# Highly Sensitive Solid-Phase Radioimmunoassay Suitable for Determination of Low Amounts of Cholera Toxin and Cholera Toxin Antibodies

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A direct solid-phase radioimmunoassay procedure was developed for the determination of cholera toxin and cholera toxin antibody. The reported method employed anti-choleragenoid antibody attached to polystyrene tubes as a solidified binder for cholera toxin. The binding of radioiodinated cholera toxin on its solidified antibody was inhibitable by unlabeled cholera toxin and cholera toxin antibody. With the help of this method, the heat stability of cholera toxin was also studied. Radioiodinated cholera toxin was shown to be labeled in both of its subunits. The stability of the iodinated cholera toxin at the reported specific radioactivity is remarkable. It was found that the labeled cholera toxin can be used in the solid-phase radioimmunoassay even 4 months after iodination.

Radioimmunoassay methods have been used successfully for more than 15 years to measure concentrations of a variety of biological materials. Originally, these methods were used to measure hormones in cases in which unlabeled hormones competed for the antibody-binding sites with labeled ones.

Several radioimmunoassay methods were also developed for the estimation of bacterial antigens. In addition various bacterial and nonbacterial toxins were determined with the help of these methods, for example, staphylococcal enterotoxins A and B (3, 4, 7, 8, 14, 16, 17, 18), staphylococcal enterotoxin $C_2$ (20), tetanus toxin (13), toxin of *Clostridium botulinum* type A (2), and epsilon toxin of *C. perfringens* type D (1, 19).

An enzyme-linked immunosorbent assay was developed (15) for the determination of cholera toxin and cholera toxin antibody. This test is, however, comparatively insensitive, and the smallest detectable concentration of cholera toxin by this method was reported to be 90 ng/ml.

To be able to determine smaller amounts of cholera toxin, it was necessary to develop a more sensitive method. This paper describes a radioimmunoassay procedure based on the use of solidified choleragenoid antibody, an assay that, in principle, is similar to that reported for hormones (5, 6). The described method allows the determination of cholera toxin in a low nanogram-per-milliliter range.

## MATERIALS AND METHODS

**Radioiodination of cholera toxin.** Two different purified cholera toxin preparations, Schwarz/Mann, lot BZ-2487, and Behringwerke, batch 251074 F1, were used for radioiodination. Iodination was done by the chloramine-T method, by the method of Greenwood et al. (12). Here, the details of iodination of cholera toxin from Behringwerke are given. $Na^{125}I$ was purchased from the Radiochemical Centre Ltd., Amersham, England. For iodination, 15 µg of cholera toxin dissolved in 15 µl of complete phosphate-buffered saline, pH 7.2, was used. A 25-µl amount of iodination buffer (0.5 M sodium phosphate buffer, pH 7.5) was added. Then, 10.5 µl of $Na^{125}I$ and 50 µl of chloramine-T (100 µg) were added. The iodination was performed at room temperature (~22°C) for a period of 30 s and terminated by the addition of sodium metabisulfite. After the addition of carrier KI and human serum albumin, the mixture was chromatographed on a Sephadex G-25 column.

**Preparation of solid-phase bound anti-choleragenoid.** Two different monospecific cholera toxin antiserum samples were used. One antiserum sample, horse serum anti-choleragenoid, was obtained from the National Institutes of Health. The designation of this sample was "Finkelstein antitoxin", lot 1970, and was prepared for the National Institutes of Health by Finkelstein (9). The other antiserum sample, sheep anticholera toxin no. 5911, was obtained from the Swiss Serum Institute. Both antisera were monospecific and were prepared against purified cholera toxin. Polystryene tubes were coated in one case with whole sheep immune serum, whereas in the other case immunoglobulin fraction (obtained by sodium sulfate precipitation) from horse serum was used. The polystyrene tubes (11 by 70 mm) were purchased from Greiner and Söhne.

Polystyrene tubes were left to coat overnight (for about 15 h) at 4°C and then for an additional 3 h at room temperature (22°C). After the termination of the coating procedure, the tubes were washed thoroughly twice with 3-ml portions of 0.90% (wt/vol) NaCl con-

taining 0.50% (wt/vol) Tween 20 and then three times with incubation buffer that consisted of 0.05 M sodium phosphate buffer (pH 7.4) containing 0.90% (wt/vol) NaCl, 0.30% (wt/vol) human serum albumin, 0.05% (wt/vol) NaN$_3$, and 0.50% (wt/vol) Tween 20.

**Solid-phase radioimmunoassay procedure.** A 500-μl amount of cholera toxin-containing samples (at the indicated concentrations), as well as 500 μl of ^{125}I-cholera toxin (~2 ng/ml), were added to antibody-coated tubes. The tubes were left to incubate at 4°C for about 18 h. After the termination of the incubation, the liquid was removed by aspiration, and the tubes were washed three times with 3-ml portions of 0.90% NaCl solution containing 0.50% Tween 20. After the last wash, the tubes were dried by suction and placed in a gamma counter.

**SDS-polyacrylamide electrophoresis of radioiodinated and non-radioiodinated cholera enterotoxin.** Sodium dodecyl sulfate (SDS) electrophoreses of ^{125}I-cholera toxin and unlabeled cholera toxin were performed in thin layer of polyacrylamide gel. The total concentration of separation gel amounted to 10.32%; acrylamide concentration was 10.00%, and the degree of cross-linkage was 3.00%. Separation gel buffer was 0.38 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 8.9. The total concentration of spacer gel amounted to 3.13%; acrylamide concentration was 2.50%, and the degree of cross-linkage was 20.00%. Spacer gel buffer was 0.06 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 6.9. The following polymerization catalysts were used: 657 μM ammonium peroxydisulfate (0.015%) and 1.33 μM riboflavin (0.00005%). The concentration of

N,N,N',N'-tetramethylethylenediamine was 13.37 mM (0.20%). The polymerization was done for 30 min. SDS concentration in the separation and spacer gels and in the electrode buffer amounted to 0.10%. A concentration of 0.025 tris(hydroxymethyl)aminomethane (pH 8.4) served as an electrode buffer. Before SDS-polyacrylamide electrophoresis, the cholera toxin samples (in spacer buffer containing 0.6% mercaptoethanol and 0.3% SDS) were incubated in a boiling-water bath for 3 min and then immediately cooled in an ice bath to room temperature.

## RESULTS AND DISCUSSION

After iodination and chromatography on a Sephadex G-25 column, radioiodinated cholera toxin was subjected to SDS electrophoresis. Figure 1 shows the distribution of radioactivity essentially into two peaks, which corresponds with the position of protein-staining bands (detected with Coomassie brilliant blue) shown in Fig. 2. By extrapolation from the migration rates of marker proteins, the apparent molecular weights of both cholera toxin subunits were estimated. The molecular weights of the B subunits were found to be in the range from 10,000 to 18,000. These values are slightly higher than those reported in the literature (10). The molecular weight of the A subunit was estimated to be about 22,000, which corresponds well with published data. Radioiodinated cholera toxin was found to be very stable and was usable in
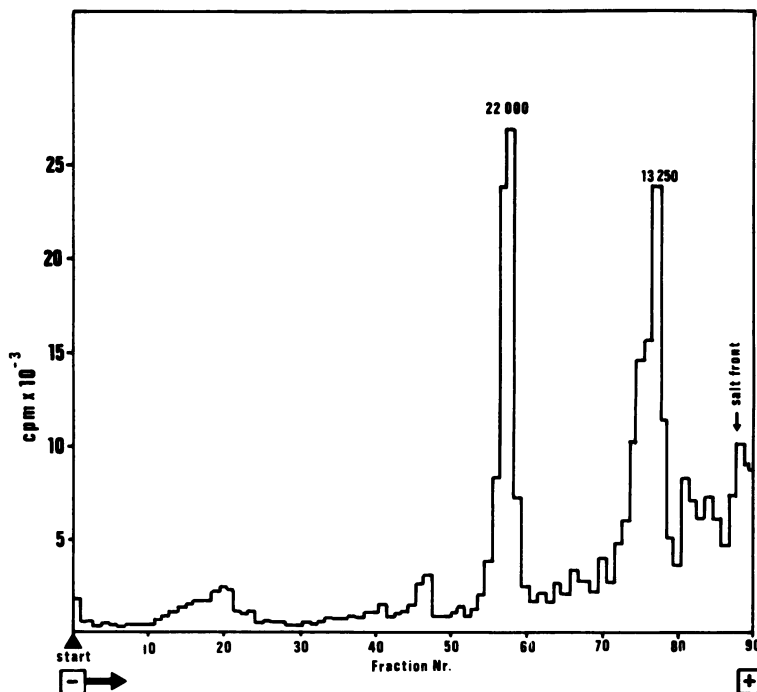


FIG. 1. *SDS-polyacrylamide gel electrophoresis of radioiodinated cholera toxin. The apparent molecular weights of the two radioactive peaks were evaluated by extrapolation from migration of marker proteins.*
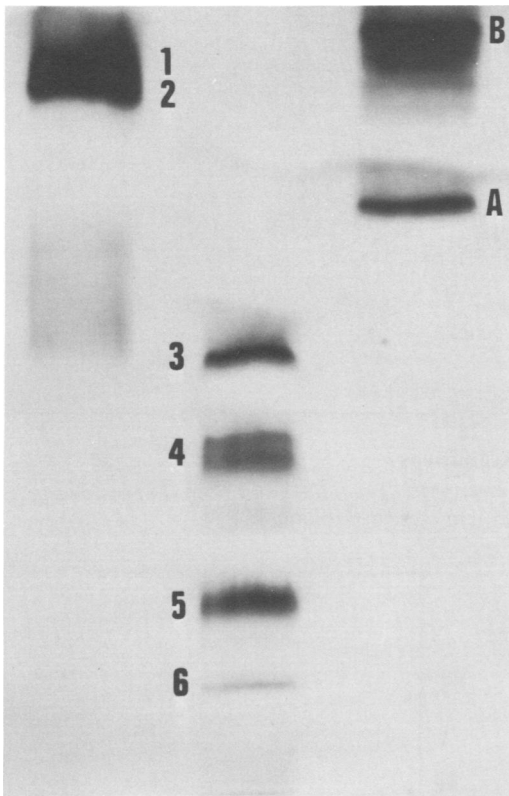
FIG. 2. *SDS-polyacrylamide gel electrophoresis of non-radioiodinated cholera toxin. Coomassie brilliant blue dye was used to stain proteins. A, Subunit A of cholera toxin; B, subunit B of cholera toxin. 1 through 6 refer to the following molecular weight markers: 1, cytochrome C (molecular weight, 12,500); 2, myoglobin (molecular weight, 17,200); 3, carbonic anhydrase (molecular weight, 29,000); 4, pepsin (molecular weight, 35,000); 5, bovine albumin (molecular weight, 67,000); 6, phosphorylase A (molecular weight, 94,000).*

our radioimmunoassay system even after 4 months of iodination.

The titer selection of cholera toxin antibodies (horse serum anti-choleragenoid) used for coating was made from the results of the experiments summarized in Fig. 3. In all subsequent work unless otherwise specified, anti-cholera toxin antibodies in dilution of 1:30,000 were used for coating the polystyrene tubes.

With the help of the two materials mentioned (radioiodinated cholera toxin and its solidified antibody), a solid-phase radioimmunoassay method was developed for the determination of cholera toxin. Figure 4 shows a standard curve, that is, the inhibition of $^{125}$I-cholera toxin uptake to its antibody (equine) by an increasing amount of unlabeled cholera toxin. As can be seen, the sensitivity of this method reaches to low nano-

gram-per-milliliter concentration ranges. A similar standard radioimmunoassay curve was obtained when anti-cholera toxin antibody raised in another animal species (sheep) was employed.

In carefully conducted experiments (11), it was observed that by a brief heating (2 min at 60°C and higher) of cholera toxin, a high-molecular-weight polymer, so called "procholeragenoid" was formed. This polymer (29S to 31S) with greatly reduced toxicity was found to be immunogenic in mice and was shown to be immunoreactive in radial diffusion assay. Its immunoreactivity was shown, however, to be lower in comparison with that of choleragen and choleragenoid. The lower immunoreactivity obtained by the radial diffusion assay was probably due to lower diffusion rate of procholeragenoid in the agar gel and not to heat inactivation. By the radial diffusion method it was, therefore, not possible to ascertain the role of heat treatment on a possible reduction of immunoreactivity of cholera toxin.

It was of interest to see whether the influence of heat on cholera toxin immunoreactivity could be more advantageously monitored by the use of a solid-phase radioimmunoassay—a method independent on the diffusion properties of molecules. Cholera toxin was incubated for 30 min at the following three temperatures: 40, 60, and 80°C. The heat-treated (as well as the untreated) cholera toxin was then used to inhibit the uptake of radioiodinated cholera toxin to its antibody. Figure 5 compares the extent of inhibition of unheated with heated cholera toxin. It can be seen that temperatures up to 60°C did not cause any significant change in immunoreactivity of the heated toxin. Heat treatment of cholera toxin at 80°C caused almost complete destruction of the immunoreactivity of this toxin. On the basis of the influence of temperature on the biological and immunoreactive properties of cholera toxin, it seems that different molecular sites are responsible for these activities.

This solid-phase radioimmunoassay could also be used for the measurement of cholera toxin antibodies. Figure 6 shows the inhibition of $^{125}$I-cholera toxin uptake to solidified anti-cholera toxin antibody by a serially diluted anti-cholera toxin antibodies at two different hydrogen ion concentrations. The pH of the reaction mixture does not seem to exert any influence, as the slopes of the inhibition curves are essentially identical.

Immune sera as well as colostrum from pigs immunized with purified cholera toxin were also checked by this radioimmunoassay procedure. It was found that the content of cholera toxin antibodies in the above-mentioned materials could readily be estimated by this technique
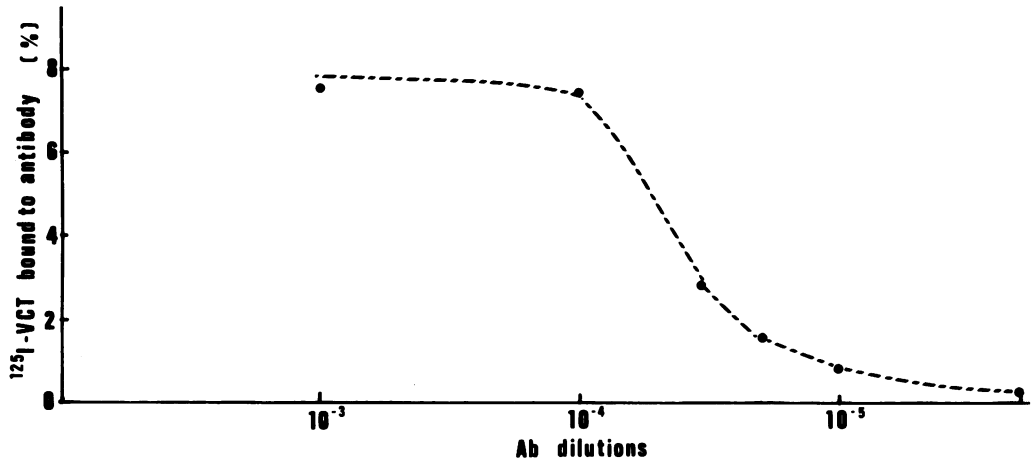
FIG. 3. *The uptake of radioiodinated cholera toxin* ($^{125}I$-*VCT) on solidified antibody. Polystyrene tubes were coated with anti-choleragenoid (Finkelstein antitoxin) at indicated dilutions.*
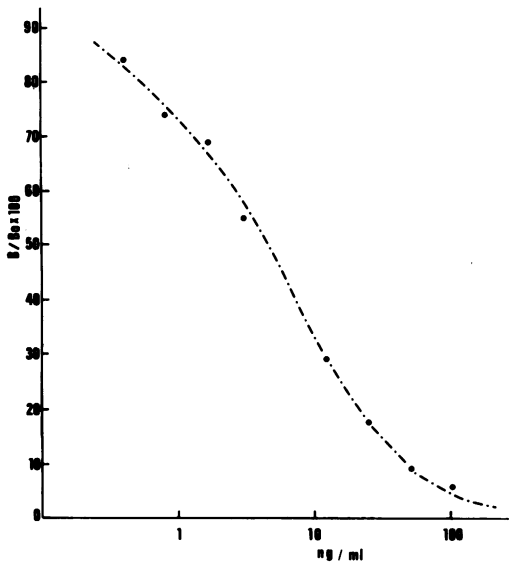


FIG. 4. *Solid-phase radioimmunoassay of cholera toxin—a typical standard curve. Finkelstein antitoxin at dilution of 1:30,000 was used.*

(50% inhibition was obtained with about 1:10,000 diluted serum and colostrum samples). It is anticipated, therefore, that this technique could well be used for the estimation of cholera toxin antibodies in various human fluids, such as in human serum and duodenal washings.

The studies concerning cross-reactivities between various toxin subunits by this solid-phase radioimmunoassay method are in progress, and the results are planned to be published in the near future.
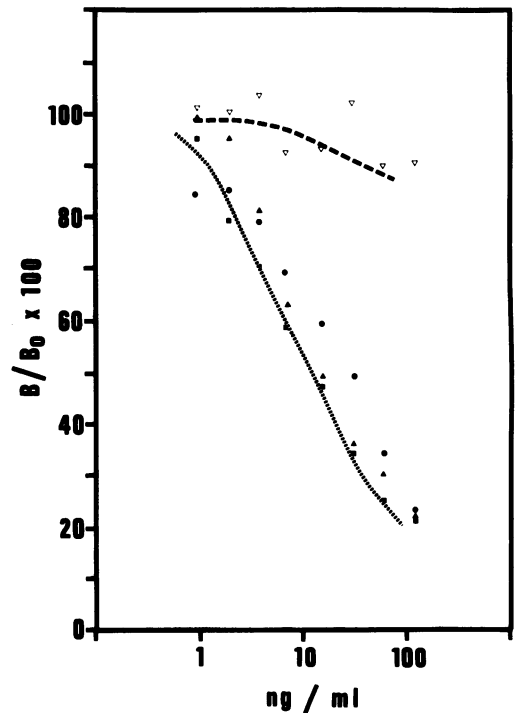


FIG. 5. *Immunoreactivity of heat-treated (and untreated) cholera toxin at: 4°C (●), 40°C (▲), 60°C (■), and 80°C (∇). For coating, anti-cholera toxin (Swiss Serum Institute) at dilution of 1:100,000 was used.*

It is hoped that this new and simple radioimmunoassay technique for the detection of cholera toxin and cholera toxin antibody will find a practical application, especially when low con-
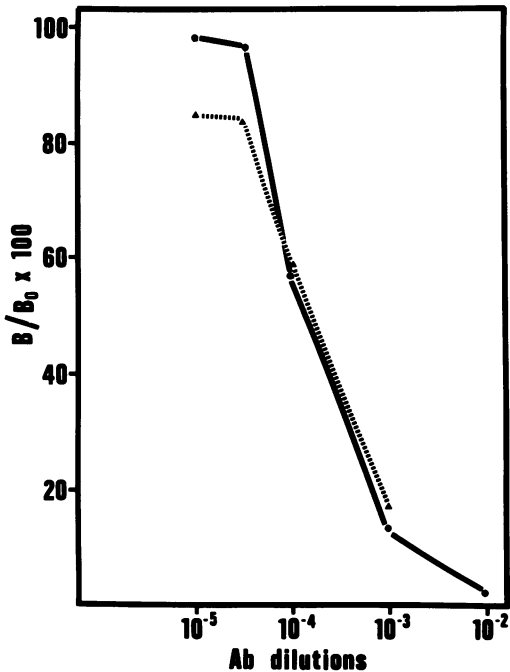
FIG. 6. *Competition between solidified and soluble cholera toxin antibody (anti-cholera toxin, Swiss Serum Institute) for cholera toxin. Symbols: pH 7.4 (●), pH 5.5 (▲).*

centrational levels of these substances have to be measured.

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