Enzyme-Linked Immunosorbent Assays for *Staphylococcus aureus* Exfoliative Toxins A and B and Some Applications

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Two enzyme-linked immunosorbent assays were developed for detection of staphylococcal exfoliative toxins A and B (ETA and ETB) with a double-antibody sandwich protocol. Antibodies against both toxins were purified by affinity chromatography from sheep antisera raised against purified ETA and ETB. These affinity-purified antibodies were free of detectable amounts of antibodies to other staphylococcal antigens and neutralized the actions of ETA and ETB. Alkaline phosphatase was conjugated to these antibodies. The enzyme-linked immunosorbent assay, which could detect at least 3 ng of ETA and ETB per ml, was used to quantitate the toxins in the culture supernatant fluids of staphylococcal strains. Thus, the kinetics of ETA and ETB synthesis and of ETA and ETB release into the supernatant fluids were determined; other determinations included the roles of carbon dioxide concentration, pH, glucose concentration, temperature, and agitation on the production of ETA and ETB.

The staphylococcal exfoliative toxins (ETs) A and B (ETA and ETB) have been implicated as the causative agents of the staphylococcal scalded-skin syndrome in humans (13, 14, 17), particularly in day nurseries and infant care units (1, 20). These toxins can be identified by an in vivo assay, the newborn mice test (13), which cannot be performed in hospital routine laboratories and which requires numerous animals to evaluate the amount of toxin present in a biological fluid or in a staphylococcal cultures supernatant; a second identification procedure is based on immunological methods and can be performed in any laboratory for the qualitative detection of ETA and ETB. Gel immunoprecipitation, which belongs to this second procedure, is the technique most frequently described (2, 15, 21); although this method can be used for the quantitation of toxin (e.g., the Mancini test), it requires a large amount of antibodies and is not very sensitive (21). Radioimmunological assays, which are very sensitive, also have been developed and adapted to the quantitation of the toxin (21); unfortunately, they also require a specialized material not available in most laboratories. The present report describes two enzyme-linked immunosorbent assays (ELISAs) specific for ETA and ETB which are many times more sensitive than the reference assay: the newborn mice test. Moreover these assays permitted us to evaluate the role of several physicochemical factors in the production of ETA and ETB by staphylococcal strains.

MATERIALS AND METHODS

Determination of protein. Protein was estimated by the method of Lowry et al. (10), with bovine serum albumin (BSA; Boehringer-Mannheim France S.A., Meylan, France) as the standard.

Preparation of ETA- and ETB-Sepharose. ET-Sepharose was prepared as described by March et al., (12) with slight modifications. Cyanogen bromide (0.4 g) (Eastman Kodak Co., Rochester, N.Y.) dissolved in 200 µl of acetonitrile was added all at once to a cooled stirring slurry containing 4 ml of Sepharose 4B (Pharmacia-France S.A., Bois-d'Arcy, France) and 8 ml of 2 M K_2CO_3, and stirring was continued for 2 min. The reaction mixture was washed first with 10 bed

volumes of cooled 1 mM HCl and then with 6 ml of 0.1 M $NaHCO_3$–0.5 M NaCl buffer (pH 8.3) (buffer A); this last wash had a maximum duration of 1.5 min, and the gel was added to 2.5 ml of ETA or ETB solutions in buffer A containing 6 mg of protein per ml. These ET preparations were purified to homogeneity by sequential ammonium sulfate precipitation, ion-exchange chromatography, and, for ETA only, chromatofocusing as previously described (16). The suspensions were gently mixed overnight at 4°C, and the uncoupled material was removed by washing the gels with 2 bed volumes of buffer A. About 50% of the ETA and ETB was bound by the activated Sepharose. The remaining active groups of the Sepharose gels were blocked by adding 1 bed volume of 1 M ethanolamine (pH 8) and mixing the gels for 4 h at room temperature. The gels, designated ETA- and ETB-Sepharose, were washed four times with alternating volumes (2 bed volumes per wash) of 0.1 M sodium acetate–0.5 M NaCl buffer (pH 4.0), buffer A, and finally with 2 bed volumes of 0.1 M glycine HCl–20 mM NaCl buffer (pH 2.8) (buffer B). The gels were stored at 4°C in buffer A containing 0.05% NaN_3 (wt/vol).

Purification of ETA antibodies. Fifteen milliliters of sheep antiserum raised against purified ETA preparations (15) were dialyzed overnight against buffer A at 4°C. The dialyzed material was applied to a column (3 by 1.3 cm) of ETA-Sepharose, and the column was washed with buffer A until there was no measurable absorbance at 280 nm. Antibodies bound to the gel were eluted by applying 2 bed volumes of buffer B to the column. One-milliliter fractions of the eluate were collected in 0.5 ml of 0.1 M glycine NaOH–20 mM NaCl buffer (pH 10) to adjust the eluted proteins to pH 8. The eluate was then dialyzed against buffer A, centrifuged, and applied to a 4-ml column of ETB-Sepharose (3 by 1.3 cm); this column was washed with buffer A. The protein-containing eluate was collected, dialyzed against 10 mM Tris-hydrochloride–150 mM NaCl buffer (pH 7.5) (buffer C), and applied to a column of protein A-Ultrogel (Industrie biologique française [IBF], Villeneuve-la-Garenne, France) (6 by 1 cm) which was washed with buffer C; the protein-containing fractions were designated ETA antibody preparation. The ETB-Sepharose was regenerated by buffer B and the protein A-Ultrogel was regenerated by 0.2 M glycine–HCl buffer (pH 2.8).

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Purification of ETB antibodies. The purification steps of ETB antibodies were similar to those of ETA antibodies, except that the first column was ETB-Sepharose-eluted with buffer B; after dialysis and centrifugation, the protein-containing eluate was applied to ETA-Sepharose which was washed with buffer A and was then applied to protein A-Ultrogel which was washed with buffer C. The protein-containing fractions were designated ETB antibody preparation.

Preparation of ETA (or ETB) antibody preparation–alkaline phosphatase conjugate. Enzyme labelings of ET antibody preparations were performed as described by Engvall and Perlman (4), with slight modifications. ETA (or ETB) antibody preparations were adjusted to 1 mg of protein per ml, and 1 ml was added to 1.5 mg of bovine intestine alkaline phosphatase (Boehringer-Mannheim) and dialyzed overnight at 4°C against phosphate-buffered saline (PBS). Glutaraldehyde was added to the mixture at a final concentration of 0.06% and left at room temperature for 6 h. After dialysis overnight against PBS, BSA and NaN_3 were added at final concentrations of 1 and 0.05%, respectively. The conjugates were stored in the dark at 4°C and were designated anti-ETA and anti-ETB conjugates.

ELISA for ETA and ETB. Sheep ET antibody preparations in 50 mM Na_2CO_3–$NaHCO_3$ (pH 9.6) (carbonate buffer) were diluted to a ratio of 1:200 in the same buffer, and 0.15 ml of the diluted antibodies was added to each well of flexible polyvinyl chloride microtiter plates (PVC-ST; Dutscher, Brumath, France). The plates were incubated for 4 h at 37°C and emptied; each well was washed three times with 0.25 ml of PBS–0.05% Tween 20 (vol/vol) (PBS-T). Then 1% BSA in carbonate buffer was added to each well (0.2 ml per well) and incubated for 1 h at 37°C. After three washes with PBS-T, the wells were filled with test samples of antigen (0.15 ml) which were diluted, if necessary, in PBS-T–0.2% BSA–0.05% NaN_3 (PBS-T-BSA–NaN_3). After overnight incubation at 4°C, the wells were washed three times with PBS-T; 0.15 ml of a 1:2,000 dilution of anti-ET conjugate in PBS-T-BSA–NaN_3 was added in each well, anti-ETA conjugate when the wells were covered with ETA antibody preparation and anti-ETB conjugate in the other case. The plates were incubated for 1 h at room temperature and washed as described above; 0.2 ml of 1 mg of disodium *p*-nitrophenyl phosphate per ml (Sigma Chemical Co., St. Louis, Mo.), in 1 M diethanolamine–0.5 mM $MgCl_2$ buffer (pH 9.8) was added to each well. The plates were incubated for 1 h at 37°C, and the absorbance at 405 nm was measured with a Kontron SLT 210 apparatus (Kontron Analytique, Trappes, France) with a reference beam at 620 nm. Controls included coating the wells with sheep normal serum in place of ET antibody preparations, adding PBS-T-BSA–NaN_3 in place of the test sample antigen, and adding non ET-producing staphylococcal culture in place of the test sample antigen.

Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets and stainings. The strains used for this set of experiments were the same as those used for ETA and ETB purifications: the ETA-producing strain 50586 was isolated in our laboratory from an outbreak of staphylococcal scalded-skin syndrome in infants; its phage pattern was 3A/3C/55/71. The ETB-producing strain TC 142 was kindly supplied by J. P. Arbuthnott (Dublin); its phage pattern was 3A/55. Both strains belonged, therefore, to phage group 2; they were coagulase positive and produced alpha toxin.

ETA- or ETB-containing staphylococcal culture supernatants were concentrated by a tangential flow ultrafiltration system (Millipore S.A., Molsheim, France) with a 10,000-molecular-weight pore-size ultrafilter and were then dialyzed against distilled water and centrifuged. Thirty-five micrograms of protein and a standard calibration kit ranging from 14,000 to 94,000 (Pharmacia) were loaded on a 1.4-mm-thick sodium dodecyl sulfate-denaturing polyacrylamide gel formed of a polyacrylamide gradient ranging from 10 to 20%. The composition of both acrylamide solutions poured into the linear gradient mixer was as described by Maizel (11), except that the 10% and 20% acrylamide solutions contained only 100 μl of ammonium peroxodisulfate (10% [wt/vol]; E. Merck AG, Darmstadt, Federal Republic of Germany) and 10 μl of *N,N,N',N'*-tetramethylethylenediamine (British Drug House [BDH], Poole, England) for each 30 ml of solution. After electrophoresis, a part of the gel was stained with Page Blue G-90 (BDH), and the remaining gel was submitted to an electrophoretic transfer of the proteins to a nitrocellulose membrane filter (0.45-μm pore size; Schleicher and Schuell, Dassel, Federal Republic of Germany), by the method described for sodium dodecyl sulfate gels in Towbin et al. (18). A part of the blot was stained with amido black (18) for control of the transfer, and the remaining nitrocellulose sheet was soaked in 3% BSA in PBS for 30 min at 37°C to saturate additional protein binding sites. After being rinsed in PBS-T, the sheet was incubated overnight with diluted anti-ETA or anti-ETB conjugate at room temperature; the dilutions were performed in PBS-T–BSA–NaN_3 solutions and were diluted to ratios of 1:1,000 and 1:20, respectively, for the anti-ETA and anti-ETB conjugates. The blots were then rinsed four times with PBS-T, and the phosphatase activity was revealed as previously described (7) with 26 mg of sodium 1-naphthylphosphate (Merck) and 38 mg of fast red salt (Sigma) in 30 ml of 0.1 M Tris-hydrochloride buffer (pH 9.3).

Neutralization assays for ETA and ETB. Samples of purified ETA or ETB containing 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 μg of protein under a volume of 20 μl were subcutaneously injected in triplicate into 1-day-old mice (SWR/J); the minimum amount of toxin necessary to cause a Nikolsky sign 6 h after injection (exfoliative dose [ED]) was determined. Neutralization titers were determined by incubating for 60 min at 37°C a 0.2-ml volume (composed of 0.1 ml of twofold serial dilutions in PBS) of either ETA or ETB antibody preparation or crude sheep anti-ETA or anti-ETB serum with 0.1 ml of toxin solution containing 1,000 EDs per ml; the injected volume (20 μl per mouse) of the incubation mixture contained 10 EDs of toxin. When no neutralization occurred with this protocol, 67, 40, 33, and 25 μl of ET solution containing 1,000 EDs per ml were mixed with undiluted ET antibody preparation to obtain a 0.2-ml mixture which was incubated as previously described. The injected volumes were 30, 50, 60, and 80 μl, respectively; 10 EDs were thus injected into the animals. All assays were made in duplicate. One antitoxic unit was the smallest quantity of ET antibody preparation or serum neutralizing the action of 1 ED.

Kinetics of ETA or ETB synthesis and of ETA or ETB release into the supernatant fluid. The strains examined for the study of kinetics were the ETA-producing strain 50586 and the ETB-producing strain TC 142. Precultures of the strains were performed in 25 ml of Trypticase-yeast (TY) medium (8) at 37°C, with agitation (150 rpm) for 2.5 h and under normal atmosphere. Production of ETA and ETB was performed in 1.5 liters of TY medium at 37°C in an atmosphere of 10% CO_2–90% air, with agitation (150 rpm) for 48 h. Twenty milliliters of culture was sampled each hour between hours 1 and 11 and each 2-h period between hours

11 and 48. Each sample was immediately centrifuged at 800 × g for 10 min; the supernatant was frozen, and the bacterial pellet was washed once with 20 ml of PBS, resuspended in 5 ml of PBS, and frozen at −80°C.

ETA and ETB concentrations were determined in the supernatant after thawing, with the ELISA procedures made in duplicate during the same time period, on two different microplates. For each plate, a standard curve was constructed. The number of CFU per milliliter in the resuspended pellet was counted on sheep blood agar after sonication in an ice bath (W-375 sonicator; Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) for 1.5 min to dissociate staphylococcal clusters.

The amounts of ET within the PBS-washed pellets were determined as follows. Pellets were washed again with 7 ml of 10 mM Tris-hydrochloride–1 M NaCl–5 mM EDTA Na_4 buffer (pH 7.5) and resuspended in 1 ml of 10 mM Tris-hydrochloride–1 mM EDTA buffer (pH 8) with 20 μg of lysostaphine (Sigma) for 30 min at 37°C with agitation. Under these conditions, more than 90% of the staphylococci was lysed as observed by phase-contrast microscopic examination. The mixture was then put in an ice bath, and 1 ml of 2% Brij 35–1% sodium deoxycholate–0.2 M EDTA Na_4 solution was added; after 10 min of agitation at 37°C, the amounts of ETA and ETB were determined in duplicate in this mixture with the ELISA protocols as previously described for the supernatants; the protein concentrations were also determined.

Role of various physicochemical factors on the production of ETA and ETB. Staphylococcal cultures (0.5 ml) containing the strains 50586 and TC 142 were performed, with agitation (100 rpm) for 18 h, within the wells of cell culture plates (Multidish; Nunclon Delta, Nunc, Denmark) in an atmosphere containing a CO_2-air mixture as previously described (15). The number of CFU per milliliter in the culture was determined as described above after culture sonication. The role of CO_2 concentration was assayed between 0 to 20% in
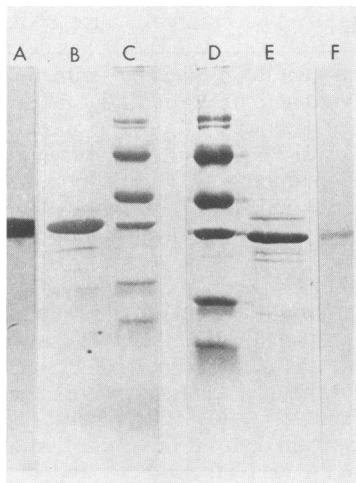

FIG. 1. Analysis of affinity-purified ETA and ETB antibody preparations. Crude ETA- and ETB-containing staphylococcal culture supernatants were submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a 10 to 20% acrylamide gradient (lanes B and E, respectively). Electrophoretic transfer of sodium dodecyl sulfate-polyacrylamide gel electrophoresis-separated crude ETA and ETB preparations to nitrocellulose sheets gave the patterns seen in lanes A and F, respectively, with the homologous anti-ET conjugates, in which only band was visible. The molecular weight calibration kit had the following values in kilodaltons (lanes C and D): 94, 67, 43, 30, 20.1, and 14.4.

TABLE 1. Neutralization titers of ETA and ETB antibody preparations and of crude sheep ETA and ETB antisera

Sample	Neutralization titer for[a]:	
	ETA	ETB
Purified toxin	1 ED = 0.1 μg	1 ED = 0.3 μg
Antibody preparations	500 ATU/ml (1 ATU = 2.3 μg of protein)	250 ATU/ml (1 ATU = 1.1 μg of protein)
Crude sheep antisera	2,000 ATU/ml (1 ATU = 46 μg of protein)	2,000 ATU/ml (1 ATU = 50 μg of protein)

[a] ATU, Antitoxic unit. See the text for an explanation of ED and ATU values determined.

TY medium and in Y medium (yeast extract [25 g/liter], NaCl [5 g/liter], $K_2HPO_4 \cdot 3H_2O$ [3.3 g/liter]). The role of the pH was assayed between pH 4.0 to 10.0 in a 15% CO_2 atmosphere and in a modified TY medium in which the salt mixture was replaced by the following buffer: citric acid (33 mM), o-phosphoric acid (33 mM), boric acid (57 mM), NaOH (343 mM), and KCl (20 mM). pH was adjusted to the desired value with HCl and was measured only at the start of cultivation. The role of glucose concentration was tested between 1 nM to 100 mM in TY medium in a 15% CO_2 atmosphere; the role of temperature was assayed between 28 and 42°C in TY medium in a 15% CO_2 atmosphere, and the role of agitation was tested at 37°C in TY medium in a 15% CO_2 atmosphere.

RESULTS

Preparation of ETA and ETB antibodies by affinity chromatography. ETA and ETB antibodies were specifically removed from sheep antisera raised against purified ETA and ETB preparations by passing the antisera through columns of ETA- and ETB-Sepharose, respectively. The bound antibodies were then eluted from the gel with 0.1 M glycine HCl–20 mM NaCl buffer. We attempted to minimize as much as possible the suppositional cross-reactions between both toxins by absorbing the eluates with the heterologous ET-Sepharose. Furthermore, to avoid nonspecific reactions between immunoglobulins and protein A contained in staphylococcal culture supernatants, sheep immunoglobulins which have a low affinity for protein A (5) were used and passed through protein A-Ultrogel. The resulting antibodies preparations were specific for the homologous ET, based on the following findings. (i) When a crude toxin preparation was submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose filter, a single protein band was stained with the homologous anti-ET conjugate. (ii) The stained band had the same molecular weight as did the purified toxin (30,000 for both toxins), as observed by comparison with the calibration kit (Fig. 1). (iii) The ET antibody preparations neutralized the homologous ET as observed with the newborn mice test: one antitoxic unit was contained in 2.3 μg of the ETA antibody preparation and in 1.1 μg of the ETB antibody preparation (Table 1).

ELISA for ETA and ETB. Double-antibody sandwich ELISA procedures were developed by coating wells of microtiter plates with sheep ET antibody preparations. Bound ET was determined by sequentially adding sheep anti-ET conjugate substrate. ETA and ETB gave a dose-dependent response in the ELISA (Fig. 2 and 3). The ELISA procedures could detect ETA and ETB amounts of as low as 1.3 ng/ml for ETA and 2.9 ng/ml for ETB with the dilutions of the ET-antibody preparations used (1:200) (Table 2). However, despite the absorption of ET antibody preparations by the heterologous ET-Sepharose, slight cross-reac-
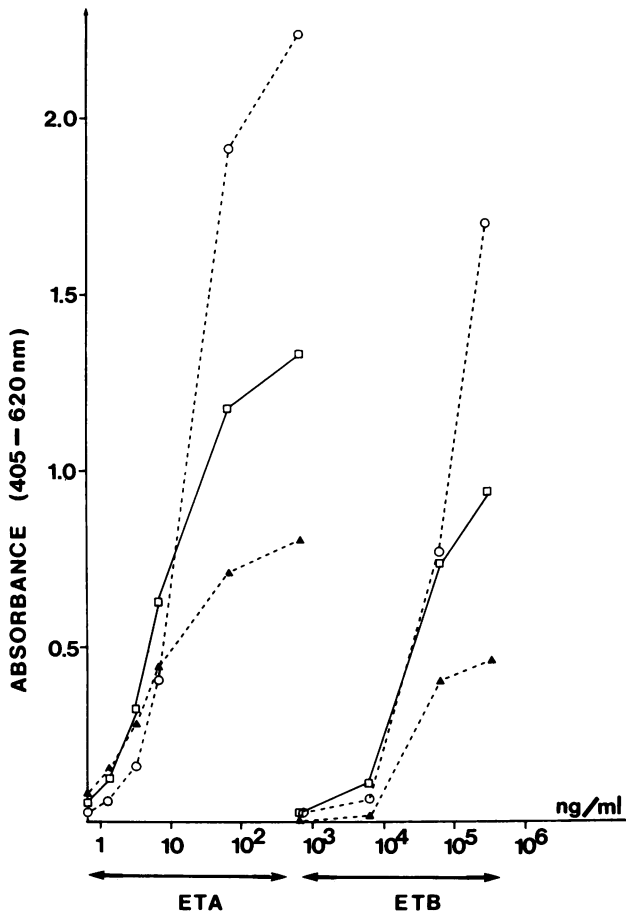
FIG. 2. Quantitation of ETA by ELISA. The wells of a microtiter plate were initially coated with dilutions of 1:50 (O), 1:200 (□), and 1:500 (▲) for the affinity-purified ETA antibody preparations. A dilution of 1:2,000 for the anti-ETA conjugate is used in all cases after the addition of ETA or ETB antigens to the coated wells.

tions remained between ETB antigen and ETA antibody preparation and between ETA antigen and ETB antibody preparation. With ETA antibody preparation, a defined optical density was obtained either with a defined ETA concentration or with a 10,000-fold-higher concentrated ETB solution (Fig. 2). With ETB antibody preparation, a defined optical density was obtained either with a defined ETB concentration or with a 100,000-fold-higher concentrated ETA solution (Fig. 3). The ELISA procedures performed with crude cultures of ET-nonproducing staphylococci did not shown any difference with the blank, even with strains producing large amounts of protein A (e.g., Cowan strain).
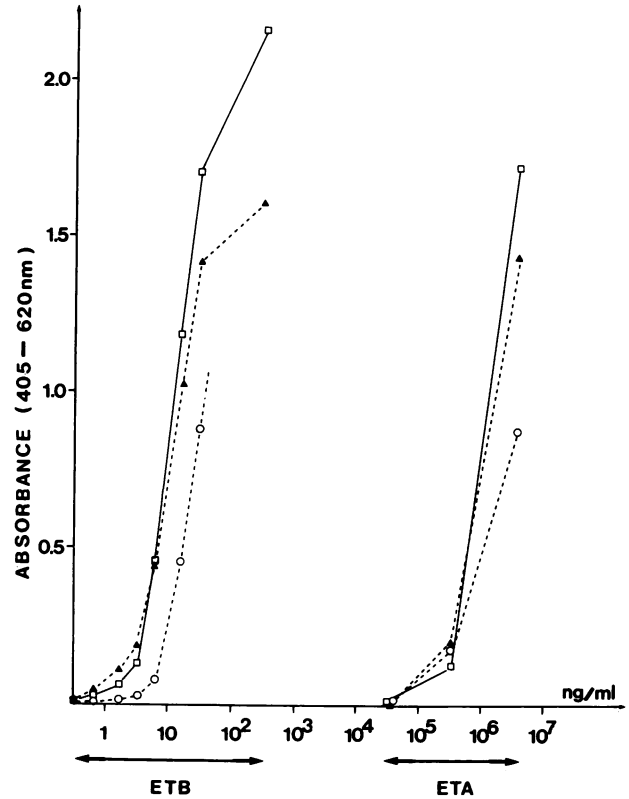

FIG. 3. Quantitation of ETB by ELISA. The wells of a microtiter plate were initially coated with dilutions of 1:50 (O), 1:200 (□), and 1:500 (▲) for the affinity-purified ETB antibody preparations. A dilution of 1:2,000 for the anti-ETB conjugate is used in all cases after the addition of ETB or ETA antigens to the coated wells.

Kinetics of ET synthesis and of its release into the supernatant fluid. The ETA and ETB contents of the growing staphylococci showed that the toxins were synthesized during the exponential growing phase. This synthesis occurred from between hours 2 and 4 to between hours 8 and 10 of the culture. During this lapse of time, the ratio of intracellular ET to intracellular protein was increased 100 times; throughout the stationary phase, this ratio remained approximately constant (5×10^{-5} for ETA and 3×10^{-3} for ETB) (Fig. 4 and 5). The ETA and ETB excretions occurred as soon as ET was detected within the bacteria; furthermore, the kinetics curves of intracellular ET per intracellular protein and of excreted ET per bacteria (obtained by the ratio of ET [nanograms per milliliter of TY medium] to the number of bacteria per milliliter of TY medium) were nearly parallel: the maximum increase of ET per bacteria in the

TABLE 2. Lowest detection limit of ET related to homologous ET antibody preparations[a]

Dilution of ETA antibody preparation	Value for ETA (ng/ml) of:					Dilution of ETB antibody preparation	Value for ETB (ng/ml) of:				
	0	0.7	1.3	3.3	6.7		0	0.6	1.4	2.9	5.8	14
1:50	0.07	0.09	0.12	**0.22**	**0.47**	1:50	0.06	0.07	0.07	0.08	0.14	**0.51**
1:200	0.06	0.11	**0.18**	**0.38**	**0.68**	1:200	0.06	0.09	0.12	**0.19**	**0.52**	**1.24**
1:500	0.07	0.13	**0.22**	**0.35**	**0.50**	1:500	0.07	0.11	0.18	**0.26**	**0.49**	**1.10**

[a] The data represented are the differences calculated from the values of optical densities at 405 and 620 nm, with air as the blank. The values with a signal-to-noise ratio of ≥3 are considered as significantly positive and are boldfaced.
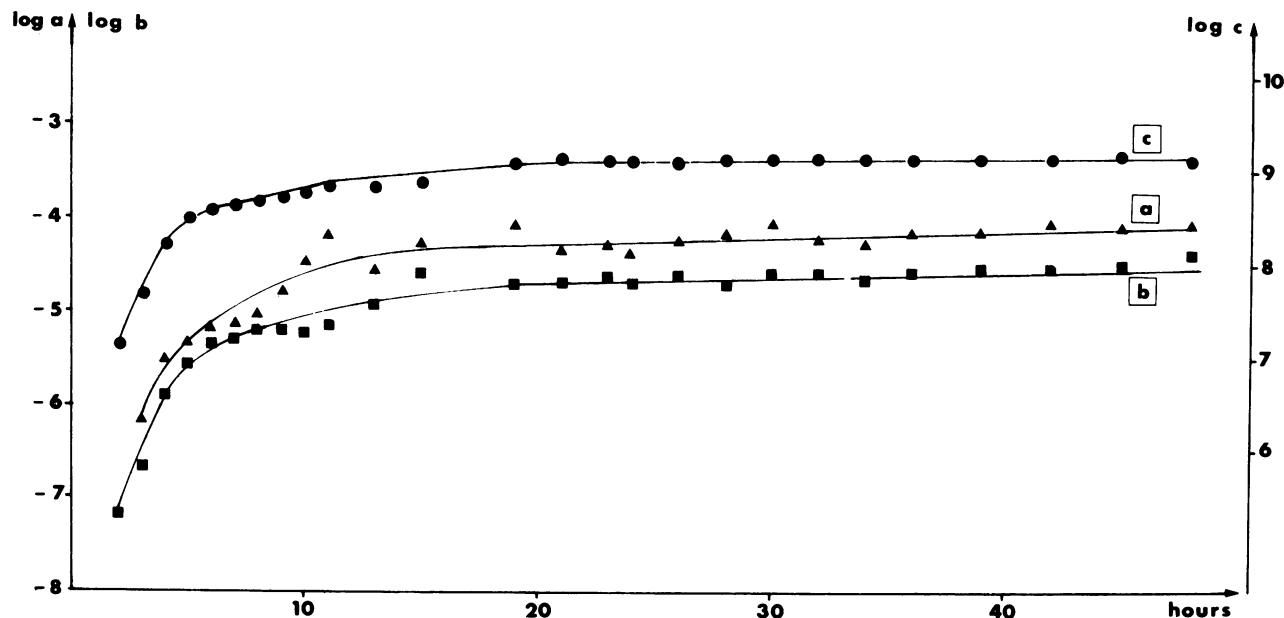
FIG. 4. Kinetics of ETA synthesis and of its release into the culture supernatant fluid by the strain 50586 under a 10% CO_2 atmosphere were calculated as follows: (a) ETA (nanograms per milliliter of staphylococcal lysate)/protein (nanograms per milliliter of staphylococcal lysate) (▲); (b) ETA (nanograms per milliliter of TY medium supernatant)/number of bacteria per milliliter of TY medium) (■); and (c) bacterial number per milliliter of TY medium (●).

culture supernatant also occurred during the exponential growing phase, and this ratio remained nearly constant throughout the stationary growing phase (Fig. 4 and 5).

Role of various physicochemical factors on the production of ETA and ETB. ETA and ETB were produced even in the absence of CO_2 in the growing atmosphere, but the maximum ET production per bacteria required a 15 to 20% CO_2

atmosphere (Fig. 6). When used in a 20% CO_2 atmosphere, the Y medium seemed to be the most satisfactory medium for ET production. The optimum pH for ETA production was between 6.5 and 10 at least; for ETB production, the optimum pH was between 6.0 and 10.0 (Fig. 7); acidic pH inhibited ET production. The glucose contained in the TY medium did not affect the ET production per bacteria, even
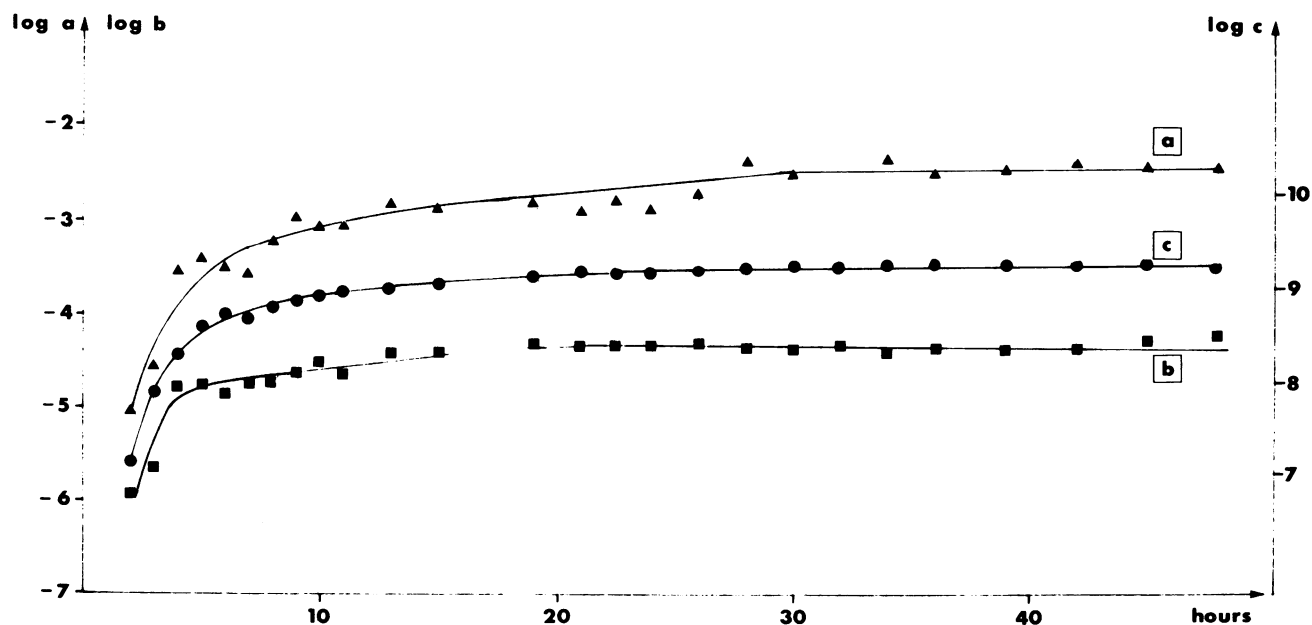

FIG. 5. Kinetics of ETB synthesis and of its release into the culture supernatant fluid by the strain TC 142 under a 10% CO_2 atmosphere were calculated as follows: (a) ETB (nanograms per milliliter of staphylococcal lysate)/protein (nanograms per milliliter of staphylococcal lysate (▲); (b) ETB (nanograms per milliliter of TY medium supernatant)/number of bacteria per milliliter of TY medium (■); and (c) bacterial number per milliliter of TY medium (●).
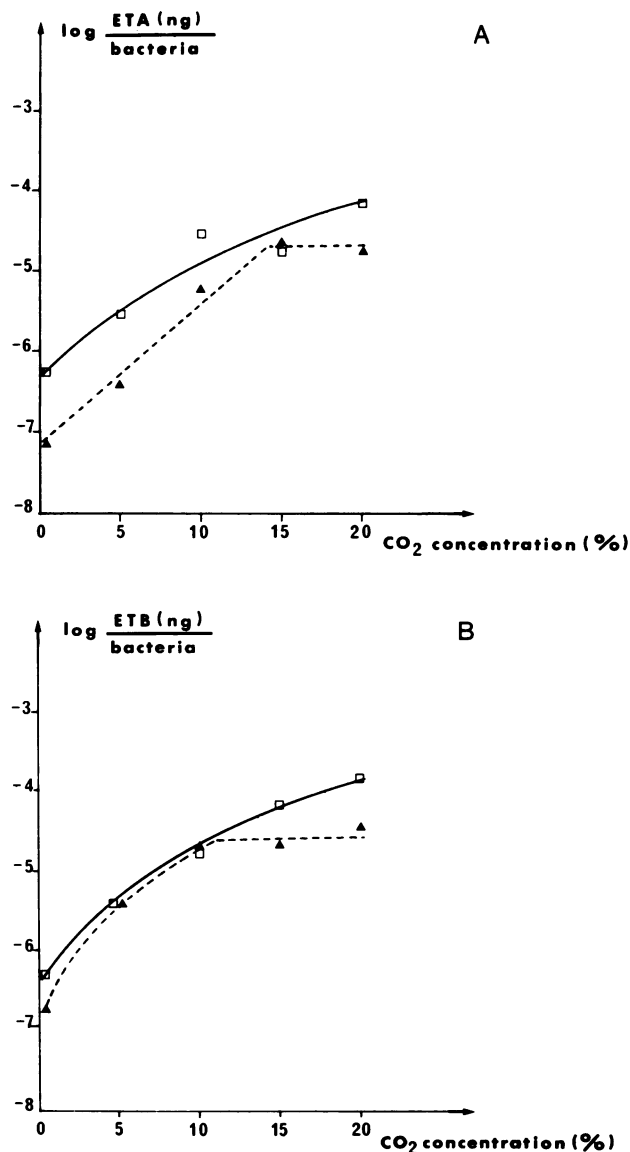
FIG. 6. Role of CO_2 concentration on the production of ETA (A) and ETB (B) in TY medium (▲) and in Y medium (□). ET production is expressed as the ratio of ET (nanograms per milliliter) to the number of bacteria per milliliter of culture supernatant.

at high concentrations (Fig. 8). The addition of glucose to TY medium (pH 6.9) did not modify the pH at the start of cultivation. Moreover, the final pH after 18 h of cultivation was the same in TY medium (pH 7.7) and in TY medium containing glucose concentrations ranging from 1 nM to 10 mM; however, 100 mM glucose in TY medium lowered the pH to 4.6 after 18 h of cultivation. Despite this acidic pH, which had an inhibitory effect on ET production per bacteria (Fig. 7), the production of ET per bacteria was not very different from that observed with lower glucose concentrations (Fig. 8). The growing temperature had no significant influence upon ET excretion per bacteria between 28 and 42°C (data now shown). Finally, the absence of culture agitation resulted in a 20-fold decrease in the number of staphylococci, but the amount of ET produced per bacteria was equal to that observed in an agitated culture.

DISCUSSION

At the present time, no simple and sensitive procedure is available for the detection of small amounts of ET (those measured by nanograms per milliliter). The lowest detection limit of the reference test, the newborn mice assay, was 1 to 5 μg/ml as observed in this work and elsewhere (21). For the Mancini test, this limit was the same (5 μg/ml) (21), whereas the radioimmunoassay had a considerably lowered limit (5 to 20 ng/ml) (3, 21). Our report describes two simple, specific, and sensitive (3 ng/ml) ELISA procedures which require affinity-purified toxin antibodies and which can be used to determine the ET concentration in culture supernatant fluids of *Staphylococcus aureus* strains or to potentially detect ET in biological fluids (sputum, blood, urine, and blister fluid). Only affinity-purified antibodies were used in this study to obtain the most specific response. However, cross-reaction occurred between ETB antigen and ETA antibody preparation and between ETA antigen and ETB antibody, although the ET antibody preparations were passed throughout a heterologous ET-Sepharose column. These cross-reactions could be explained by the presence of one or several
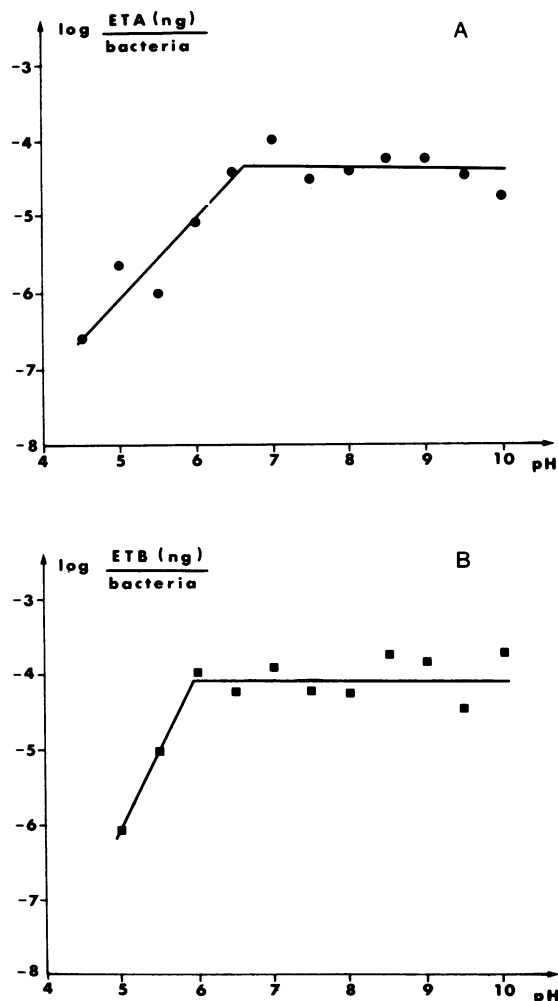

FIG. 7. Role of pH on the production of ETA (A) and ETB (B) under a 15% CO_2 atmosphere in a modified TY medium. ET production is expressed as the ratio of ET (nanograms per milliliter) to the number of bacteria per milliliter of culture supernatant.

FIG. 8. Role of glucose concentration on the production of ETA ($\bullet$) and ETB ($\blacksquare$) under a 15% CO_2 atmosphere in TY medium. The arrow at the origins corresponds to the absence of glucose in the medium. ET production is expressed as the ratio of ET (nanograms per milliliter) to the number of bacteria per milliliter of culture supernatant.
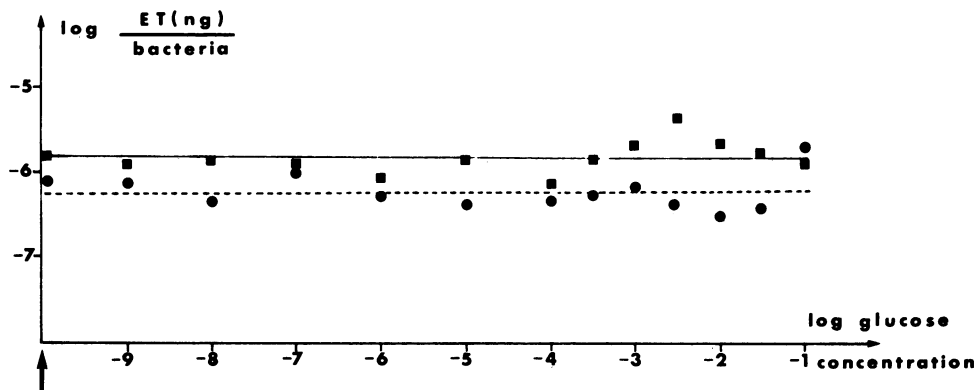
antigenic determinants common to both ETA and ETB; the resulting common antibodies would not be retained on the heterologous Sepharose-column, because these determinants could be not satisfactorily exposed or BrCN substituted on this heterologous column. When the ELISA procedure was used as a qualitative assay for determining the presence or absence of ETA or ETB in fluids, this cross-reaction could be misleading in the determination of the causative ET serotype. However, the comparison between quantitative ETA and ETB ELISA procedures permitted us to solve the problem, since the ratio of the quantitative results obtained for given ET antigen with the heterologous ELISA against the homologous ELISA was 10^{-4} to 10^{-5}. In the case of a fluid containing both types of toxins, this ratio would be considerably increased. In fact, in our laboratory, we qualitatively detected ETA and ETB in staphylococcal culture supernatants by electrosyneresis as previously described (15) with rabbit antisera. The ELISA tests could be performed even with noncentrifuged crude staphylococcal cultures for detecting ETA and ETB. Moreover, we used ETA and ETB, since in addition to the use of sheep antibodies having little or no affinity for staphylococcal protein A, these antibodies were passed through a protein A-Ultrogel column; thus, the presence of protein A in the sample did not give false-positive results.

The quantitative procedure gave similar information on the physiology of ETA and ETB synthesis. The comparisons of the kinetic curves of ETA and ETB intracellular synthesis and of ETA and ETB release into the supernatant fluid showed that the toxins were synthesized in the early stages of the growing phase and that the toxins appeared at the same time in the culture supernatants. These findings are in good agreement with a secretion process of ETA and ETB across the staphylococcal membranes.

We observed that an acidic pH (<6.5 for ETA and <6.0 for ETB) resulted in a decrease of ET production per bacteria. Regarding the role of CO_2, all authors (6, 8, 9) using a TY medium for ET production made the incubation in an atmosphere containing 10% CO_2, except Wiley et al. (19), who used heart infusion broth in an atmosphere of ca. 10 to 20% CO_2. Our results showed that for the ETB-producing TC 142 strain, the highest ETB quantity produced in TY medium was obtained in an CO_2 atmosphere of 10% or more, whereas for the ETA-producing 50586 strain, the highest ETA quantity produced was obtained in an CO_2 atmosphere of 15% or more. Therefore, it seems more satisfactory to use the TY medium (or better, the Y medium) in a 15 to 20% CO_2

atmosphere to obtain a maximal ET production. As observed by Kapral and Miller (8), elimination of CO_2 or of yeast extract from the TY medium significantly reduced the yield of ET. However, these authors found that the addition of glucose to the medium also reduced this yield. We did not observe such an effect with our two strains tested. The yield of ETA or ETB per bacteria remained constant, even with a high glucose concentration (100 mM) in which the pH became acidic (pH 4.6) after 18 h of cultivation. However, at this pH, no ET production occurs (Fig. 7). This apparent discrepancy could be related to the occurrence of two simultaneous phenomena. On one hand, bacterial growth occurred, since the initial pH was 6.9 and later pH decreased to 4.6. On the other hand, the production of ET occurred early, principally during the exponential growing phase (Fig. 4 and 5). The simultaneity of pH decrease and rapid ET production might explain the level of ET production observed even with 100 mM glucose added to the TY medium.

Finally, the incubation temperature and agitation had a role in the number of bacteria obtained after 18 h of culture, but the ET yield per bacteria remained unaffected by these conditions.

The ELISA procedures presented in this report provided a reliable and sensitive method for future detection of staphylococcal ETs in a variety of clinical samples of culture supernatant fluids. Moreover, these methods permitted the rapid detection of toxins in a few hundred samples all at once, with small volumes of samples, owing to their sensitivity. The quantitation of the toxins was also facilitated and led to a better understanding of the physiology of toxin production. In addition, these methods are simple and require equipment that is standard in most laboratories. This advantage could also facilitate the development of clinical and epidemiological surveys concerning those ET-producing staphylococci. The use of monoclonal antibodies instead of affinity-purified antibodies could eliminate the occurrence of cross-reactions between both toxins.

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