway (59, 60).

As shown in Table II, 10 fusions produced 57 mAbs that reacted with the surface of PE38. Along with three previous mAbs (70), we obtained a panel of 60 mAbs. The epitope locations of the three mAbs that had been previously produced ("P" row in Table II) were reidentified in the new mAb panel. Abs from mice with two different H2 haplotypes (H2d for BALB/c and H2a for A/J) reacted with a similar variety of epitopes, suggesting that the same B cell epitopes were recognized with distinct T cell support. Different fusions yielded mAbs with different average affinity constants from 0.4 to 65.2 $10^8$ M$^{-1}$ as determined using Biacore (each value will be shown in Fig. 7). These values are within general levels of affinities of other mAbs to protein Ags. The difference in affinities and the difference in epitopes suggest that individual mAbs are distinct.

Topographical epitope mapping and evaluation of the relationships between the epitope locations

To classify the mAbs based on the topographical relationship of their epitopes, we measured the mutual competition of all possible pairs of the epitopes. The competition between any two mAbs was almost always mutual; very few (86 of 3600, 2.4%) showed $50\%$ competition in only one direction. This indicates that the competition pattern is primarily determined by the overlapping of epitopes and not by a...
conformational change in PE38 induced by the binding of mAbs. The competition pattern was analyzed by cluster analysis (72), which gave a hierarchical tree representing the clustering of epitopes (Fig. 3A). Objective criteria for clustering can be obtained by the heights of nodes and from the bootstrap values (reproducibility of the connection). We used the stability index (SI) with these two parameters to determine the number of epitope groups (Fig. 3B) (72). We identified 7 major topographical epitope groups that can be further divided into 13 subgroups (1a, 1b, etc., as shown in Fig. 3A).

We characterized the quality of the Ab grouping of the Abs in several ways. First, to examine whether the epitopes recognized by different mAbs exhibiting no mutual competition were sufficiently distinct to allow additive binding, five mAbs reacting with four different epitopes (1b, 5, 6a, and 7) were evaluated in multiple binding assays using Biacore (Fig. 3C). In the sensorgram, the binding of one mAb to PE38 blocked binding of a second mAb to the same epitope, but allowed the additional binding of three different mAbs to distinct epitopes. The signals from four mAbs to different epitopes were additive and the total signal obtained from a mixture of the four mAbs (data not shown). We conclude that the competition assay represents the isolated location of each epitope on the surface of PE38.

Next, we evaluated the separation between epitope groups by the frequency of competition between the groups (Fig. 4A). For example, subgroup 4a that consists of eight mAbs can be evaluated for grouping quality by testing $8 \times 8 = 64$ combinations of competition; all of these showed $>50\%$ competition. This indicates an excellent overlap of the individual epitopes in this group. The overlap of Ep4a (eight members) with Ep4b (three members) can be evaluated by testing $8 \times 3 \times 2 = 48$ combinations of competition, and 19 (40\%) are positive for the competition. This indicates a modest overlap between these subgroups. Epitope 2a was excluded from this analysis because this group has only one mAb. Overlaps of (>$20\%$) are observed between only 7 pairs of epitope subgroups of 132 (5.3\%) (1a/1b, 1a/3a, 3a/3b, 4a/4b, 5/6b, 6a/6b, and 6b/7 pairs). Therefore, the epitope subgroups are clearly discrete with relatively little overlap, suggesting a limited number of B cell epitopes on the PE38 molecule. To examine whether the epitopes of the mAb panel are distributed randomly or whether they are located in clusters, we generated artificial epitopes randomly on the surface of PE38 and performed a virtual competition assay. The selection of epitopes was conducted with exposed residues on PE38 and the data were compared with the experimental epitope mapping data. The inset in Fig. 4B shows a representative competition pattern with 60 randomly created epitopes around exposed residues on the surface of PE38. Superficially, the clustering pattern did not look as discrete as the experimental data (Fig. 2). However, for an objective evaluation of the quality of the clustering (epitope independence), ROC plots were introduced in which the true positive rates (rates of competitions inside clusters) and false positive rates (rates of competitions outside clusters) in different epitope group numbers (cluster number) are shown (Fig. 4B). Each clustering gives a point on the ROC curve. Discrete locations of epitopes should produce higher true positive rates and lower false positive rates in the competitive patterns under a series of numbers of clusters. The results should produce a ROC curve that lies close to the left-top corner of the graph. As shown in Fig. 4B, the ROC curve from the experimental data is far closer to the left-top corner than the average of the ROC curves randomly generated in 10 simulations. This result indicates that the clustering pattern generated from the experimental competition data has a nonrandom distribution on the surface of PE38. Thus, the epitope locations are clustered.

**Characterization of epitopes recognized by human anti-PE38 Abs**

To determine whether immunotoxin treatment induces Ab responses to the same epitopes as those identified by the mAbs, anti-PE38 Abs from the sera of eight patients were analyzed by the competition assay used in Fig. 2. Sera from patients with pancreatic, colon cancer, or mesothelioma, who had been treated with immunotoxins LMB-9 or SS1P, were evaluated (10, 57). In these clinical trials, Ab production is routinely monitored using biosays that measure immunotoxin neutralization activity. Most patients with solid tumors produced neutralizing Abs after one cycle of immunotoxin treatment. Fig. 5 shows a competition analysis of paired serum samples. Before treatment, the sera contained almost no specific Ab to any of the PE38 epitopes, as expected from the very low neutralizing activity of pretreatment sera. In contrast, the sera obtained after immunotoxin treatment contained anti-PE38 Abs that were specific for the PE38 epitopes.

**FIGURE 6.** Neutralizing activity of anti-PE38 mAbs. Abolishment of immunotoxin-dependent cell killing by incubation with each mAb. The percentage of neutralization is the percentage of recovery of cellular protein synthesis compared with immunotoxin-treated cells in the absence of mAb. Bars represent SDs of four replicate cultures. The association of neutralizing activity to Ep1a is significant ($p < 0.001$) by nonparametric statistics (Mann-Whitney $U$ test).
Abs to every topographical epitope as shown by their ability to inhibit the binding of the corresponding mAbs to each epitope. Some differences in competition values within the positive range were recorded depending on the patients and the epitopes. These results show that immunotoxin treatment induces human Abs against the epitopes identified by the mouse mAb panel.

**Characteristics of anti-PE38 mAbs**

The association of the topographical epitopes with toxin neutralization activity and with the binding kinetics of individual mAbs was also examined. Fig. 6 shows the neutralization of an immunotoxin directed to CD25 (M1dsFv-PE38) by incubating it with an excess amount of each mAb. The sensitivity of this neutralization assay is similar to those used in our clinical trials (8, 9, 12). Only 6 of 60 mAbs neutralized the immunotoxin cytotoxic activity by >50% (nos. 43, 62, 57, 34, 83, and 7); Ab concentration was kept sufficiently high so that failure of a mAb to neutralize the immunotoxin was not the result of poor binding due to the lower affinity. Of the six mAbs, three (nos. 43, 62, and 57) are assigned to the same epitope subgroup, Ep1a, and all mAbs assigned to Ep1a showed neutralization activity.

The binding kinetics of each mAb were measured by capturing mAbs through an anti-Fc secondary Ab that had been covalently attached on a Biacore chip and by flowing each immunotoxin over the mAbs in solution to avoid possible alterations of the paratopes and epitopes. As shown in Fig. 7, the mAbs show ranges of association rate constants (1.1–160 × 10^4 M^{-1} s^{-1}), dissociation rate constants (0.1–218 × 10^{-4} s^{-1}), and affinity constants (0.1–174 × 10^8 M^{-1}) except one mAb (no. 57) showing an extremely low dissociation rate constant (0.003 × 10^{-4} s^{-1}) resulting in 5.3 × 10^{11} M^{-1} for the affinity constant).

We statistically evaluated the results of neutralization assay and binding kinetics data to determine whether any of these characteristics are associated with specific topographical epitopes. We find that Ep1a is significantly associated with the neutralizing activity of the mAbs binding to this epitope. In addition, Ep1a is significantly associated with slower dissociation rates of the mAbs resulting in high affinity. The neutralization activity is not explained by the simple increase of the affinity because mAbs that react with different

**FIGURE 7.** Binding kinetics of anti-PE38 mAbs. Binding kinetics was analyzed using a Biacore biosensor under conditions that allowed both paratopes and epitopes to be recognized in native PE38. The connection of the association rate, dissociation rate and affinity constants with the topographical epitopes was analyzed by nonparametric statistics (Mann-Whitney U test). †, Significantly higher; ‡, significantly lower (p < 0.01). A high dissociation rate constant of mAb 11 (218 × 10^{-4} s^{-1}) is shown as 100 × 10^{-4} s^{-1} in this figure for simplification.

**FIGURE 8.** Residual reactivity (the difference in affinity) of mAbs to mutants of PE38. A. Examples of the competition assay using mAbs 35 and 42 and wild-type PE38 (BL22 immunotoxin). Fifty-percent inhibition concentrations (IC50) were calculated from each curve. B. A plot of the IC50 as a function of the binding affinity (Kd) of each mAb used for the competition assay. The IC50 of each mAb were in general agreement with the affinity values (correlation coefficient between IC50 and Kd = −0.75). mAbs 35 and 42 are shown in this graph as a red closed circle and blue closed circle, respectively. C and D. Examples of the determination of residual reactivity in competition assays using mAbs 35 and 42. These two mAbs with different affinities both belong to Ep1b group. The IC50 values of E285A, E327A, and wild-type PE38 were 32, 330, and 32 ng/ml for mAb 35 (shown in C), and 510, 5100, and 500 ng/ml for mAb 42 (shown in D), respectively. Despite the differences in the IC50 values between the two mAbs with different affinity, the ratios of the IC50 (residual reactivity = ratio of the affinity) to each mutant were close (1.00 for no. 35 and 0.98 for no. 42 to E285A; 0.10 for no. 35 and 0.10 for no. 42 to E327A) because of the proximity of the epitopes. We used the residual reactivities for pairs of mAbs and mutants to evaluate the location epitopes (reported in Fig. 9).
epitopes with similar affinity do not neutralize immunotoxins efficiently. Ep1b and Ep3b are recognized by mAbs with lower affinities and those with higher affinities, respectively, although the changes in association and dissociation rate constants are not significant. Ep2c and Ep 5 and 7 are associated with slower and faster association rates of the mAbs, respectively, although the differences are not large enough to significantly change the affinities.

Localization of topographical epitopes using PE38 mutants

To locate each epitope on PE38, we produced a panel of 41 point mutants of PE38 (see Materials and Methods) and tested their reactivity with 40 representative mAbs that cover all 7 major epitope groups and 12 of 13 subgroups. Each mutant immunotoxin was tested for its reactivity with each mAb. We developed an assay that quantified the interaction in solution where both PE38 and mAbs should be in a native state. This assay gives a competition curve with a pair of mAbs and each immunotoxin mutant (Fig. 8A). Under these conditions, the 50% inhibition concentration (IC50) indicates the apparent Ab affinity (81). A good correlation (correlation coefficient = −0.75) was observed between the competition values and the affinities that had been determined in a BLAcore analysis (Fig. 8B).

Fig. 8, C and D, shows two examples of competition assays using two mAbs 35 and 42. These two mAbs both belong to the Ep1b group but exhibit different binding affinities to PE38 (3.3 × 10^8 M^-1 Kₐ for no. 35 and 3.8 × 10^7 M^-1 Kₐ for no. 42). As expected, wild-type PE38 showed different IC₅₀ for these two mAbs (32 ng/ml for no. 35 and 500 ng/ml for no. 42) (Fig. 8A). In similar competition assays (Fig. 8, C and D), the binding of each mAb to the E285A mutant was comparable with its binding to wild-type PE38 (IC50 = 32 ng/ml for no. 35 and 510 ng/ml for no. 42), while reactivity of the mAbs with the E327A mutant was 10% of wild type as shown by shifts of the competition curves to 10-fold higher concentration ranges (IC50 = 330 ng/ml for no. 35 and 5100 ng/ml for no. 42). Consequently, the IC50 ratios of the two mAbs to each mutant compared with wild type were similar (the ratios to E285A were 1.00 for no. 35 and 0.98 for no. 42; the ratios to E327A were 0.10 for no. 35 and 0.10 for no. 42). In this study, residual reactivity was determined as the ratio of the IC50 in this assay. These values were used for a comprehensive assessment of loss of reactivity depending on the location of epitopes. We determined the residual reactivities for all pairs of mutants and mAbs.

Residual reactivity of each mAb to individual mutants of PE38

The results of 40 mAbs assayed against 41 mutants are summarized in Fig. 9 in a matrix format. Each mAb bound to most of the PE38 mutants with the same affinity as to wild-type PE38 but failed to bind to a few key mutants (62 of 1636, 3.8%).

FIGURE 9. Residual reactivity of each mAb to individual mutants of PE38. Residual reactivities of each PE38 mutant (columns) with each mAb (rows) are shown. Orange, blue, and gray cells indicate <0.1 residual reactivity, >0.1 residual reactivity, and not tested (NT), respectively. The mutants are aligned by the location of the mutated residue from the N terminus (left) to the C terminus (right). The exposed areas of each mutated residue are shown in Å² in the PE38 model. The next column to the mAb names shows the IC₅₀ of the wild type (BL22-immunotoxin) in this assay.
Figure 10. Locations of the epitope residues on PE38 structure. The mutated amino acids that decreased the binding to mAbs in an epitope-specific manner (<10% binding to more than two mAbs assigned for the same epitope). We identified 14 PE38 mutants whose mutated residues affected the binding of some mAbs in this manner. The locations of 7 major epitope groups (9 of 12 subgroups tested) were identified by the 14 mutants. Two subgroups of Ep1 were identified by a single Q332A mutant, indicating the proximity of Ep1 subgroups. Ep3a and Ep3b were also identified by another single mutant (R313A). With some mutations, the loss of reactivity was detected for all the mAbs assigned to the same topographical epitope (footnote c in Table III). It is likely that the side chain of these residues form an important core for each topographical epitope structure and their mutation commonly reduced the binding of all the mAbs assigned for the same topographical epitopes. We identified such key residues for Ep1, 2c, 3, 5, and 7. None of the 14 mutations rendered an immunotoxin inactive (Table III). These mutants exhibited toxicity for Ag-positive cells in the same range as the wild-type immunotoxin protein although small gains and losses of cytotoxicity with some mutants were recorded.

### Location of epitopes on the PE38 structure

Because the loss of reactivity is related to the location of the topographical epitopes on PE38, we were able to locate each epitope group by mutations that affected mAb binding. Fig. 10 shows the location of the 14 aa residues listed in Table III. As expected, mutated residues identifying the same epitope are generally located on a restricted area on the structure model. The epitopes identified by the mutant panel (shown in color) were located at the surface of both domains of PE38 (II and III). Mutants that were not recognized by any mAb are shown in gray. The gray residues are often located between epitopes, indicating that this mutant analysis also showed that epitopes of mAbs make clusters and are not evenly distributed on the PE38 surface.

### Discussion

To characterize the humoral response to PE38, we injected mice with several PE38-based immunotoxins and derived hybridomas from the responding B cells. The activities of 57 new and 3 existing mAbs were then mapped to the surface of PE38. Mutual competition experiments indicate the presence of 7 major epitopes, which can be further divided into 13 subgroups. These epitopes were also recognized by human Abs induced in patients treated with immunotoxins. Among the 13 subgroups, epitope E1a was found to be the major neutralizing epitope. Also, when binding affinities were measured, Abs to E1a included some of the highest affinity mAbs that we isolated. Because our eventual goal is to modify PE38 and render it less immunogenic, we were gratified to learn that the B cell repertoire sees only 7 major epitopes and only 1 of these provokes strongly neutralizing Abs. Thus, the number of epitope sites that will need to be modified seems “manageable.” In part, this may be due to the existence of antigenic hot spots. Our computer analysis and the mapping experiments using a series of

### Table III. Epitope-related residues of PE38 identified by the corresponding point mutants with reduced reactivities with mAbs against each epitope

<table>
<thead>
<tr>
<th>Topographical Epitope</th>
<th>Mutation</th>
<th>% Cytotoxic Activity (IC50) of the Mutant to Wild Type in Cytotoxicity Assays</th>
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<tbody>
<tr>
<td>Ep1 a, b E327A</td>
<td></td>
<td>96</td>
</tr>
<tr>
<td>Ep1 c E331A</td>
<td></td>
<td>126</td>
</tr>
<tr>
<td>Ep1 Q332A&quot;</td>
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<td>121</td>
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<tr>
<td>Ep2 b Not identified</td>
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<td></td>
</tr>
<tr>
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<td>77</td>
</tr>
<tr>
<td>Ep2 R538A&quot;</td>
<td></td>
<td>141</td>
</tr>
<tr>
<td>Ep3 a, b R313A&quot;</td>
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<td>128</td>
</tr>
<tr>
<td>Ep3 D324A&quot;</td>
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<td>94</td>
</tr>
<tr>
<td>Ep4 a E431A</td>
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<tr>
<td>Ep4 b Not identified</td>
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<td></td>
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<tr>
<td>Ep5 R412A</td>
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<td>100</td>
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<tr>
<td>Ep5 R490A&quot;</td>
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<td>85</td>
</tr>
<tr>
<td>Ep5 R576A</td>
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<td>91</td>
</tr>
<tr>
<td>Ep6 a R513A</td>
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<td>Ep6 b Not identified</td>
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<tr>
<td>Ep7 K590A&quot;</td>
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* Point mutant that showed <10% residual reactivities against more than two mAbs of the same epitope group. When a mutation was not shared by two epitope subgroups, the subgroups are shown together.
* IC50 of wild-type IT (BL22) to Raji cells = 0.21 ng/ml.
* Mutation that inhibited all the tested mAbs to the corresponding epitope.

Conversely, each mutant bound normally to most of the mAbs but lost its reactivity with a few mAbs and those were usually assigned to the same topographical epitopes. Overall, the mutations that reduced binding are specific for each epitope group, indicating that the structures of these mutants are altered in a restricted area recognized by the mAbs assigned to the same topographical epitope. Importantly, this also confirms that the structure of the rest of the molecule is not affected.

Table III lists the mutations that reduced the binding to mAbs in an epitope-specific manner (<10% binding to more than two mAbs assigned for the same epitope). We identified 14 PE38 mutants whose mutated residues affected the binding of some mAbs in this manner. The locations of 7 major epitope groups (9 of 12 subgroups tested) were identified by the 14 mutants. Two subgroups of Ep1 were identified by a single Q332A mutant, indicating the proximity of Ep1 subgroups. Ep3a and Ep3b were also identified by another single mutant (R313A). With some mutations, the loss of reactivity was detected for all the mAbs assigned to the same topographical epitope (footnote c in Table III). It is likely that the side chain of these residues form an important core for each topographical epitope structure and their mutation commonly reduced the binding of all the mAbs assigned for the same topographical epitopes. We identified such key residues for Ep1, 2c, 3, 5, and 7. None of the 14 mutations rendered an immunotoxin inactive (Table III). These mutants exhibited toxicity for Ag-positive cells in the same range as the wild-type immunotoxin protein although small gains and losses of cytotoxicity with some mutants were recorded.

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point mutants confirmed that the location of each epitope was clustered and not random. The most notable success in deimmunizing therapeutic proteins has been the humanization of mouse mAbs by various means including mutagenesis and CDR grafting, where regions specific to mouse Ig were replaced with their human counterparts (44, 82). These successes have focused attention on the foreignness of proteins as a major obstacle to achieving success in the clinic (3, 5). Here, we present evidence for the presence of a limited number of epitope clusters on the surface of a bacterial protein, PE38. Our results suggest that a foreign protein may be made less immunogenic by replacing structures that are likely to be epitopes with those that are unlikely to become epitopes, although we have not identified the structural characteristics that determine the tendency to become epitopes. In this study, we successfully produced PE38 point mutants that abolish the binding of anti-PE38 mAbs in an epitope-specific manner. We also produced many mutants that caused no change in the binding of any of the mAbs. A comprehensive comparison of the structural difference between the areas altered by these mutations might help to disclose common structural features that determine the likelihood of a region becoming an immunogenic site.

The 14 mutants reported here with diminished binding to mAbs (Table III) are predicted to be less antigenic forms of PE38 in an epitope basis. The mutants will be used in the development of immunotoxins with reduced immunogenicity. Deimmunization of functional proteins is only possible if mutant versions retain biological activity. We found that all the 14 individual PE38 mutants retained substantial cytotoxic activity, despite the change of hydrophilic residues to either alanine or glycine. Ep1a that was shown to be associated with strong neutralizing activity (Fig. 6) was mapped to a region around Q332. It is particularly interesting that mutant Q332A does not bind to any of Ep1a mAbs (all are neutralizing) and yet retains full cytotoxicity. This residue is distant from the six functionally important regions previously identified: 276–279 (60), around W281 (83), 350–355 (84), around Y470 (85), around E553 (59), and 609–613 (86). Another potentially important mutation is R490A that abolished the binding of Ep5 mAbs. We recently reported that this mutation increases specific cytotoxicity of some PE38-based immunotoxins (87). This mutant could be useful both to diminish antigenicity and to increase the efficacy of immunotoxins.

In this study, we characterized Abs to epitopes exposed on the native surface of PE38, which should be useful for the prediction of Ab formation in patients treated with immunotoxins. Our approach to the selection of mutants and the quantitative assay that measures residual reactivity enabled us to scan the whole protein surface for B cell epitopes with a relatively small number of mutants. The identification of topographical epitopes on the surface of protein Ags followed by determining their location using point mutants could be the basis for a strategy to deimmunize foreign proteins.

Acknowledgments

We thank Dr. Sookhee Bang for preparation of the R490A mutant of PE38, and Anna Mazzuca and Dawn A. Walker for their editorial assistance.

Disclosures

I. Pastan and D. J. FitzGerald are partial owners of a non-U.S. patent on the use of PE38 to produce immunotoxins. The patent is currently licensed to Neophrm and IVAX for use in countries outside the United States.

References


