Production of Rabbit Antisera to the Staphylococcal Enterotoxins

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ABSTRACT

An immunization method for production of antisera to the staphylococcal enterotoxins in rabbits is presented. The bleeding schedule is tailored to the enterotoxin type. About 0.5 mg of staphylococcal enterotoxin is used per rabbit and serum harvest begins 11 weeks after the initial injection. Proposed are subcutaneous injections of 50, 100 and 300 μg of toxin on days 0, 3, 8, 24 and 28, respectively. Serum harvest ranges from a 4-week period for enterotoxin A to 8 or more weeks for enterotoxin E. Immunizations with all toxin types using the proposed or similar injection programs produced antibody titers from about 20 to over 100. Individual variation in response of rabbits in the same group was generally 3- to 5-fold, and in extreme instances, 10-fold. Immunization experiments were augmented by booster experiments in which the rabbit variable was held relatively constant by sequentially testing different schedules and doses on the same group of animals.

Staphylococcal food poisoning, one of the most common types of foodborne disease in the United States, is caused by the presence of staphylococcal enterotoxins in ingested food. To date all methods for identification and quantification of the known enterotoxins are based on the availability of specific antibodies for each of the enterotoxin types, A through E (SEA, etc.). The enterotoxins are low molecular weight proteins (27,000 to 30,000 daltons) of high toxicity and relatively low antigenicity. Initially, a lengthy series of inoculations was used to immunize rabbits starting with several injections of enterotoxoids followed by several injections of toxin (3). Later, immunizations were done beginning with very small amounts of enterotoxin (2 to 5 μg) with stepwise increases to doses of as much as 1 to 4 mg using various injection schedules (2,4,7,8,9,12,13,14), two of which required relatively small amounts of toxin, 164 μg (7) and 220 μg (13). In addition to these methods, one was reported in which two injections of 10 μg given 6 weeks apart resulted in acceptable antibody titers (18,19). The requirements of time and numbers of rabbits to obtain meaningful results limit the number of experiments that can be done within a reasonable number of years. Usually it is not possible to test a new immunization procedure or each of the published methods with all toxin types. In addition, the lack of uniform definition and methodology for determination of serum titers makes it difficult to compare and evaluate immunization procedures.

The purpose of this communication is to present a method of immunization that evolved during the years of experience of preparing specific antisera to the staphylococcal enterotoxins in the Food Research Institute. The method requires only moderate amounts of enterotoxin to produce high-titered antisera from responsive rabbits and antisera usable titers from moderately responsive rabbits. Immunization data are presented for all of the identified staphylococcal enterotoxins.

MATERIALS AND METHODS

Staphylococcal enterotoxins

The staphylococcal enterotoxins used for production of the antisera were purified (95 to 98%) at the Food Research Institute (FRI) [SEA (7), SEB (16), SEC (6), SECj (1), SECf (unpublished), SED (10), SEE (5)]. Purity was determined by the presence of a single band on SDS gels, and by the presence of only one precipitin band when a range of concentrations of the pure toxin was tested on double diffusion plates (15) against the undiluted antiserum produced against the pure toxin.

Rabbits

Female New Zealand white rabbits (2 to 3 kg) were purchased from licensed commercial producers.

Preparation of inocula using Freund's adjuvant

Complete Freund's adjuvant (cFa) (Difco) was used in the initial injections. Thereafter, either cFa or Difco's incomplete adjuvant (iFa) was used. The toxin dose in 1 ml of either physiological saline solution or phosphate buffered saline solution was mixed to a hard emulsion with 1 ml of Freund's adjuvant (Fa) by pumping back and forth between two sterile syringes connected by a female-female Lurelock adapter. One-half of the emulsion was injected subcutaneously into the shoulder area near the neck on each side of a rabbit.

Preparation of inocula using potassium alum

As an alternative to using Fa, the staphylococcal enterotoxin was absorbed with nascent alumina by a method using AIR (SO3)2×12 H2O which was adapted from Chase (11). One mg of toxin adsorbed by the floc was slurried in a Vortex mixer with 1 ml of physiological saline solution contain-
ing 1 mg of enterotoxin; the mixture was injected subcutaneously into both shoulders of two rabbits.

**Immunization methodology**

Normally 6 to 8 rabbits were started in each group. Only small amounts of toxin were used in the 2 to 5 initial injections, the amount depending on the toxicity of the enterotoxin type, with a gradual increase until the rabbit's undiluted serum gave a precipitin line on a double gel diffusion plate vs. 2 μg/ml enterotoxin. Those animals that did not respond to the experimentally determined threshold dose of 20 μg were discarded. The rabbits were weighed before each injection and at the same time each day on the two following days to check for weight loss (up to 200 g). The next injection was postponed if the weight loss was not regained by the scheduled date. The threshold injections were followed with two to three booster injections, generally of 2 to 5 times the amount of the preceding one; all were given within an 8- or 14-day period. Test bleedings were taken weekly from the marginal ear vein until the serum antibody titer approached 20, after which full bleedings of up to 50 ml were taken by syringe from the ear artery at semi-to-biweekly intervals. The immunization schedules are given in Table 1.

**Boosting procedures**

Immunized rabbits at their normal weights and with serum titers in the range of 5 to 15 were given booster injections. The normal time between successive boosting procedures was about 3 months, depending on how long the serum titer remained above 15. Three different boosting procedures were used:

1. (a) one to three-step boosts with cFa and/or icFa,
2. (b) one-step boosts of toxin adsorbed on Al(OH)3, and
3. (c) a combined two-step boost, using Fa for the first injection and toxin adsorbed on Al(OH)3 for the second. With SEA the same boosting procedure was used twice with the same group of rabbits to demonstrate reproducibility.

**Determination of antibody titer**

Antibody titers were determined by the method of Weiss and Robbins (20). This is a 7-day quantitative single gel diffusion tube procedure using 2-fold dilutions of the test serum. The coefficient of variation in our hands is 3%. It was routine to make overnight estimates of titer by visual comparison of the two nearest precipitin zone lengths to that in a standard single gel diffusion tube (standardized serum-agar at a titer of 1, overlaid with crude toxin at 10 μg/ml). Such estimates generally varied from the calculated value by only 10-20%, and allowed a prompt decision on whether to follow with a full or a test bleeding. A serum diluted to a titer of 1 will produce a balanced line with 1 μg/ml of enterotoxin on a double gel diffusion plate. This is similar to the microslide titer used by Bradstreet et al. (7) and therefore, the titers they report should be comparable to those given in this paper. In general, serum antibody titers to the staphylococcal enterotoxins lie within the range of 2 to 256 when they are determined by gel precipitation methods.

**RESULTS AND DISCUSSION**

**Immunization schedules**

Results from the various immunization schedules are presented in Table 1. To facilitate comparisons with later experiments, the data in Table 1 are given only for those rabbits that were used in all procedures reported in this paper for that group; however in the text, results for all rabbits started are discussed.

The size of the initial injection is limited by the toxicity of the enterotoxins. In our experience with SEB and SEC, no rabbit died with an initial dose of 10 μg, although one died after an initial injection of 20 μg of SEB. In previous immunizations with SEA, 3 of 17 rabbits died after an initial injection of 5 μg. For this reason the recommended initial dose for the general immunization procedure was set at 1 μg of toxin, a level that has not been lethal to rabbits in this laboratory.
The good response to SEA (Table 1, schedule 1) at a total dose of 250 μg is attributed to the relatively large number of injections (seven). Subsequent experiments also indicated that the antibody titer to SEA is dependent on frequent injections of SEA for effective immunogenicity. On the other hand, the results from schedules 2, 3, 4, and 5 (Table 1) show that 4 injections appear to be adequate for SEB and the SECs, provided they were given over a period of 35 d. The relatively low titer of 21 for SEB (Table 1, schedule 9) obtained with 350 μg given in 4 doses over a 21-d period contrasts with twice the titer from less than half the toxin when given in 4 doses over 70 d (Table 1, schedule 2). Later experiments showed that the rabbits used for the injections in schedule 9 were not poor responders.

In schedules 2 and 5 for SEB and SEC₂ (Table 1), the increase to 50 μg for the second injection resulted in the loss of two of the SEC₂ rabbits with several others (both SEB and SEC₂) recovering very slowly from excessive weight loss. When the initial 10-μg injection was followed by a 20- or 25-μg injection, weight loss was not a problem.

To produce a satisfactory antibody titer with SED, an amount higher than 230 μg appears to be required because the highest individual titer was 13 with an average of 9 for 5 rabbits (Table 1, schedule 11) while injections totaling 440 μg resulted in a high titer of 49 with an average of 30 for the 3 rabbits used (Table 1, schedule 7).

The purpose of schedule 10 (Table 1) was to determine if the threshold dose would be effective as a boosting dose. It was not with SED. Of 4 rabbits immunized, the highest titer obtained was 12 with an average of 6. Poor results were obtained for SEB also when the threshold dose was used for boosting (data not shown).

Schedule 8 (Table 1), which was used with SEE, was designed with the features found important for the other enterotoxin types. It included multiple injections, a 13-d rest before and a 39-d rest after the second series of injections (threshold cluster) and a total dose of 564 μg, which was high enough to produce satisfactory titers even with SED. The resulting average peak titer for the 7 surviving rabbits (of 8 started) was 80. A conservative approach for SEA and SEE would be to lower the level of the initial sequence to 1, 2, 5, 10 and 20 μg and take particular care that any weight loss be regained before a subsequent injection is given. The weight data (not shown) indicate that a 50-μg dose given 42 d after the threshold dose should pose no toxicity threat for SEA and SEE.

From an overall review of the immunization schedules used for SEB and the SECs it would appear that a streamlined but effective immunization program for these toxins would be 10, 20, 30, 60 and 300 μg of toxin given at 0, 24, 28, 63 and 70 d. The 60 μg at 63 d allows the attainment of a 300-μg dose level at 70 d without exceeding a safe increment of 5-fold increase in each dosage of the series at this stage.

A summary of 3 immunization schedules is presented in Table 2. The first is recommended as being applicable to all staphylococcal enterotoxins and is based on the successful trials shown in Table 1. The second schedule, adapted to the greater toxicity of SEA and SEE, probably could be used with all of the enterotoxin types. Injections found unnecessary for SEB and SEC are eliminated in the third schedule.

**Booster experiments with SEA**

Compared with SEB and SEC, SEA has been a weak immunogen. The most effective method for preparing the toxin for the booster injections was to absorb SEA on an aluminum hydroxide floc and suspend the floc in a solution of SEA. The results of sequentially boosting the 4 rabbits whose immunizations are recorded in schedule 1, Table 1, with various levels of SEA using for adjuvant (a) alum only, (b) Fa with a small first dose followed by a larger second dose using alum and (c) Fa only are summarized in Table 3. The procedure ofchoice is the three-step one with injections of 50, 100 and 350 μg over an 8-d period (schedules 6 and 7, Table 3) because it produces results comparable to the best alum procedure with only half as much toxin.

**Booster experiments with SED**

In a series of three challenge experiments using the 8 rabbits previously injected with SED (same rabbits as the ones listed in Table 1), each rabbit was given 630 μg of SED per experiment within an 8-d period. A different group (2, 3 and 3 rabbits) received either 1, 2 or 3 injections in each of three 8-d periods. A greater difference in titers between responsive and moderately responsive rabbits was observed than differences resulting from the number of injections used (Fig. 1). Nevertheless, the highest serum titers were obtained in both the responsive and moderately responsive rabbits with three injections.

**Titer response**

The titer of the rabbits immunized by multiple injections of toxin with Fa was found to divide into three patterns: (a) high peaking or steadily rising titers during the collection period; (b) moderately arched, increasing and then decreasing titers; and (c) essentially constant low level titers. These patterns reflect the individual rabbit’s responsiveness at the particular time to the stimuli given. In our experience with known effective schedules, the rabbit’s initial antibody response pattern is generally predictive of future performance, particularly for categories 1 and 3. As can be seen from Table

<table>
<thead>
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<th>Table 2. General and specialized immunization schedules.</th>
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<td><strong>Enterotoxins</strong></td>
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<td>------------------</td>
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<tr>
<td>General schedule</td>
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<tr>
<td>For SEA and SEE</td>
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<td>For SEB and SEC</td>
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TABLE 3. Effect of booster injections of SEA on serum antibody titers.¹

<table>
<thead>
<tr>
<th>Schedule</th>
<th>Method</th>
<th>Injections (μg)</th>
<th>Total dose (μg)</th>
<th>Individual Peak titers</th>
<th>Ave.</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>With Alum</td>
<td>Day 0</td>
<td>Day 3</td>
<td>Day 8</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>one-step</td>
<td>50 + 500b</td>
<td></td>
<td></td>
<td>550</td>
</tr>
<tr>
<td>2</td>
<td>one-step</td>
<td>500 + 500b</td>
<td></td>
<td></td>
<td>1000</td>
</tr>
<tr>
<td>3</td>
<td>two-step</td>
<td>100d</td>
<td>500 + 500b</td>
<td></td>
<td>1100</td>
</tr>
<tr>
<td>4</td>
<td>two-step</td>
<td>100d</td>
<td>400 + 500b</td>
<td></td>
<td>1000</td>
</tr>
<tr>
<td>5</td>
<td>three-step</td>
<td>35</td>
<td>60</td>
<td>100</td>
<td>195</td>
</tr>
<tr>
<td>6</td>
<td>three-step</td>
<td>50</td>
<td>100</td>
<td>350</td>
<td>500</td>
</tr>
<tr>
<td>7</td>
<td>three-step</td>
<td>50</td>
<td>100</td>
<td>350</td>
<td>500</td>
</tr>
</tbody>
</table>

¹Same rabbits as in Table 1.
²Soluble toxin + alum precipitated toxin.
³This rabbit was not included in this experiment.
⁴This injection was given with Fa.

1, if both very responsive rabbits and poor responders are in the same group, the range of peak titers is about 10-fold, if one extreme is missing, the range is 3- to 5-fold, and if both extremes are missing, the range is reduced to about 2-fold. The response of a group of rabbits will be dependent on the proportion of rabbits falling into each of the three categories. This can easily mask the role played by the different procedures being studied.

The maximum serum titers were reached at different times after the booster injections and remained high for different periods, each toxin type having its own characteristic pattern. This can be seen in Fig. 2 where titer variation with time is shown for an individual rabbit for each toxin type. The du-
ration of elevated serum titer is shortest for SEA and increases in the order SED, SEC, SEB and SEE. To maximize the harvest of the rabbit’s best antisera, the following schedule is indicated: 6 semi-weekly bleedings for SEA and 6 weekly bleedings for the other toxin types, starting at 1 week after the final injection for SEA and SED, 1 1/2 weeks for SEC and 2 weeks for SEB and SEE.

The practice of boosting previously immunized rabbits produces immune sera within 2 weeks compared to 11 for the initial immunization, and by selecting only the most responsive rabbits, results in a saving of toxin and work while yielding over 100 ml of good quality serum per animal. For the different enterotoxins the following challenge doses have proven effective: for SEA, SED, and SEE, 50, 100, and 350 μg given over an 8-d period; for SEB, SEC and SEE, 100 μg followed a week later by 500 μg.

Whereas individual antisera to the same immunogen differ in terms of class, specificities and affinities, the composite reaction of the immunoglobulin population of different sera can be measured with the single gel diffusion tube assay and the sera equated on that basis to give the same response. For the two main uses of antisera to the staphylococcal enterotoxins, quantitation of enterotoxin by a single gel diffusion tube assay and detection of enterotoxin by double gel diffusion methods, the antibody titers described here allow the substitution of one serum for another. However, single gel diffusion tube titers do not necessarily equate sera for use in the radioimmunoassay or the enzyme linked immunosorbent assay (ELISA).

We recommend the method presented here for the following reasons: high volume and quality of sera produced, moderate time span, moderate number of injections, need for little special attention to the animals and much less toxin required than by classical methods. Additionally, the method has been tested successfully with all of the known enterotoxins. Acceptable titers have been achieved in most of the rabbits used. Antisera produced in repeated series of boostings and bleedings have been very satisfactory as reagents in immunodiffusion methods. Also, they have been used successfully in ELISA, RIA and blotting techniques.

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REFERENCES