Stability of $^{125}$I-Labeled Staphylococcal Enterotoxins in Solid-Phase Radioimmunoassay

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Staphylococcal enterotoxins, Types A, B, and C, were labeled with $^{125}$I by the chloramine-T method at approximately two levels of specific activity, 40 and 4 $\mu$Ci/µg of protein. Toxins labeled with high specific activity showed extensive dissociation of $^{125}$I when stored at different temperatures, including -23 C. In contrast, toxins labeled with low specific activity did not show any significant loss of $^{125}$I when stored at -23 C for as long as 2 months. Enterotoxins, whether labeled with high or low activities, formed aggregates immediately upon labeling. Aggregate formation increased in high-activity-labeled toxins on storage at -23 C, and low-activity-labeled toxins showed no significant increase in aggregate formation, even after 2 months at -23 C. The aggregated forms of the enterotoxins were either devoid of antigenic activity in solid-phase radioimmunoassay or they possessed significantly reduced antigenic activity. Thus, a decrease in binding of $^{125}$I-labeled enterotoxin to specific antibody in solid-phase radioimmunoassay results mainly from (i) loss of $^{125}$I on storage, and (ii) formation of aggregates with reduced antigenic activity.

Our adaptation of the solid-phase radioimmunoassay procedure to staphylococcal enterotoxins in food (8) has provided a simple, sensitive, and specific method for their determination. The $^{125}$I-labeled enterotoxins used in this procedure are, however, subject to decomposition as the result of the natural isotopic decay of $^{125}$I. Alteration of the enterotoxin during iodination and storage could lower the precision and sensitivity of the radioimmunoassay procedure and could change the specificity of the test because of the unpredictable behavior of the decomposition products.

This paper describes the labeling of staphylococcal enterotoxins A (SEA), B (SEB), and C (SEC) at two different levels of specific activity by the chloramine-T method. The release of $^{125}$I and the possible alterations of the enterotoxin molecules at different storage temperatures were studied as a function of time to obtain a more stable reagent for enterotoxin solid-phase radioimmunoassay studies.

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MATERIALS AND METHODS

Purified enterotoxins. Purified SEA, SEB, and SEC were supplied by M. S. Bergdoll, University of Wisconsin, Madison. The toxins contained less than 5% impurities (1). The toxins were dissolved in 0.15 M phosphate-buffered saline (PBS) (0.07 M phosphate, 0.07 M NaCl), pH 7.2, at a concentration of 1 mg/ml and stored frozen until the time of labeling.

Radioactivity. Carrier-free Na $^{125}$I, free from preservatives and reducing agents, was obtained in 0.1 M sodium hydroxide from New England Nuclear Corp., Boston, Mass., at a concentration of 250 mCi/ml. The activity was used as received, or a 1:10 dilution in 0.1 M sodium hydroxide was prepared.

Gels. Sephadex G-25 and -75 dextran gels were supplied by Pharmacia Fine Chemicals, Inc., Piscataway, N.J. The gels were prepared for use according to the manufacturer’s instructions.

Iodination of enterotoxins. SEA, SEB, and SEC were labeled by the reaction of 50 µg of toxin with 2.5 or 0.25 mCi of $^{125}$I and treated with 5 µg of chloramine-T for 2 or 10 min. Several minor changes have been made in the previously described procedure (9). (i) All reagents were prepared in PBS. (ii) Hydrochloric acid, 0.1 M, was substituted for phosphoric acid. (iii) Phosphate buffer volume was increased from 130 µl to 150 µl. $^{125}$I-labeled enterotoxin was separated from unbound $^{125}$I by gel filtration through 5 g of Sephadex G-25. The labeled enterotoxin was diluted to 10 ml and contained a final concentration of approximately 5 µg/ml in a solution containing 1% bovine serum albumin and 0.1% sodium azide in PBS. Further dilutions of labeled toxin were prepared in the same buffer.

Measurement of protein-bound activity. The percentage of enterotoxin-bound $^{125}$I was estimated by trichloroacetic acid precipitation (7).
Column chromatography. Aliquots (1 to 3 ml) of the stored, labeled enterotoxins were analyzed for aggregate formation by fractionation on a Sephadex G-25 column (1.1 by 24 cm) equilibrated with PBS containing 0.02% sodium azide to separate dissociated $^{131}$I from enterotoxin-bound $^{131}$I. The protein fraction was collected and fractionated at 5 C on a Sephadex G-75 column (2.5 by 35 cm) equilibrated with PBS containing 0.02% sodium azide. The radioactivity of the column eluates (2 ml) was counted.

Radioimmunoassay test. The preparation of enterotoxin antisera, the preparation of antibody-coated polystyrene tubes, and the technique of solid-phase radioimmunoassay have been described elsewhere (9).

Counting equipment. Radioactivity was measured with a Packard Auto-Gamma Counter (model 5320). This system has a counting efficiency of approximately 60% and a background count rate of approximately 50 counts/min.

RESULTS

Staphylococcal enterotoxins were labeled with $^{125}$I with an efficiency ranging from 81 to 93%. The molar ratio of iodide to enterotoxin ranged from 1.3 to 1.6. At least 97% of the radioactivity of the labeled proteins was precipitable by trichloroacetic acid after gel filtration (Table 1, time of labeling data).

To study the loss of $^{131}$I, SEA, SEB, and SEC were labeled with $^{125}$I at high specific activity and stored at three temperatures (room, 1 C, and -23 C). The release of $^{125}$I on storage was monitored by measuring the quantity of radioactivity precipitated by trichloroacetic acid. Representative data are presented in Table 1. All the toxins showed a loss of $^{131}$I at the three temperatures of storage with time. The rate of loss was least in toxins stored at -23 C. $^{131}$I-labeled SEA, for example, had 97% of its activity bound at the time of labeling, but only 71% was bound after 71 days at -23 C. $^{131}$I-labeled SEB and $^{131}$I-labeled SEC showed greater stability in that 91 and 87% of their respective activities were still protein bound after 71 days at -23 C. The free $^{131}$I from the trichloroacetic acid test behaved in an identical manner as $^{131}$I chromatographed on Sephadex G-25 and anion-exchange resin.

Data on the comparative stability of a preparation of SEA labeled at high (37 $\mu$Ci/ug of protein) and low (3 $\mu$Ci/ug of protein) specific activity and stored at -23 C are presented in Fig. 1. There was a 25% release of $^{131}$I from the 37-$\mu$Ci activity preparation at 60 days, but the 3-$\mu$Ci activity preparation showed no significant release even at 100 days of storage. Since the only variable in these labeled preparations was the level of specific activity, it appears that SEA labeled with low specific activity is significantly more stable than that labeled with high activity. A similar pattern was observed with SEB and SEC labeled at high and low levels of specific activity (data not shown).

Enterotoxins labeled with $^{131}$I by chloramine-T formed aggregates during labeling and storage. This result is indicated in Fig. 2, which contains chromatographic data for high-specific-activity $^{131}$I-labeled SEA (40 $\mu$Ci/ug of protein) on a Sephadex G-75 column. The data are depicted as fractions from toxin chromatographed immediately after labeling (Fig. 2A) and after storage for 60 days (Fig. 2B) at -23 C. The formation of aggregates is indicated by a peak or peaks preceding the peak of principal activity that contains $^{131}$I-labeled SEA in the monomeric form. The aggregate peak(s) in this experiment represents approximately 8% of the protein activity immediately after labeling and 16% after 60 days of storage. The amount of aggregate formed immediately after labeling is essentially the same from experiment to experiment; but after 60 days of storage at -23 C, the aggregate(s) may represent as much as 40% of the protein-bound activity (data not shown).

Furthermore, aggregate formation in toxins labeled with low specific activity (approximately

| Time | Activity bound (%) | SEA | SEB | SEC | SEA | SEB | SEC | SEA | SEB | SEC |
|------|--------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|      | Room temp          | 1 C | -23 C |      | 1 C | -23 C |      | 1 C | -23 C |      |
|      |                    |     |       |     |     |       |     |     |       |     |
| At the time of labeling | 97 | 98 | 97 | 97 | 98 | 97 | 97 | 98 | 97 |
| After 14 days | 80 | 93 | 90 | 84 | 95 | 93 | 89 | 96 | 94 |
| After 29 days | 67 | 88 | 84 | 72 | 91 | 88 | 82 | 94 | 92 |
| After 43 days | 54 | 82 | 79 | 63 | 88 | 84 | 77 | 92 | 89 |
| After 57 days | 48 | 79 | 76 | 59 | 86 | 82 | 74 | 91 | 88 |
| After 71 days | 43 | 77 | 73 | 55 | 84 | 79 | 71 | 91 | 87 |

*Specific activities of labeled enterotoxins: SEA, 38 $\mu$Ci/ug; SEB, 42 $\mu$Ci/ug; SEC, 48 $\mu$Ci/ug.
4 μCi/μg of protein), although initially similar in amount to that of toxins with high specific activity, does not increase in amount on storage for as long as 60 days at –23 C. Aggregates were not found in untreated enterotoxins, so their presence in radiolabeled enterotoxins resulted from the labeling procedure (data not shown).

Table 2 contains data on the immunoreactivity of the monomeric and aggregate fractions of high-specific-activity 125I-labeled enterotoxins. Immunoreactivity was measured by determining the percentage of uptake of labeled toxins by antibody-coated polystyrene tubes (8). The activity added to the tubes corresponded to 0.001 μg of labeled enterotoxin in all cases. The aggregate fraction of 125I-labeled SEA had little antigenic activity, since only 5% of it was bound by antibody and 57% of the monomeric fraction was bound. The aggregate fraction of 125I-labeled SEB was slightly more active than that of 125I-labeled SEA, but it was substantially lower than that of its corresponding monomeric fraction. The most active aggregate fraction was that of 125I-labeled SEC, with 31% bound by antibody. But even here the monomeric form was more active, with 73% bound by antibody-coated tubes. Thus, labeled, aggregated enterotoxins are either devoid of or have significantly reduced antigenic activity when compared to the labeled toxin in the monomeric form.

Data on the binding of 125I-labeled enterotoxin of high and low specific activity by antibody-coated polystyrene tubes after storage at –23 C are presented in Table 3. A decrease in percentage of binding of radioactivity of the high-specific-activity preparations was observed when compared to the binding of low-specific-activity preparations of the same age.

Table 2. Percentage of uptake of monomeric and aggregate forms of 125I-labeled enterotoxins by antibody-coated polystyrene tubes

| Labeled enterotoxin | Monomeric fraction | Aggregate fraction |
|---------------------|--------------------|--------------------|
| SEA                 | 57                 | 5                  |
| SEB                 | 45                 | 11                 |
| SEC                 | 73                 | 31                 |

*The enterotoxins are the same as those labeled with high specific activity in Table 1. The toxins were stored for 60 days at –23 C before separation of monomeric and aggregate forms on Sephadex G-75 columns.
This decrease is due to the greater amount of unbound $^{125}$I (Table 1) in the high-specific-activity enterotoxin preparations and to the formation of larger amounts of aggregates by these toxins when compared to low-specific-activity enterotoxin preparations. Aggregated radiolabeled enterotoxins have reduced immunoreactivity (Table 2). When compared shortly after labeling, the use of a low-specific-activity preparation did not change the sensitivity of the radioimmunoassay method as reported earlier (9).

**DISCUSSION**

The data presented here show that $^{131}$I-labeled enterotoxins undergo decomposition by a loss of $^{125}$I and aggregate formation. The extent of decomposition depends on the specific activity of the preparation, time of storage, and storage temperature. The loss of $^{125}$I by enterotoxin is not unique. Other $^{131}$I-labeled proteins have been reported to exhibit similar behavior (3, 4). Berson and Yalow have described it as "the decay catastrophe" (2), a term that refers to the situation resulting from the presence of two radioactive atoms present in one molecule of the labeled compound. One of the radioactive atoms disintegrates and disrupts the molecule. We have obtained preliminary data suggesting the presence of diiodotyrosine in our labeled preparations (unpublished data).

Since it has recently been reported that chloramine-T brings about the formation of high-molecular-weight complexes, the initial enterotoxin aggregate formation is probably due to the action of chloramine-T (10). The extent of complex formation has been shown to be related to the weight ratio of chloramine-T to protein and to the nature of the protein (10). In our procedure, the quantity of chloramine-T used represents the minimal amount required for efficient labeling (> 80%) of enterotoxin. The continued formation of aggregates by the high-specific-activity preparations during storage was probably due to radiolysis since this phenomenon was not observed in low-specific-activity preparations. All other conditions of labeling were the same except the levels of radioactivity. Other investigators (5, 6), who found that the chloramine-T method of radiolabeling enterotoxins was unsatisfactory, probably erroneously labeled the enterotoxins with high specific activity, since satisfactory labeling was obtained with specific activities approximating 4 $\mu$Ci/$\mu$g of protein by another labeling procedure (5). Also, these investigators could have used excessive amounts of chloramine-T (5, 6).

When enterotoxins were labeled at low specific activity (approximately 4 $\mu$Ci/$\mu$g of protein) and stored at $-23^\circ$C, a satisfactorily labeled antigen was available for radioimmunoassay for at least 2 months.

We believe that this information may be applied in the study of other radiolabeled protein antigens.

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