Protection against the staphylococcal enterotoxin-induced intestinal disorder in the monkey by anti-idiotypic antibodies

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Communicated by M. Lindauer, December 23, 1987 (received for review October 4, 1987)

ABSTRACT The staphylococcal enterotoxin serotype B (SEB)-induced enteric intoxication and the immediate-type reaction in the skin of unsensitized monkeys was used to define whether agents competing with SEB for target cell receptors may inhibit pathophysiological effects. For this purpose a duodenal provocation test was developed by use of a pediatric gastroscopy, allowing the evaluation of the influence of antagonists on the intestinal disorder upon SEB challenge at the same duodenal site. First, carboxymethylation of histidine residues of SEB caused a complete loss of emetic and skin-sensitizing activity without changing the immunological specificity. However, carboxymethylated SEB is a strong inhibitor of enteric intoxications and immediate-type skin reactions upon SEB challenge. Second, after immunization of BALB/c mice with monoclonal anti-SEB antibodies, monoclonal anti-idiotypic antibodies (anti-Id) were obtained by the "hybridoma technique" and purification by idiotype-affinity chromatography. Anti-Id specifically inhibited the binding of horseradish peroxidase-labeled anti-SEB to the ligand, and SEB blocked as well the interaction of these two antibody species, indicating a high degree of binding-site selectivity. Anti-Id completely protected against emetic response and diarrhea upon duodenal provocation with SEB and inhibited immediate-type skin reactions as well. Further, anti-Id acted as an antagonist without triggering biologic functions themselves. This shows that anti-Id constitute a useful tool to protect against a bacterial toxin-induced intestinal disorder.

Staphylococcal enterotoxins (SE) are responsible for one of the most common types of food poisoning in humans (1). All SE produce emesis and diarrhea in humans and other primates as a result of oral administration, whereas the toxin appears to have little, if any, clinical effect in other laboratory animals (1). Although considerable efforts have been expended on attempts to define the pathogenesis, so far very little information has been available on the mode and cellular site of SE action in the gastrointestinal tract.

Recently, however, evidence was provided that unsensitized monkeys develop an immediate-type reaction in the skin upon intradermal challenge with SE serotype B (SEB; ref. 2). As shown by a series of experiments, SEB administered intradermally causes skin reactions by affecting mast cells (2). This type of nonimmunological mast cell stimulation by SEB offered a new approach, providing a model for investigating the mechanisms of SEB action. In addition, evidence was provided that carboxymethylation of SEB resulted in a loss of toxicity associated with the complete abrogation of skin-sensitizing activity without changing the immunological specificity of the toxin. It has been established that carboxymethylated SEB (CM-SEB) could compete with SEB for binding sites on the target-cell surface (2).

To define whether SEB exerts its effect on mast cells by binding to specific cell-surface receptors or whether a less specific type of ligand-cell membrane interaction is involved, we have focused attention on the functional aspects of anti-receptor antibodies induced through the anti-idiotypic route as a useful tool to recognize receptors on the target cell membrane.

Such an approach has already been successfully applied with hormones in the identification of insulin receptors (3) and β-adrenergic receptors (4), respectively, on cell membranes. In a first series of experiments (5), some of us demonstrated that polyclonal anti-idiotypic antibodies (anti-Id) with a high degree of binding-site selectivity raised in BALB/c mice against monoclonal anti-SEB antibodies (anti-SEB) completely antagonize immediate-type reactions in the skin of unsensitized monkeys. Since differences may exist in the response of the skin and the gastrointestinal tract to SEB in the monkey, the relevance of the skin test data is unclear. To gain a better understanding of the mechanisms underlying SEB-induced enteric intoxication, we therefore performed duodenal provocation tests with SEB via a pediatric gastro scope to define the influence of CM-SEB and of anti-Id on the intestinal disorder. In this context, the use of monoclonal rather than polyclonal anti-Id was preferred to ensure a stable source of antibodies with a single activity and availability in large quantities. We will demonstrate that CM-SEB and monoclonal anti-Id, the latter even in the picomolar range, completely protected against emetic response and diarrhea in the monkey following SEB challenge. This is the evidence for complete protection against a toxin-induced intestinal disorder by anti-Id in primates.

MATERIALS AND METHODS

Reagents. Ketamine hydrochloride (ketavet; Parke, Davis); Evans blue, Alu-Gel-S, and polyoxyethylene sorbitan monolaurate (Tween 20; Serva, Heidelberg); bovine serum albumin and 2,6,10,14-tetramethylpentadecane (pristane; Roth, Karlsruhe, F.R.G.); egg albumin, o-phenylenediamine dihydrochloride, and compound 48/80 (Sigma); complete Freund's adjuvant (Difco); myoglobin sperm whale (Serva); 2,4-dinitrofluorobenzene (EGA Chemie, Steinheim, F.R.G.); horseradish peroxidase (Serva); mouse isotype-specific antisera (Miles Laboratories, Naperville, IL); and oxirane acrylic beads (Europol C; Röhm Pharma, Weiterstadt, F.R.G.) were obtained from the

Abbreviations: SE, staphylococcus enterotoxins; SEB, SE serotype B; CM-SEB, carboxymethylated SEB; anti-Id, anti-idiotypic antibodies; HRP, horseradish peroxidase; anti-SEB, monoclonal anti-SEB antibodies.

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indicated sources. Heat-killed *Listeria* monocytogenes and ascites fluid of BALB/c origin containing monoclonal anti-*Listeria* monocytogenes antibodies of the IgG3x isotype were a gift from Stefan Kaufmann of our institute. Rat IgE anti-dinitrophenyl antibody and dinitrophenyl-conjugated bovine serum albumin was donated by Horst Mossmann of our institute.

**Animals.** Cynomolgus monkeys (*Macaca fascicularis*) (body weight, 2.5–3 kg; provided by the Rijksinstituut voor Volksgezondheid, Bilthoven, The Netherlands) were selected from a colony of primates that were free of disease and of SEB-specific antibodies. Laboratory animals were cared for in accordance with published animal protection procedures (6). BALB/c (H-2d) mice were obtained from our own breeding stock, maintained on standard diet and water ad libitum, and used routinely at age 6–8 weeks.

**Toxin.** SEB was highly purified by the method described previously (7). To prepare CM-SEB, the procedure of Harris and Hill (8) was slightly modified. Bromoacetic acid (0.4 M) was dissolved in water and adjusted to pH 7.0 with 5 M NaOH. An aliquot of this solution was then added to an equal volume of 0.6% SEB in 0.5 M potassium phosphate buffer (pH 7.0). The solution was incubated in the dark for 14 days at room temperature. At the end of the reaction period, the solution was dialyzed at 4°C against several changes of distilled water, and the protein concentration was determined by amino acid analysis on a Chromakon 500 analyzer (Kontron, Eching, F.R.G.) after hydrolysis in 6 M HCl (Ultrapur, Merck) for 24 hr at 110°C. The integration constants used for carboxymethylated derivatives of histidine have been described (9).

**Preparation of Monoclonal Antibodies.** Monoclonal anti-SEB antibodies (anti-SEB) were obtained after immunization of BALB/c mice with highly purified SEB by the hybridoma technique (10) and were purified by affinity chromatography as described by Bamberger et al. (5). Polyclonal anti-Id, with specificity against the binding sites of anti-SEB, were prepared and purified by published methods (5). For production of monoclonal anti-Id, BALB/c mice primed with dinitrophenyl-substituted ovalbumin were immunized with monoclonal anti-SEB lightly substituted with dinitrophenyl by the method of Schuler et al. (11) with slight modifications (12). Finally the mice were rechallenged 4, 3, and 2 days before sacrifice with 100 μg i.p. of dinitrophenyl-substituted anti-SEB. Splenic lymphocytes (1 × 10⁸) from one immunized mouse were fused with 2 × 10⁶ P3X63-Ag8.653 mouse myeloma cells by standard techniques (10) as described in detail (5). Supernatants of hybridomas were screened for anti-Id in inhibition enzyme immunoassay. After the cloning and recloning of selected hybridomas by limiting dilution, pristane-pretreated BALB/c mice were injected i.p. with 1 × 10⁶ to 2 × 10⁶ cells in 0.5 ml of phosphate-buffered saline (10 mM sodium phosphate/140 mM NaCl, pH 7.2; PBS).

**Purification of Monoclonal Anti-Id.** The IgG-enriched fraction of anti-Id was obtained from ascitic fluid by precipitation with 2 M ammonium sulfate and subsequently absorbed with preimmune BALB/c IgG cross-linked to Eupergit C following approved techniques (5). Finally, anti-Id was purified by elution from a monoclonal anti-SEB-Eupergit C column (5). Isotyping of purified anti-Id by ELISA using subclass-specific antisera yielded antibodies of the IgG1κ isotype.

The preparation of F(ab')₂ fragments from monoclonal anti-Id used in this study has been described by Parham (13).

**Enzyme Immunoassay of Anti-Id.** Monoclonal anti-SEB or preimmune BALB/c IgG was conjugated to horseradish peroxidase (HRP) by the peridate method of Wilson and Nakane (14), and the anti-SEB-HRP or BALB/c IgG-HRP conjugate was separated by HPLC on an Ultropac TSK-G 3000 column (LKB). Wells of microtiter plates (Nuncimmunoplates) were coated with 100 μl of SEB (1.6 μg/ml) or anti-Id (2 μg/ml) at 37°C for 3 hr and further incubated at 4°C overnight or were coated with heat-killed *Listeria* monocytogenes (diluted 1:100) and kept at 60°C for 12 hr. After incubation, coating solutions were replaced for 1 hr with 2% bovine serum albumin in PBS. The plates were washed several times with PBS/0.05% Tween 20, and then 10–30 ng of HRP-conjugated anti-SEB per ml or ascites fluid containing monoclonal antibodies specific for *Listeria* monocytogenes (diluted 1:50) was added together with serial dilutions of anti-Id or of SEB. The plates were incubated for 1 hr at 37°C and rinsed five times with PBS/0.05% Tween 20; bound HRP-conjugated anti-SEB was determined with o-phenylenediamine as indicator.

**Direct Skin Test.** A series of skin-testing experiments was performed in unsensitized monkeys that had been anesthetized by i.m. injection of ketamine hydrochloride (11 mg/kg) and maintained unconscious for 30 min. Prior to any form of challenge, each monkey received an i.v. injection (2 ml/kg) of 1% Evans blue. Two minutes after this, duplicate 0.05-ml samples of SEB were mixed with preimmune BALB/c IgG and injected intradermally into the shaved anterior aspect of the thorax and abdomen. To evaluate the effect of CM-SEB monoclonal anti-Id, or monoclonal anti-SEB on the response to intradermal SEB, CM-SEB or purified antibodies were added to the agonists at different molar ratios, and the mixture (0.1 ml) was tested for its ability to elicit immediate-type hypersensitivity reactions in the skin of monkeys. The size of any blueing reaction giving a threshold of 5-mm diameter was recorded 15 min later.

**Intraduodenal Provocation Test.** A series of intraduodenal provocation tests were performed in unsensitized monkeys in the last 10 min of a 30-min anesthesia (11 mg of ketamine hydrochloride i.m. per kg of body weight) by use of a pediatric gastroscope (GIF-P10, Olympus, Munich). In a few cases, the animals were challenged with 0.1 ml of either 5 μM SEB or 25 μM CM-SEB, respectively, or with a mixture of both (0.1 ml total) at a molar ratio of 1:5 by injection into the duodenal mucosa with a sclerotherapy needle (diameter 0.18 mm) as shown in Fig. 1. In general, however, to study the anti-Id effect, purified anti-Id, anti-SEB, or preimmune

![Fig. 1. X-ray film showing the pediatric gastroscope positioned with its tip in the descending part of the duodenum. The arrow marks the injection needle pushed through the biopsy channel.](image-url)
IgG were added to SEB at different molar ratios and applied onto the mucosal surface of the duodenum via a plastic tube (inside diameter, 0.18 mm). In addition, the persistence of these antibodies on target cell receptors was evaluated by their administration onto the mucosal surface 24 hr before challenge with SEB on the same site in the descending part of the duodenum, ≈1.5 cm below the papilla of Vater. To mark the duodenal site precisely for an additional application of agents, a biopsy was taken immediately beneath the original application site. The clinical course was followed for a period of 24 hr.

RESULTS

Carboxymethylation of SEB. The rate of carboxymethylation of SEB was similar to that observed by Harris and Hill (8) and specific for histidine residues under the conditions used. Alkylation proceeds in 14 days to such an extent that the six histidine residues present in our SEB preparation (7) were derivatized to carboxymethylhistidine. This chemical modification of SEB caused a conversion from alkaline components to more acid species and an increase in microheterogeneity (2).

By testing CM-SEB for biological activity, it could be shown that the modification of six histidine residues caused a total loss in toxicity of the molecule.

Specificity of Anti-Id. The idiotypic specificity of anti-Id was confirmed in the inhibition of anti-SEB binding to solid-phase SEB by anti-Id. In Fig. 2a, the inhibiting capacity of monoclonal anti-Id is compared with that of polyclonal antibodies (5), both being highly efficient in impeding the binding of HRP-conjugated anti-SEB to the ligand in a concentration-dependent manner. Even at 8 pM, monoclonal anti-Id was able to effect 50% inhibition of anti-SEB binding, and its inhibiting capacity was remarkably greater than that of polyclonal anti-Id. As expected, preimmune BALB/c IgG consistently lacked any capacity to compete for binding. Moreover, in a competitive inhibition ELISA, the binding of HRP-conjugated anti-SEB to solid-phase anti-Id could be inhibited almost 50% by 1 μM SEB, but there was no such inhibition when myoglobin sperm whale was substituted for SEB (Fig. 2b) as control. The idiotypic specificity of anti-Id was further confirmed in inhibition experiments where anti-Id were unable to block the binding of monoclonal antibodies to heat-killed Listeria monocytogenes.

Effect of CM-SEB on SEB-Induced Reactions. Previous studies (2) have indicated that CM-SEB could compete with SEB for binding on the target-cell surface in the skin of unsensitized monkeys. From the data in Fig. 3, it is apparent that SEB promoted immediate-type skin reactions, whereas CM-SEB consistently failed to do so, even when tested at doses up to 100 μM. However, immediate-type skin reactions were completely inhibited in the presence of CM-SEB when used at a 5 × molar excess.

Further, we examined the possibility that CM-SEB might be a useful tool to influence the SEB-induced intestinal

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**Fig. 2.** Assessment of anti-Id activity by the ELISA technique. (a) Comparison of the ability of polyclonal (a) and monoclonal (●) anti-Id or preimmune IgG (■) to inhibit the binding of HRP-conjugated anti-SEB to solid-phase SEB. (b) Inhibition of the interaction between HRP-conjugated anti-SEB and solid-phase monoclonal anti-Id by SEB (●) or myoglobin sperm whale (■).

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**Fig. 3.** Effect of chemical modification of histidine residues on intradermal and emetic activity of SEB. CM-SEB, SEB, or an incubation mixture of both at a molar ratio of 5:1 were administered intradermally in a total volume of 0.1 ml. Enteric intoxication was induced by injection of SEB, CM-SEB, or both into the duodenal mucosa using a 5 × molar excess of CM-SEB in all experiments. Vomiting is documented by the number of monkeys showing positive reactions versus the number of animals challenged. The data are the mean ± SD of 6–10 experiments.
disorder. Indeed, the data in Fig. 3 show clearly that CM-SEB administered intraduodenally completely protected against emetic response and diarrhea after challenge with SEB when used at a 5:1 molar ratio.

Effect of Anti-Id on SEB-Induced Reactions. To examine whether the monoclonal anti-Id reacts with the ligand binding site on the target cell surface, we first tested its effect on the SEB-induced immediate-type skin reaction. In this context, increasing concentrations of anti-Id were used to compete with the action of SEB at different SEB doses (0.1–2.5 μM). Anti-Id at 1 pM to 1 nM impeded the immediate-type reaction in the skin of unsensitized monkeys in a concentration-dependent manner (Fig. 4). However, preimmune IgG did not have any significant effect at equimolar concentrations. To examine the specificity of anti-Id, we performed competition experiments with nonimmunological and immunological stimuli capable of inducing immediate-type reactions in the skin of monkeys. However, anti-Id lacked any capacity to impede skin reactions mediated by compound 48/80 or dinitrophenyl-conjugated bovine serum albumin in skin sites presensitized with rat IgE anti-dinitrophenyl antibody. The effect of anti-Id was next tested on the SEB-induced enteric intoxication by administration onto the mucosal surface of the duodenum. The most striking finding was the observation that, like its effect on the skin of unsensitized monkeys, anti-Id at 100 pM behaved as an antagonist and totally abolished the intestinal disorder upon challenge with SEB (Table 1). Even at concentrations as low as 10 pM, anti-Id was able to delay the emetic response up to 6 hr in comparison with controls (2 hr). However, anti-Id itself, at the concentration range used, lacked any capacity of triggering biologic functions. Exactly the same results were obtained by using the F(ab')2 fragment from anti-Id. Since some of us have reported (5) inhibitory capacity of anti-SEB as well on SEB-induced immediate-type skin reactions, the effect of these antibodies was assessed on the enteric intoxication. From the results it is clear that anti-SEB has inhibitory activity, but it is less potent than that of anti-Id (data not shown).

From the data presented in Table 1, it should be expected that anti-Id reacts with the target cell receptor in the intestinal mucosa in a ligand-like fashion. If so, the inhibitory effect of anti-Id on the SEB-induced intestinal disorder might be studied as a function of time. The effect of anti-Id was analyzed at 100 pM to 1 nM and at a concentration of 5 μM of the challenging ligand. Duodenal sites treated with up to 1000 pM anti-Id 24 hr before SEB challenge became totally unresponsive (Table 1). Even at concentrations at 100 pM, anti-Id induced a delay of the emetic response up to 8 hr. However, treatment of duodenal sites with anti-SEB lacked any inhibitory capacity (data not shown).

DISCUSSION

The impetus for these studies arose from the observation that SEB provokes an immediate-type reaction in the skin of unsensitized monkeys that could be completely inhibited in the presence of CM-SEB or polyclonal anti-Id (2, 5). Therefore, it was certainly reasonable to expect that CM-SEB and the anti-Id reagent might act as antagonists of the toxin and as such would block the intestinal disorder upon challenge with SEB as well. To dissect these effects on the intestinal (duodenal) level, the use of a monoclonal anti-Id as a stable source of antibodies with a single activity would be of considerable advantage.

In the first part of this study, we report the preparation and characterization of CM-SEB and monoclonal anti-Id, the latter generated against monoclonal anti-SEB by the hybridoma technique. The detoxification of SEB was based on the finding that carbamoylation of SEB type A resulted in a loss of toxicity without changing immunological specificity (15). A corresponding treatment of SEB with bromoacetate at pH 7.0 for 14 days derivatized all histidine residues and abrogated the toxicity completely.

The monoclonal anti-Id of the IgGlκ isotype induced against monoclonal anti-SEB was purified by idioype affinity chromatography. Before testing, anti-Id was sequentially absorbed on preimmune BALB/c IgG and SEB to remove any anti-isotypic or anti-SEB antibodies that could interfere with our tests. That anti-Id specific for the binding site of monoclonal anti-SEB may act as an internal image of the toxin was substantiated by showing that monoclonal anti-Id, even at picomolar concentrations, competed with SEB for binding to HRP-conjugated anti-SEB in a dose-dependent fashion. Its inhibiting capacity was much greater than that of a polyclonal anti-Id generated previously (5), suggesting that only a small fraction of the latter antibody molecules function as the immunological internal image of SEB. The reaction between solid-phase anti-Id and HRP-conjugated anti-SEB was also blocked in a dose-dependent manner by the SEB ligand, indicating a high degree of binding site selectivity. The specificity of anti-Id was further demonstrated by its inability...
to compete with the binding of monoclonal antibodies to heat-killed *Listeria monocytogenes*.

Most important in this study was the development of a duodenal provocation test by use of a pediatric gastroscope in the monkey, allowing the evaluation of the influence of CM-SEB and of anti-Id on the intestinal disorder upon SEB challenge at the same duodenal site. In this context it has to be stated that the animals showed no reaction on the duodenal mucosa by this technique. We have presented detailed evidence for the failure of CM-SEB to elicit enteric intoxication and immediate-type reactions in the skin of unsensitized monkeys. It is apparent from the results, however, that in fact CM-SEB specifically inhibits the response to SEB at binding sites of skin reactions. Correspondingly, CM-SEB injected in combination with SEB into the duodenal mucosa completely protected against emetic response and diarrhea when used at a 5 × molar excess. The data strongly support the view that CM-SEB competes with SEB for the binding to target-cell receptors. That SEB may act by affecting mast cells is reflected in the ultrastructural morphology of these cells at sites of skin reactions (2). So far, however, it has not been delineated whether skin mast cells are activated for mediator release in a direct or indirect way. Recent studies, however, have clearly indicated that cysteinyl leukotrienes are important mediators in the pathophysiology of the SEB-induced enteric intoxication (16). Moreover, evidence was provided that excretion of intestinal leukotriene metabolites with stool competes with their enterohepatic circulation (17). Since the observation by Sege and Peterson that anti-Id against anti-insulin induces a physiological response (3), several reports have demonstrated antagonistic effects of anti-Id as well (18, 19). Of particular importance, therefore, was the finding that anti-Id indeed, but not preimmune IgG, was highly efficient in inhibiting immediate-type reactions. Comparison of the inhibitory capacity of polyclonal (5) and monoclonal anti-Id indicated that the latter was 10²-fold stronger. Inhibition was also observed when anti-SEB was substituted for anti-Id, but the former was less efficient. The most striking of our findings was the observation that 100 pM anti-Id applied onto the mucosal surface of the duodenum completely protected against enteric response and diarrhea when challenged with SEB at the same duodenal site. Monkeys whose duodenal sites were exposed to 1000 pM anti-Id 24 h before SEB became totally unresponsive as well. Most notable is the fact that only picomolar concentrations of anti-Id are required to compete with 10³- to 10⁴-fold higher concentrations of SEB for binding to its receptor. Further, anti-Id did not inhibit immediate-type skin reactions mediated by IgE antibody or induced with 48/80, demonstrating its specificity. However, treatment of duodenal sites with anti-SEB 24 h before challenge with the toxin lacked any inhibitory capacity. This is in keeping with corresponding studies in the skin of the monkey. Further, the findings provide evidence that at least some amount of anti-Id is passing the duodenal barrier without being seriously degraded. At least in the monkey system used, the anti-Id acts exclusively as antagonist of SEB and as such will block the binding of the natural ligand and also inhibit the toxic effects.

That anti-Id, even at the picomolar range, exerts a prolonged blocking effect on the intestinal intoxication promoted by SEB indicates a considerable affinity and persistence of anti-Id at binding sites on target cells in the intestinal mucosa. Whether the receptors recognized by anti-Id are SEB-specific cannot at present be answered because at least polyclonal anti-Id also impeded the immediate-type skin reaction provoked by SE type A and C₁ (5). Whether this is secondary to the action of anti-Id at a site other than that which binds SEB is not clear. However, it cannot be excluded that several SE receptors may actually exist on the surface of cutaneous mast cells in the cynomolgus monkey. There is some indication that mucosal mast cells might be the targets for SEB in the intestinal mucosa, but one has to define whether the toxin affects the mediator release by acting directly on these cells. Alternatively, mast cell secretion may also be mediated by neuropeptides released from peripheral terminals of sensory nerve fibers (20) upon stimulation with SEB.

We presented here evidence that a monoclonal anti-Id against monoclonal anti-SEB displays the characteristics of a competitive antagonist and as such will protect against the intestinal disorder upon challenge with a bacterial exotoxin. Therefore, monoclonal anti-Id able to recognize SEB-specific receptors may be regarded as an original and promising tool to protect against SEB-provoked enteric intoxications. Further, by using the anti-Id approach, internal images of bacterial toxins could be produced, and this concept may be applied to the detection and characterization of specific receptors on the targets in the intestinal mucosa.

We are indebted to Doris Scheel for carboxymethylation of SEB and to Brigitte Sextl, Christa Thorwarth, and Iris Hahn for expert laboratory assistance. Gabriele Prosch is gratefully acknowledged for preparation of the manuscript. We are grateful to Olympus Co. for making the pediatric gastroscope GIF-P10 available to us for our intraduodenal provocation tests. This work was supported by grants from the Fraunhofer-Gesellschaft, Munich, and the Deutsche Forschungsgemeinschaft, Bonn (Grant Ha 403/13-1).