Anti-Idiotypic Antibody-Induced Protection against *Clostridium perfringens* Type D

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A monoclonal antibody (BALB/c mouse) with specificity for a neutralizing epitope on the epsilon-toxin produced by *Clostridium perfringens* type D was used to raise anti-idiotype antibodies (anti-Id) in different strains of mice and rabbits. These were purified and used in cross-immunization studies to induce anti-(anti-idiotype). All strains of mice and rabbits immunized with BALB/c-derived anti-Id showed a high-titer antibody response directed towards the active site of the toxin. This protected the animals against toxin challenge and against an oral dose of the vegetative organisms. Animals immunized with other anti-Id preparations showed no specific antibody response and were not protected. Guinea pig peritoneal macrophages have a cell surface receptor for the toxin, and incubation of these cells with BALB/c anti-Id allowed them to survive toxin challenge, indicating that occupation of the receptors by the anti-Id prevented binding by the toxin. In conclusion, we have shown that an internal-image anti-Id preparation will induce protective immunity in syngeneic and xenogeneic animals and furthermore that immunity to a single epitope on the exotoxin is sufficient to protect against the toxin and clinical sequelae evoked by the disease-causing organism itself.

Vaccination against toxin-secreting organisms has traditionally been achieved with toxoids. However, with new developments in biotechnology, it is now possible to minimize the components needed for a vaccine by identifying individual epitopes on the toxin and assessing their ability to confer protective immunity. One such approach to vaccination invokes the use of anti-idiotype antibodies (anti-Id) as surrogate antigens. Anti-Id recognize variable-region determinants on specific antigen-binding antibodies. As some of these anti-Id are intimately associated with the antigen-binding region, they will mimic the three-dimensional structure of the original antigen. Nisonoff and Lamoyi (18) proposed that these so-called "internal-image" antibodies could be used in a vaccine to replace the original antigen and evoke an antigen-specific immune response. The epsilon-toxin of *Clostridium perfringens* type D has been selected for this study to test the hypothesis.

*C. perfringens* produces at least 12 different toxins (15, 26), 4 of which, alpha, beta, iota, and epsilon, are considered major toxins and are used to group the species into five toxigenic types, A, B, C, D, and E, respectively. Epsilon-toxin is the major toxin produced by *C. perfringens* type D (8), although other toxins are produced in smaller amounts. The toxin is responsible for a rapidly fatal enterotoxemia in sheep, cattle, and goats and has marked dermonecrotic, edematous, and neurotoxic activity in a range of animal species (10). It is produced as a relatively inactive prototoxin that is converted to the lethal toxin after cleavage by proteolytic enzymes such as trypsin and chymotrypsin (17).

While the precise mode of action of the toxin is still unknown, it has been shown to cause increased intestinal permeability, enhancing its absorption into the bloodstream (1). After absorption, the toxin has been found to bind to the vascular endothelium of the brain, Henle’s loops and distal convoluted tubules of the kidney, and the sinusoids of the liver (4). As epsilon-toxin has been shown to bind to cell surface receptors, it is assumed that cells in these areas bear the receptor for the toxin (3).

In this study, we have used a formalinized epsilon prototoxin to raise polyclonal and monoclonal antibody (MAb)-secreting hybridomas. One of these MAbs, ASC12, is specific for a neutralizing epitope on the toxin, in that incubation of activated toxin with ASC12 enabled mice to receive 40 mouse lethal doses (MLD; based on an intraperitoneal [i.p.] lethal dose of 1.6 μg/kg of body weight) without any apparent harmful effects. This MAb was used to raise anti-Id in mice and rabbits, and these anti-Id were assessed for the presence of internal-image antibodies and the ability to afford protection against toxin and organism challenges in vivo and in vitro.

**MATERIALS AND METHODS**

Toxin and microorganisms. *C. perfringens* type D strain NCTC 8346 was grown in Trypticase-peptone-yeast extract-glucose (TPYG) medium (6) for 18 h at 37°C. Incubations and all subsequent manipulations were carried out under anaerobic conditions. Cultures were then centrifuged at 5,000 × g for 10 min, the supernatants were discarded, and the sediments were suspended in a total final volume of 5 ml of corn oil. In order to assess the number of viable organisms, 0.1 ml was removed and added to 0.9 ml of TPYG medium. Further 10-fold dilutions were made in TPYG, and these dilutions were plated as 0.1-ml volumes spread over the surface of duplicate plates of reinforced clostridial agar (Oxoid Ltd., Basingstoke, Hampshire, U.K.). The plates were incubated at 37°C for 18 h; colonies were then counted, and viable cells were counted.

Spores were produced by inoculating 5 × 10⁸ vegetative cells onto 130 ml of sporulation medium (20) in Roux bottles. These were then incubated under anaerobic conditions for 48 h at 37°C. Spores were harvested from the surface of the medium with glass beads and, after centrifugation and suspension in distilled water, the residues were heated to 70°C for 1 h in a water bath. Viable cells were counted as

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described above, and resting spores were stored at 4°C until use.

Purified epsilon-prototoxin was kindly donated by the Wellcome Foundation.

**Animals.** Female 8- to 10-week-old mice were used. BALB/c, C3H, and DBA/2 mice were obtained from Charles River Laboratories, Margate, Kent, U.K. NZB and CBA mice were supplied by OLAC Ltd., Bicester, Oxford, U.K. Young adult male White Dutch rabbits were obtained from Porcasso Animal Breeding Ltd., Heathfield, Sussex, U.K., and male Dunkin-Hartley guinea pigs were obtained from Interfauna, Huntington, Cambridge, U.K.

**Production of MAbs to epsilon-toxin.** Epsilon-prototoxin was treated as follows. Prototoxin was suspended in phosphate-buffered saline (PBS) at 400 μg/ml, and formaldehyde was added to a final concentration of 3 mM. After incubation for 7 days at 37°C, the reaction was stopped by the addition of L-lysine to 1 mM final concentration, and residual formaldehyde was removed by exhaustive dialysis against PBS.

BALB/c mice were immunized with 20 μg of prototoxoid i.p. followed by booster doses on days 7, 14, and 28. Spleen cells from hyperimmune animals were then fused with X63.Ag8-653 mouse myeloma cells by the method of Gelfter et al. (7). Ten positive hybridomas were subcloned three times by limiting dilution and then expanded in vitro. Ascites from each hybridoma were prepared by priming BALB/c mice with 0.5 ml of pristane (Sigma Chemical Co., Poole, Dorset, U.K.) i.p., and 10 days later the mice were inoculated with 10° hybridoma cells. Ascites were purified with caprylic acid (Sigma) (23), and the purified MAbs were stored at −70°C.

Prototoxin was converted to the active toxin by trypsin cleavage as described previously (9, 26), and toxin neutralization studies were carried out by i.p. injection of mice with 40 MLD of activated toxin which had been incubated with a 10-fold molar excess of MAb. One MAb, ASC12 (immunoglobulin G1 kappa chain [IgG1(k)]), gave complete protection against this dose of toxin and was selected for anti-idiotypic studies, while a second MAb, A6A11 [IgG1(k)], gave no protection and was used in the purification and characterization of anti-Id.

**Production of anti-Id.** Purified ASC12 and keyhole limpet hemocyanin (KLH; Sigma) were mixed together in PBS (pH 7.4) to give final concentrations of 5 and 0.25 mg/ml, respectively. Glutaraldehyde (grade 1; Sigma) was added to a final concentration of 0.08%, and the reaction was allowed to proceed at room temperature until turbidity developed. The reaction was stopped with L-lysine (0.1 M final concentration), and samples were exhaustively dialyzed against PBS at 4°C.

BALB/c (H-2d), DBA/2 (H-2d), and C3H (H-2k) mice as well as White Dutch rabbits were used to raise anti-Id. Mice were immunized with 75 μg of ASC12-KLH copolymer i.p. on days 0, 7, 14, 28, 35, and 49. The first two injections were given in incomplete Freund adjuvant (Sigma), and subsequent injections were given in PBS. Rabbits were immunized with 300 μg of the copolymer in incomplete Freund adjuvant, intradermally, at several sites over the dorsal surface on day 1, followed by an identical boost on day 14 and then every subsequent 28 days.

Animals were bled 28 days prior to each injection for estimation of antibody titer. Due to the frequency with which samples were taken from mice, a method was developed to obtain 50 to 100 μl of blood consistently and with minimum stress and trauma to the mouse. An anesthetic cocktail was used, consisting of a mixture of Hypnorm (Fentanyl base [0.2 mg/ml] and Fluanisone [10.0 mg/ml]; Crown Chemical Co. Ltd., Lamberhurst, Kent, U.K.) and Hypnovel (midazolam hydrochloride [5 mg/ml]; Roche Products Ltd., Welwyn Garden City, Hertfordshire, U.K.). Both were mixed with an equal volume of distilled water before being added together at a 1:1 ratio. The anesthetic was administered i.p. at a dose rate of 7.5 ml/kg of body weight (a typical 20-g mouse received 150 μl). Marked vasodilation was induced, allowing bleeding from a tail vein with a small-gauge needle. The tails were not traumatized, allowing subsequent sampling at or near the original site. Rabbits were bled from an ear vein under a local anesthetic.

**Estimation and purification of anti-Id.** Anti-Id titers were estimated by enzyme-linked immunosorbent assay (ELISA). Fab fragments were prepared by the method of Porter (21) and were bound to micro-ELISA plates (Immulon 2; Dynatech Laboratories Ltd., Billingshurst, West Sussex, U.K.) at 5 μg/ml in 0.1 M carbonate buffer (pH 9.6) at 4°C overnight. Residual binding sites were blocked with 1% (wt/vol) dried milk powder in PBS (Blotto) for 1 h, and serial dilutions of serum were then allowed to react with the Fab fragments for 1 h at 37°C. A horseradish peroxidase-conjugated anti-mouse (Fc specific; Sigma) or anti-rabbit (Sigma) immunoglobulin antibody was used as the detection system (at dilutions of 1:1,500 and 1:3,000, respectively, in Blotto) for 1 h at 37°C. Plates were developed with ARTS substrate [55 mg of 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma), 10 μl of 30% (wt/vol) hydrogen peroxide in 100 ml of 0.1 M phosphate-citrate buffer (pH 4.3)], and the A414 of samples was read on a Titertek Multiskan MCC (Flow Laboratories Ltd., Rickmansworth, Hertfordshire, U.K.). Plates were washed with PBS containing 0.02% Tween 20 (Sigma) between each stage on a Titertek microplate washer 120 (Flow Laboratories). A response of three times above background (unimmunized control animal sera) was considered positive.

Polyclonal anti-Id were purified from hyperimmune mouse and rabbit sera by affinity chromatography. ASC12 and A6A11 were coupled to cyanogen bromide-activated cross-linked agarose (Sigma) by the manufacturer’s instructions at a ratio of 1 mg of swollen gel to 10 to 15 mg of protein and placed in individual columns. Filtered sera were diluted 1:1 in PBS and repeatedly passed through the A6A11 column to remove any anti-allotypic and anti-isotypic antibodies that were present. The sera were then passed through the ASC12 column, and bound anti-Id were eluted with 200 mM glycine hydrochloride buffer (pH 2.5). After dialysis against PBS, protein concentrations were calculated from the A308 of samples.

**Anti-Id immunizations.** Purified polyclonal anti-Id (Ab2) were conjugated to KLH, and animals were immunized as indicated in Table 1 with the regimen detailed above. In this way, iso-, allo-, and heterotypic anti-Id were used to immunize BALB/c, DBA/2, C3H, CBA, and NZB mice and rabbits. Anti-epsilon-toxin antibody titers were detected by ELISA with the protocol detailed above except that epsilon-prototoxin (2 μg/ml) was used as the ligand.

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<thead>
<tr>
<th>Source of anti-Id</th>
<th>Recipient of anti-Id</th>
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<tr>
<td>BALB/c mouse</td>
<td>BALB/c, C3H, DBA/2, NZB, and CBA mice, rabbit</td>
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<tr>
<td>C3H mouse</td>
<td>BALB/c, C3H, and DBA/2 mice</td>
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<td>DBA/2 mouse</td>
<td>BALB/c and C3H mice</td>
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<td>Rabbit</td>
<td>BALB/c mice</td>
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**TABLE 1. Anti-Id protocols**
FIG. 1. Groups of four mice or two rabbits were immunized with BALB/c-derived anti-Id, and the subsequent anti-epsilon-toxin antibody response over time was measured by ELISA. Data represent the antibody titers for pooled sera from each group. Animals immunized with the other anti-Id preparations and unimmunized control groups showed no detectable anti-epsilon response.

**In vitro assay for the detection of internal-image antibodies.** Buxton (5) has shown that guinea pig peritoneal macrophages (GPPMs) are sensitive to epsilon-toxin, and these cells were prepared by washing the peritoneal cavities of freshly killed Dunkin-Hartley guinea pigs with PBS. After being washed, GPPMs were suspended in Dulbecco modified Eagle medium (GIBCO Ltd., Paisley, Scotland) supplemented with 10% fetal calf serum (HyClone; GIBCO) and L-glutamine (2 mM final concentration; Flow Laboratories). Samples (200 μl) of a suspension of macrophages (5 × 10^6 cells per ml) were placed in the wells of eight-chamber tissue culture slides (Miles Scientific, Stoke Poges, Slough, U.K.). Cells were incubated at 37°C for 2 h to allow adherence and were then washed and incubated overnight with fresh medium containing anti-Id preparations at 100 μg/ml. After further washing, GPPMs were challenged with activated toxin at a concentration of 1 or 0.1 μg/ml in medium.

After 60 min, cells were counted with an acridine orange-thamid bromide vital stain. All counts were corrected as follows to give the percentage of viable cells after treatment with toxin: final viable count divided by initial viable count times 100. Mean counts and standard error of the mean were then calculated for at least four samples.

**Challenge with toxin and organisms.** Immunized animals and corresponding control groups were challenged in one of three regimens.

(i) Mice and rabbits were injected with 10 MLD of activated epsilon-toxin i.p.

(ii) BALB/c mice were given an oral dose of 5 × 10^6 viable vegetative organisms. Mice were anesthetized with Halothane (Cooper Animal Health Ltd., Crewe, Cheshire, U.K.), and 0.1-ml volumes of the suspension were given orally with modified blunt-ended needles.

(iii) BALB/c mice were given an oral dose of 2 × 10^6 viable spores in 0.1 ml as detailed above.

The mean time to death and standard error of the mean were then calculated for each group of animals.

**RESULTS**

**Production of anti-Id.** The A5C12-KLH copolymer was used to induce an anti-Id response in BALB/c, C3H, and DBA/2 mice and in rabbits. After 52 days, all animals showed an anti-Id titer of >1:100,000. Blood was then taken from animals within each group, and the serum was removed by centrifugation. Hyperimmune sera were then passed through the A6A11 and A5C12 immunoaffinity columns to remove the anti-Id, and in all cases, the yield of anti-Id was approximately 1 mg/ml of serum. These anti-Id preparations were coupled to KLH and used to raise Ab3 in animals (Table 1).

**Specificity of Ab3.** All animals immunized with anti-Id conjugates were assessed for the presence of anti-epsilon-toxin antibody. Immunization of mice with C3H-, DBA/2-, or rabbit-derived anti-Id induced no detectable anti-epsilon response. However, immunization of all strains of mice (BALB/c, C3H, DBA/2, NZB, and CBA) with BALB/c-derived polyclonal anti-Id resulted in a high-titer anti-epsilon response (Fig. 1). The mouse strain haplotype did not appear to influence the animals’ ability to respond to this anti-Id preparation, although syngeneic BALB/c mice consistently produced a higher-titer anti-epsilon response than all other strains. Rabbits immunized with this preparation also produced anti-epsilon-toxin IgG.

The specificity of the anti-epsilon Ab3 was determined by a modification of the ELISA with epsilon-prototoxin as the ligand. The binding of prototoxin by Ab3 and A5C12 could be inhibited (>95%) by preincubation of prototoxin with a 10-fold molar excess of A5C12 Fab fragments, whereas the binding of antiserum raised against epsilon-prototoxoid was not significantly reduced (<8%). By comparison, preincubation of prototoxin with excess A6A11 Fab fragments did not significantly (<10%) inhibit the binding of Ab3, A5C12, or anti-epsilon antiserum. This indicates that whereas the anti-epsilon antiserum is polyclonal in nature and was not inhibited by preincubation of prototoxin with MAb, Ab3 is essentially monospecific, with specificity for the same epitope as that recognized by A5C12.

**In vitro protection by anti-Id.** The ability of anti-Id preparations to provide protection against the cytopathic effects of the toxin in vitro were assessed by using GPPMs. Purified anti-Id was allowed to react with cells overnight before the addition of medium containing either 1 or 0.1 μg of activated toxin per ml. Figure 2 shows that addition of toxin caused cell death in >90% of GPPMs. Preincubation of cells with
nonspecific mouse IgG or anti-Id raised in C3H or DBA/2 mice did not inhibit cell lysis. However, preincubation with BALB/c anti-Id reduced cell death by >50%.

Immunofluorescence studies to determine whether the protection afforded by the BALB/c anti-Id was due to binding of internal-image antibody to the cell surface receptor of the toxin were unsuccessful due to high background staining of the GPPMs by the anti-mouse IgG-fluorescein isothiocyanate conjugate used in the study.

**Toxin challenge of anti-Id-immunized animals.** Toxin neutralization studies have shown that A5C12 is a protective MAb and that immunization of animals with the anti-Id derived from it (in BALB/c mice) induced antibodies which are specific for the same epitope. The importance of the epitope in conferring immunity to toxin and live-organism challenges was investigated by challenging animals with 10 MLD of activated toxin i.p. Animals immunized with BALB/c-derived anti-Id were all afforded significant protection against the challenge (Fig. 3). BALB/c mice and rabbits were completely protected, and the other strains of mice showed an increase in time to death of at least a factor of three. However, animals immunized with other anti-Id preparations and the unimmunized control groups succumbed to the lethal effects of the toxin within 2 h.

The results indicate that protection against a toxin challenge was achieved only when BALB/c-derived anti-Id was used to induce Ab3. A summary of the toxin challenge experiment therefore allows the identification of protective (Fig. 4A) and nonprotective (Fig. 4B) immunization pathways.

**Protection against live-organism challenge.** BALB/c mice immunized with BALB/c-derived anti-Id were challenged with an oral dose of \(5 \times 10^8\) viable *C. perfringens* type D vegetative organisms. Of 13 unimmunized control mice, 9 succumbed within 48 h, while the remainder survived with no apparent ill effects. The 13 immunized animals were completely protected and showed no signs of distress.

In contrast, oral challenge with viable spores was unsuccessful, as no mortality was observed in either the control or immunized group of BALB/c mice when challenged with \(2 \times 10^8\) spores, presumably because the organisms were excreted before they were able to germinate and become established.

**DISCUSSION**

The results from this study demonstrate that anti-Id can be used to generate an immune response specific for a bacterial exotoxin in animals which have not been exposed to the toxin. This conforms with Jerne's hypothesis (11, 12) that the immune system can be manipulated by the injection of idiotypic antibodies.

The polymerization of the A5C12 myeloma protein to a protein with antigenic determinants recognized as foreign by the recipient (KLH) produces a potent immunogen. The mechanism of enhancement of immunogenicity by copolymerization is uncertain. It could result in increased phago-

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**FIG. 2.** GPPMs were challenged with either 1.0 or 0.1 μg of activated epsilon-toxin per ml after an overnight incubation with various anti-Id preparations. Controls included cells incubated with nonspecific mouse IgG, untreated cells incubated with toxin, and cells treated with neither antibody nor toxin. Data indicate the mean values, with standard errors, for at least four samples.

**FIG. 3.** Groups of mice or rabbits which had been immunized with various anti-Id preparations and unimmunized control groups were challenged with 10 MLD i.p. of activated epsilon-toxin. Data represent the mean and standard error of the mean for times to death of mice (four per group) or rabbits (two per group). BALB/c mice and rabbits receiving BALB/c anti-Id were completely protected against toxin challenge.
cytosis and presentation of the complex, or it could involve the production of antibodies to the carrier protein, which then bind to the copolymer and somehow increase its immunogenicity. Anti-Id were evoked with this preparation even in groups of mice (BALB/c and DBA/2) which possess the same haplotype as the donor of the original MAb (BALB/c). In contrast, immunization with monomeric ASC12 failed to induce an anti-Id response in these animals (data not shown).

The polyclonal anti-Id from each group of animals was successfully isolated from the cross-linked antibody-agarose columns. As ASC12 and A6A11 are both BALB/c-derived MAbs from the same fusion event and are of the IgG1(κ) subclass, they possess the same heavy- and light-chain constant regions. The purified anti-Id were specific for ASC12 Fab fragments only and not for A6A11 Fab fragments when screened by ELISA. This indicates that the antibodies are specific for antigenic determinants within the variable region of ASC12 and are not directed towards determinants within the constant regions.

In vitro studies with GPPMs showed that treatment of the cells with BALB/c-derived anti-Id protected them from subsequent toxin challenge, resulting in 60% survival. Treatment of cells with other anti-Id preparations was relatively ineffective in protecting against toxin challenge and resulted in only 8% viability. There is strong evidence to suggest that epsilon-toxin binds to a cell surface receptor (3). Other workers have shown that anti-Id can recognize the cell surface receptors of proteins and viruses (19, 24), and our results suggest that ASC12 is specific for the cell-binding site of the toxin and that internal-image antibodies, present only as a proportion of the BALB/c-derived anti-Id, prevent killing of the GPPMs by blocking receptor sites on the cell surface. However, while incubation of toxin with ASC12 reduced lysis to control levels (data not shown), treatment of cells with anti-Id did not provide complete protection. This can be attributed to two effects: (i) the internal-image subset of the BALB/c anti-Id represents only a small fraction of the antibody present and therefore will not be able to block all cell receptors; and (ii) the affinity of the anti-Id for the receptor will vary, and thus epsilon-toxin will be able to displace any low-affinity antibodies from the receptor and so effect cell lysis.

When BALB/c anti-Id was used to generate Ab3 in mice and rabbits, antibodies specific for epsilon-protoxin were detected, and these animals were protected from subsequent toxin challenge. This confirms the existence of a public or cross-reacting internal-image component within this preparation which is able to induce a specific response in syngeneic, allogeneic, and xenogeneic animals by mimicking the epitope on epsilon-toxin which is responsible for cell binding.

An effective organism challenge was administered by the delivery of vegetative cells in corn oil. It is assumed that this protected them from both the aerobic environment and the acidic conditions of the stomach, allowing them to pass into the duodenum, where the oil emulsified, releasing the organisms to colonize the gut. Immunization of BALB/c mice with BALB/c-derived anti-Id protected them from an oral dose of viable organisms, presumably by circulating antibody binding to and neutralizing epsilon-toxin as it entered the blood from the gut.

Burr and Sugiyama (2) have shown that oral challenge of mice with spores of C. botulinum was most effective after treatment of the animals with metronidazole to reduce the gut flora. It is therefore probable that the oral challenge with spores of C. perfringens was unsuccessful because of limited germination, caused by the presence of competing organisms. Thus, there was insufficient accumulation of toxin in the gut to cause symptoms of intoxication.

While other workers have shown that immunization with anti-Id can provide protection by mimicking epitopes of pathogenic organisms (13, 14, 16, 25), these have generally been epitopes which are present on the surface of the bacterium or virus. Reck et al. (22) have shown that monkeys can be protected against the emetic effects of staphylococcal enterotoxin type B by passive administration of an anti-Id preparation directly into the duodenum. In contrast, we have shown that active immunization with anti-Id induces immunity against a single epitope on a bacterial exotoxin and that this is sufficient to protect animals from acute challenge with the toxin-secreting organism itself.

ACKNOWLEDGMENTS

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