Assay of Staphylococcal Enterotoxin B by Latex Agglutination (33364)

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Gel-diffusion techniques have become well-established techniques for the quantitative assay of staphylococcal enterotoxins (1). While efforts were made to improve their speed and sensitivity (2), which remain limiting factors, it was not until recently that alternative serodiagnostic methods were reported from this laboratory (3, 4) and by other workers (5, 6) based on use of erythrocytes sensitized with staphylococcal enterotoxin B (SEB). However, these hemagglutination–inhibition reactions did not provide marked advantage in terms of sensitivity, and presented new problems in regard to the preparation, standardization and stability of reagents.

Singer and co-workers have shown that dispersions of polystyrene latex particles will adsorb serum macromolecules and, thereupon, be rendered suitable for various agglutination tests. First used for the detection of rheumatoid factors (7), the wide applicability of latex-fixation tests has since been amply demonstrated. Thus, the present report describes a procedure for the quantitative detection of SEB by means of latex particles coated with specific antitoxin. This approach was found to provide satisfactory sensitivity, simplicity, and rapidity.

Materials and Methods. Polystyrene latex particles (0.17 or 0.22μ, Dow Chemical Co., Midland, Michigan) were diluted in borate–saline buffer (BSB) of ionic strength 0.04 and pH 8.2 (3.1 g of H_3BO_3, 2.0 g of NaCl and 6.25 ml of 1 *N* NaOH in 1 liter), to give a suspension of 0.1% latex solids. Specific antiserum to SEB was added to 100-ml aliquots of the suspension, giving final antiserum concentrations of 1:700, 1:800, 1:900, 1:1000, 1:1100, and 1:1200. After incubation at 56° for 1.5 hr with gentle, brief swirling every 15 min and storage at 4° for 12 hr, mixtures were evaluated with dilutions of toxin ranging from 1×10^{-4} to 1×10^{-1} μg/ml by the technique described below. Additional suspension was then prepared with the concentration of antiserum optimal for sensitivity. Latex particles sensitized in this way will permit determination of 2 to 4 $\times 10^{-4}$ μg of SEB/ml, and may be stored at 4° for up to 3 months without change.

Antiserum was prepared in rabbits with pure (99+%) SEB (obtained along with specimens of lower purity toxin through the courtesy of Dr. E. Schantz, Fort Detrick, Md.) by the method of Silverman (8), or by a slightly modified procedure using Freund's complete (*Mycobacterium butyricum*) or incomplete adjuvant. Acceptable sensitivity was attained with all antisera prepared in this laboratory usually near 1:1000 dilution. The concentrations of antiserum in trial suspensions may have to be changed if this sensitivity cannot be achieved within the cited range of dilutions.

Microassay equipment consisted of V-bottom plates, sealers, 50- and 25-μl dropping pipettes, 50-μl diluting loops, microassay "reader," (all from Cooke Engineering Co., Alexandria, Va.), and 100-μl microsampling pipettes (Corning). An automated microassay device (Astec, Inc., Orange, Conn.) was also used. Equipment contacting SEB should be disposable where possible, and diluting loops must be carefully rinsed in running water to preclude carry-over from samples containing relatively high concentrations of toxin. Standard solutions were prepared with pure or 42% SEB.

The BSB containing 0.07% bovine serum albumin (BSA) (fraction V powder, Nutritional Biochemicals Corp., Cleveland, Ohio) was added in 50-μl drops to wells 2 through 12. Samples were delivered into wells 1 in aliquots of 100 μl. Twofold dilutions were made simultaneously in all 8 rows of the plate by carrying 50 μl serially from wells 1 through 12. Finally, 25 μl of sensitized latex

was added to all wells and mixed by gentle tapping of the sides of plates. Plates were sealed and left overnight at about 23° in a vibration-free area. Serial dilution through 2 plates or, preferably, to avoid cumulative errors of dilution, an initial single-step large dilution was used when SEB occurred in concentrations greater than 0.2 μg/ml. This ensured that at least 1 "negative" comparison well without visually discernible agglutination was found in each row.

The end point of the titration was taken to be the last well in the series still showing a detectable difference from the first negative or from blank control wells. In negative or blank wells, "buttons" of settled latex particles were well defined, whereas they were smaller and surrounded by a halo of agglutinated particles at the end points. Adequate determination of end points required good, even illumination from directly above and visual acuity of observers. The amount of toxin in test samples was calculated with the equation: SEB/ml of original solution = detection limit $\times$ 2^{n-1}, where the detection limit is the lowest quantity of SEB that can be determined under the particular conditions, and n is the number of the well in which the end point of a given sample was found. Analogous graphical methods were also employed.

Results and Discussion. Systematic investigation of reaction variables resulted in the development of a technique for the assay of SEB that is both simple and highly sensitive. In agreement with an earlier report (8), latex particles of about 0.2-μ diameter afforded an advantage over larger particles. BSB–BSA, pH 8.2, μ 0.04, was selected because others gave prozones, diminished sensitivity, or spontaneous agglutination, and did not give complete recovery of SEB. The success of the procedure also hinged critically on careful adjustment of concentration of antiserum for the sensitization of latex, and use of increments smaller than conventionally employed during the determination of optimal levels of antiserum. Removal of excess antiserum by centrifugation (9) yielded preparations of latex less sensitive than those prepared as described.

TABLE I. Effect of Bovine Serum Albumin.

BSA (%)	SEB[a] (μg/ml)	End point[b] (well no.)	Conc of SEB at end point[c] (μg/ml)
0	.04	—	No observable
	.004	—	end point
	.002	—	do.
.01	.04	8	.00032[d]
	.004	4	.0005 [d]
	.002	3	.0005 [d]
.04	.04	9	.00016
	.004	4	.0005
	.002	3	.0005
.07	.04	9	.00016
	.004	6	.00013
	.002	5	.00013
.10	.04	9	.00016
	.004	6	.00013
	.002	5	.00013
.20	.04	9	.00016
	.004	6	.00013
	.002	5	.00013
.40	.04	9	.00016
	.004	6	.00013
	.002	4	.00025
.80	.04	8	.00032
	.004	6	.00013
	.002	4	.00025

[a] Concentrations of samples before dilution with buffer and latex suspension.

[b] Based on duplicate assays.

[c] Concentrations at the end point before dilution, given by $C_n = C_0/2^{n-1}$, where n is the well number at the end point.

[d] End point difficult to detect; buttons very small.

With earlier preparations of sensitized latex particles, it was found that BSA increased the sensitivity of the assay, but that spontaneous agglutination was induced at about 0.4% or more. Subsequently, it developed that optimally sensitized particles gave no clearly detectable end point at all unless BSA was present in the diluent (see Table I), and that some decrease in sensitivity, but not spontaneous agglutination, was noticeable at and above 0.4%. The presence of BSA during sensitization, however, must be avoided since it will preclude coating of

the latex particles with antitoxin and yield a useless preparation.

Recovery of low concentrations of SEB in BSB, e.g., 0.1 μg/ml or less, was quite unsatisfactory when samples were held for as short a period as 1 day at 4 or −27°. By comparison, 0.4 μg of SEB/ml in BSB with 0.07% BSA was recoverable without loss within the precision of the method for 24 (but not 65) days; 0.04 μg/ml for 15 (but not 20) days; 0.004, 0.0004, and 0.0002 μg/ml for 5 (but not 12) days. There was no demonstrable benefit from keeping these aseptically-prepared samples at −27 vs 4°. However, microbial contamination of samples would appear to make freezing a prerequisite should storage before assay be unavoidable.

It should be emphasized that the end point is not based on an all-or-none phenomenon, but that the technique derives its sensitivity to a major extent from utilization of conditions conducive to the detection of small differences in agglutination. The end point was taken at that dilution of SEB which gave the last detectable partial agglutination in the dilution series. One reason for adoption of the microassay procedure, aside from considerations of economy and speed, was that such end points were more easily discernible in wells of microassay plates than in tubes.

In one of several tests of the realiability, 10 samples each of 5 concentrations, 0.04, 0.03, 0.02, 0.01, and 0.004 μg of SEB/ml, were read by 6 individuals in random sequence; each sample was read three times during the course of the experiment for a total of 30 end point determinations per reader. Samples containing 0.02 μg/ml were arbitrarily taken as standards (other concentrations as standards gave virtually identical results).

In practice, estimates of C_0, the concentration of undiluted samples, are based on duplicate assays. Thus, any pair of the 30 observations made by an individual reader for any given value of C_0 may be considered valid duplicate assays upon which to base an estimate of C_0. Each of the sets of 30 observations generates a set of 435 *pairs* of observations which are completely independent of the true concentration. If an acceptable estimate is defined to be one that falls within ± 1 well of C_0, Table II shows the .90 confidence lower bound for the probability of correct estimates. Since routine laboratory technicians and procedures were employed, i.e., an effort was made to assess the method under realistic conditions, it appears that the reliability is satisfactory. Differences in individual performances did occur, indicating that some readers, e.g., R_3 and R_6 were more proficient than others. Table II is based upon data that include experimental errors in addition to those of end point estimates. This means that failures in producing acceptable estimates were not necessarily solely attributable to faulty judgement of readers.

TABLE II. The .90 Confidence Lower Bound Estimates for the Probability that an Estimate Will Fall in Acceptable Range—by Reader by Concentration.

Conc	Acceptable range	R_1	R_2	R_3	R_4	R_5	R_6
.010	[.005, .020]	.879	.829	.994	.994	.994	.994
.025	[.0125, .0500]	.991	.994	.994	.788	.841	.991
.075	[.0375, .1500]	.938	.979	.976	.933	.994	.994
.100	[.050, .200]	.958	.976	.991	.938	.921	.994
	Av reliability	.942	.945	.989	.928	.938	.993

Figure 1, data for which were obtained by using a degree of care not possible for routine tests, indicates that instrumented reading of end points could provide greater sensitivity than the visual method, although in practice it was evident that experimental errors ultimately became limiting. This implies that detection of end points by instruments can be completely successful only if the entire process is automated to reduce these errors. Visual estimations of end points cannot be expected to extract the maximum of information because, beyond the visual end point, differences between "buttons" are too small for discrimination by the unaided eye. The extrapolated curve becomes asymptotic in the region below 7×10^{-6} μg/ml. Down to that level, and under proper conditions, instruments with better resolving power than the eye should give better sensitivity.

During the course of this study, a number of preparations of antiserum, toxin (from 10

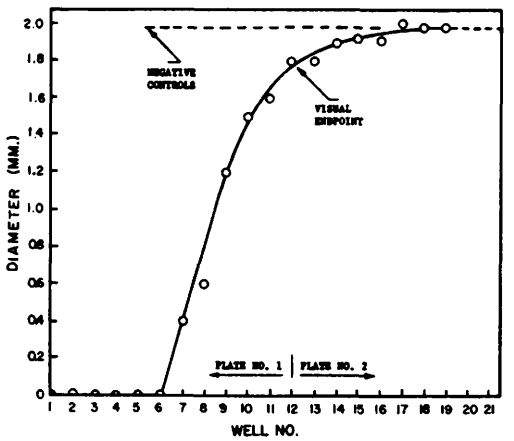

FIG. 1. Size of "buttons" as a function of concentration of toxin. A solution containing 0.42 μg of SEB/ml was carried through two assay plates in twofold serial dilution. At the *visual* end point, well no. 12, the solution has been diluted 1:2048, giving a "button" corresponding to that obtained from 2×10^{-4} μg of SEB/ml (or 1.54×10^{-5} μg of SEB actually in the well). Diameters were measured with the aid of a stage micrometer.

to 99+% pure) and latex particles were tested without adverse indications. The method evidently is not dependent upon unique, coincidental properties of any of the reagents.

In the well representing the end point of a titration with sensitivity of 2×10^{-4} μg/ml, there are approximately 0.027 molecules of SEB/latex particle. This low ratio indicates that reaction conditions described in this report are essentially optimal.

Summary. Staphylococcal enterotoxin B (SEB) may be assayed by a simple and rapid latex-fixation test, with sensitivity improved by several orders of magnitude over conventional methods. Latex particles, coated with specific antitoxin under carefully controlled conditions, were exquisitely sensitive indicators of toxin. The minimal detection limit, 2×10^{-4} μg of SEB/ml of sample, equivalent to 1×10^{-5} μg/well in the assay plate, can apparently be extended to an even lower value with instrumented reading of end points. Titrations require only 100 μl of sample.

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The Free Amino Acids of Brain and Liver during Fetal Life of *Macaca mulatta** (33365)

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The relative concentrations of all free amino acids which can be part of protein molecules of animal tissue probably influence the rate of protein synthesis at the cellular level. The other ninhydrin positive compounds in the cell detected during free amino acid analysis should indicate the metabolic activity of these low-molecular weight compounds.

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