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Staphylococcal Enterotoxin-B in Cultured Human Kidney Cells

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<b>13. ABSTRACT (Maximum 200)</b>  Staphylococcal enterotoxin-B (SEB) is a common enterotoxin that can cause diarrhea and death in man. Since, we found that digalactosylceramide is most probably a putative receptor for SEB in cultured human kidney proximal tubular cells, it was used to develop a specific and sensitive assay for SEB (enzyme linked receptor-based immunodot) in human fluids, plasma and urine. Digalactosylceramide was immobilized on a polyvinylidene difluoride membrane and the membrane was subsequently incubated with primary and secondary alkaline phosphatase conjugated antibodies. A positive reaction was discerned as a blue spot. As little as 1 ng/ml SEB could be detected in the assay. SEB did not bind to structurally related glycosphingolipids, indicating specificity. This assay was specific for SEB, but not for SEA and TSST-1. Further work is in progress to study the application of this method in the field. Our findings will be of potential value for the food industry, and to determine toxemia in our soldiers.			
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Introduction: We have previously shown that glycosphingolipids may serve as putative receptor for Staphylococcal enterotoxin-B (SEB) (1,2). Therefore, during the course of the study this year, we have attempted to develop a suitable immunodetection assay for SEB in human fluids, i.e., human plasma and urine. Additional studies have been performed to determine the biochemical mechanism of action of SEB in cultured human proximal tubular cells, namely apoptosis.

Methods and Results: A rapid, simple, and inexpensive sandwich enzyme-linked receptor based immunodot assay was developed for the detection of Staphylococcal enterotoxin-B (SEB) in human fluids by using purified glycosphingolipid digalactosylceramide (diGalCer) receptor for SEB. Three microgram of diGalCer was immobilized on a polyvinylidene secondary alkaline-phosphatase, the membrane was subsequently incubated with primary and secondary alkaline phosphatase labeled antibodies. A positive reaction was discerned as a blue spot. As little as 1 ng/ml of SEB could be detected in this assay. SEB did not bind to structurally related glycosphingolipids, such as glucosylceramide, galactosylceramide, and lactosylceramide in this assay. Of five monoclonal anti-SEB antibodies and commercial anti-SEB antiserum tested, latter was the most sensitive in our assay. The specificity of SEB assay was assessed by comparison with structurally related toxins, e.g., Staphylococcal enterotoxin-A, and toxic shock syndrome toxin 1 (TSST-1). TSST-1 was not detected in the assay. Only at very high concentration of SEA some cross reaction was found.

In conclusion, we believe that this assay may be widely applicable because it is highly specific for SEB, it does not require special equipment, and the results can be obtained within a few hours with the naked eye. Since the receptor for SEB has a long-shelf life under adverse conditions, it can be easily stored and used for a long time (Appendix #1).

SEB induced apoptosis in cultured kidney cells. We have begun a series of experiments to determine the pathological processes involved in SEB induced cell death. In particular, we previously found that upon incubation of cells with SEB resulted in a time and concentration-dependent loss of cell proliferation. Our more recent studies indicate that this may due to programmed cell death. In particular, we are focusing on our studies to show whether SEB by itself or in combination of factors would induce the activity of neutral sphingomyelinase (N-SMase). N-SMase cleaves sphingomyelin to ceramide and phosphocholine. Ceramide in turn, induces apoptosis. In this regard, our preliminary results indicate that SEB indeed, induces sphingomyelinase activity and programmed cell death in cultured human kidney proximal tubular cells. We have extended these studies in an in vivo model. We have incubated rabbit kidney with SEB and are beginning to assess its effect on apoptosis by the TUNEL assay and several other histochemical assays, such as DNA ladder assay and chromatin condensation.

Structure/function relationship of SEB binding to human kidney cells. In our previous studies, we found that SEB peptide sequence KKKVTAQEL may be important in SEB induced cell proliferation and may be crucial in developing a neutralizing antibody to SEB. The binding of peptide sequence 93-112 in PT cells was on the order of 7-fold less than peptide sequence 130-160 (peptide #9), and 24-fold less than peptide sequence 191-120 (peptide #12). These studies have been reported previously. Since peptide #9 exerted a concentration-dependent inhibition of cell proliferation, and since scientists at Walter Reed (3) have prepared numerous mutant peptides of this particular peptide, we will begin a systematic study on the effects of mutation of peptide #9 on the binding, metabolism and apoptosis in cultured human kidney proximal tubular cells.

Conclusions: During this one year period, we have developed a suitable and sensitive assay for detecting SEB in human fluids. We are beginning to assess the SEB induced apoptosis in cultured human kidney proximal tubular cells as well as rabbit kidney. Our studies on mutant peptides representing the 130-160 amino acid sequence of SEB will reveal additional novel information regarding structure/function relationships of SEB induced toxemia in man. We do not expect and accordingly, do not recommend any further changes in our future direction of research to address this problem further.

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A NOVEL RECEPTOR BASED IMMUNOASSAY TO DETECT  
STAPHYLOCOCCUS ENTEROTOXIN B (SEB) IN BIOLOGICAL FLUIDS

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## ABSTRACT

A rapid, simple, and inexpensive sandwich enzyme-linked receptor based immunodot assay was developed for the detection of staphylococcal enterotoxin B (SEB) in human fluids by using purified glycosphingolipid digalactosylceramide (diGalCer) receptor for SEB. Three microgram of diGalCer was immobilized on a polyvinylidene difluoride membrane and the membrane was subsequently incubated with primary and secondary alkaline-phosphatase labeled antibodies. A positive reaction was discerned as a blue spot. As little as 1 ng/ml of SEB could be detected in the assay. SEB did not bind to structurally related GSLs, such as glucosylceramide (GlcCer), galactosylceramide (GalCer), and lactosylceramide (LacCer) in this assay. Of five monoclonal anti-SEB antibodies (MAB) and commercial anti-SEB antiserum (AS) tested the latter was the most sensitive in our assay. The specificity of SEB assay was assessed by comparison with structurally related toxins, e.g. staphylococcal enterotoxin A (SEA), and toxic shock syndrome toxin 1 (TSST-1). TSST-1 was not detected in the assay. Only at very high concentration of SEA some cross reaction was found.

In conclusion, we believe that this assay may be widely applicable because it is highly specific for SEB, it does not require special equipment, and the results can be obtained within few hours with the naked eye. Since the receptor for SEB has a long-shelf life under adverse climatic conditions, it can be easily stored and used for a long time.



## INTRODUCTION.

Infections caused by staphylococci remain an important cause of morbidity and mortality. The virulence factors associated with the toxinogenic diseases of *Staphylococcus aureus* are the staphylococcal enterotoxins. Among them staphylococcal enterotoxin B (SEB) attracts the most attention because of its implication in immunological reactions. SEB has been shown to be able to stimulate mitogenic activity in T-cells (17). This phenomenon appears to involve specific binding of the toxin to major histocompatibility complex (MHC) class II molecules and subsequent stimulation of the T-cell via the TCR-V-beta elements (7).

Glycosphingolipids (GSL) are composed of carbohydrates, fatty acid and sphingosine. They are components of the eukaryotic cell membrane. Recently, GSL have been implicated in various biological phenomena. For example, GSL have been shown to be involved in cell proliferation (2,22), cell migration (6,15), and apoptosis (programmed cell death) (11). Most importantly, GSL have been shown to serve as receptors for numerous bacterial toxins and viruses (1,5,14). For example, a ganglioside GM<sub>1</sub>, has been long established to serve as a receptor for cholera toxin (23).

We have recently shown that digalactosylceramide (diGalCer), a glycosphingolipid present in the human kidney and proximal tubular cells can specifically bind SEB but not structurally related toxins, staphylococcal enterotoxin A (SEA) and toxic shock syndrome toxin 1 (TSST-1) (4). The specificity of binding to the diGalCer receptor and physiological function was established subsequently. Therein, we found that SEB induced the uptake of [<sup>14</sup>C]choline and increased the synthesis of

phosphatidylcholine, in contrast SEA and TSST-1 failed to stimulate phospholipid biosynthesis (10).

Since the above delineated an important role of the receptor in mediating the metabolic action of SEB, we rationalized and designed a rapid assay for this toxin employing receptor based technology. In this communication we report a novel assay for the detection of SEB in human and mammalian fluids. We believe that this assay would be widely applicable because of the long-shelf life of the receptor under adverse climatic conditions.

## MATERIALS AND METHODS

### *Materials*

Polyvinylidene difluoride (PVDF) membrane was obtained from Millipore Corp., Bedford, MA. SEB, SEA, TSST-1 and monoclonal mouse anti-SEB antibodies (MAB), clones 2B, 3B, 6B, 12B, and 18B were obtained as a gift from Drs. Peter Gemski and Marti Jett, Washington, DC. Anti-mouse and anti-rabbit alkaline-labeled polyclonal antibodies were from Boehringer Mannheim Corp., Indianapolis, IN. Bovine serum albumin (initial fractionation by heat shock) was from Sigma Chemical Company, St. Louis, MO. Rabbit anti-SEB antiserum (AS) and other chemicals were also obtained from Sigma Chemical Company. Digalactosylceramide (diGalCer) was prepared from human Fabry kidney and characterized as described previously (4).

### *Immunodot blot assay*

The following procedure for the detection of SEB on PVDF membrane has been developed.

One microliter of diGalCer (3  $\mu\text{g}/\mu\text{l}$ ) dissolved in chloroform-methanol (2:1) was applied to premarked (4 mm diameter) spots on PVDF membrane and dried at room temperature. Pieces of the membrane were placed to the wells of 96-microtiter plate, and the membrane was incubated for 30 min in a 3% solution of bovine serum albumin (BSA) in phosphate-buffered saline (PBS) at room temperature. Then it was washed with PBS (3 x 10 min), and incubated in 400  $\mu\text{l}$  of solution of SEB for 60 min at room temperature (all following incubations were performed in the volume of 400  $\mu\text{l}$ ). After washing 3 times with PBS and once with 3% BSA in PBS (10 min), the membrane was incubated with primary antibodies (anti-SEB diluted 1:40,000 or MAB diluted 1:1,000 with 3% BSA in PBS) for two hours at room temperature. Then the membrane was washed 3 times with PBS and once with 3% BSA, and incubated with alkaline phosphatase-labeled antibodies (diluted 1:1,000 with 3% BSA in PBS) for 2 h at room temperature. After washing 3 times with PBS it was incubated with the solution of alkaline phosphatase substrate (100 mM Tris-HCl, 100 mM NaCl, 5 mM  $\text{MgCl}_2$ , pH 9.5; 5-bromo-4-chloro-3-indolyl-phosphate (BCIP); 4-nitro blue tetrazolium chloride (NBT); 300:2:1) for 10 min in the dark. Reaction was stopped by washing with water.

The addition of Tween-20 to the wash buffer (0.05% Tween 20 in PBS) did not improve the sensitivity and worsened the staining probably because of extracting diGalCer from the PVDF membrane, and hence was omitted in our procedure.

In an attempt to amplify the sensitivity of the assay we have applied the biotin-streptavidin system. Although the background was raised significantly, we failed to raise

the sensitivity, probably because of non-specific interactions of the components of biotin-streptavidin system with diGalCer.

#### *Quantitation of results*

The quantitative comparison of staining was performed by scanning densitometry on a Protein DNA Imager (PDI Inc., Huntington Station, NY) equipped with image processing software.

### RESULTS AND DISCUSSION

This study elaborated a novel approach to detect small quantities of SEB in mammalian fluids employing a receptor based immunodot assay.

We have determined the specificity of binding of SEB to diGalCer and structurally related GSL, such as glucosylceramide (GlcCer), galactosylceramide (GalCer), and lactosylceramide (LacCer). The chemical structure of these GSLs is summarized in Table 1. We have used a MAB 12B at a dilution 1:1,000. As shown in Fig. 1, SEB did not significantly bind to GlcCer, GalCer, and LacCer at a concentration of 1-2  $\mu\text{g/ml}$  that produced strong binding to diGalCer. These results confirm our previous report on the binding of [ $^{125}\text{I}$ ]SEB to various glycosphingolipids immobilized on a microtiter plate (4). An additional advantage of our current method is the use of non-radioactive reagents to detect SEB.

The effect of different dilutions of antibodies on the immunodetection of SEB after binding to 3  $\mu\text{g}$  of diGalCer in our assay is shown in Fig. 2. Three of five MAB, namely 2B, 3B, and 12B at a dilution of 1:1,000 were more sensitive than 6B and 18B.

But polyclonal anti-SEB AS even at a dilution 1:10,000 was more sensitive (7-folds) than the monoclonal antibodies in detecting SEB.

Polyclonal anti-SEB AS obtained from a commercial source bound to the complex diGalCer-SEB (concentration of SEB was 1  $\mu\text{g}/\text{ml}$ ) in a saturable fashion. However, non-specific binding of anti-SEB AS to diGalCer (10  $\mu\text{g}$ ) in this assay increased with increase in the concentration of the AS (Fig. 3). Accordingly, in subsequent studies we have chosen the AS dilution of 1:40,000 to decrease the non-specific binding and at the same time to retain sensitivity. Similarly, we have determined that the minimal concentration of diGalCer which retains the sensitivity but eliminates nonspecific binding of AS (at the dilution 1:40,000) is 3  $\mu\text{g}$  per assay.

Next, we studied the effect of concentration of SEB on its detection with anti-SEB MAB: 2B, 3B, and 12B (diluted 1:1,000), and anti-SEB AS (diluted 1:40,000) after binding to 3  $\mu\text{g}$  of diGalCer. We found that the commercial anti-SEB AS is most sensitive in the assay at all concentration ranges of SEB (Fig. 4). However, at higher concentrations of SEB (0.5  $\mu\text{g}/\text{ml}$ ), it is feasible to use MAB (e.g. 12B) when definitive results on the nature of intoxication is desired.

We have also assessed the possible cross reactivity of structurally related toxins, SEA and TSST-1 with diGalCer (3  $\mu\text{g}$ ) and anti-SEB AS (1:40,000) in our assay. At low concentrations (0.5-1  $\mu\text{g}/\text{ml}$ ), SEA and TSST-1 did not bind to diGalCer (Fig. 5). Only at very high concentration of SEA (5  $\mu\text{g}/\text{ml}$ ) we found some nonspecific binding to diGalCer. These findings suggest that our assay may be suitable for use in detecting SEB in samples contaminated with other structurally related toxins.

These findings are consistent with our previous reports demonstrating that [<sup>125</sup>I]SEB did not compete for binding with SEA and TSST-1 in our microtiter plate assays and in cultured human kidney proximal cells (4). The latter was accompanied by a marked stimulation of phosphatidylcholine synthesis by SEB but not by SEA and TSST-1 (10).

To study the possibility of measuring SEB in mammalian biological fluids we added SEB exogenously to human urine or serum samples (100-10,000 ng/ml) and used commercial anti-SEB AS at a dilution of 1:40,000 (the amount of diGalCer was 3 µg). The concentration of SEB which could be detected in the serum in our assay was 500 ng/ml. At the same time 100 ng of SEB per ml in the urine sample could be easily detected with anti-SEB AS (Fig. 6). The recovery of the enterotoxin from urine was about 80% of the amount added compared with SEB added to water. Subsequently, we found that as little as 1 ng/ml of SEB added exogenously to urine was detectable employing our assay (Fig. 7). However, it was not as sensitive at low concentrations (1 ng/ml) of toxin compared to higher concentrations of toxin (20 ng/ml) (Fig. 7) indicating that this method may be suitable for use in detecting small quantities of toxin in urine. In the case of very low concentrations of toxin, an overnight incubation with primary anti-SEB antibodies would be more effective in raising the sensitivity of the assay. Our experiments do not explain why the sensitivity of the assay for SEB in human urine is higher than in human serum. A possible reason may be that serum has a number of lipid binding proteins including albumin which may bind to diGalCer, thus preventing it from interacting with SEB.

In conclusion, various procedures have been developed to detect staphylococcal enterotoxins in food (3,8,9,13,18-21). Such methods employ microslide immunodiffusion assay, passive and reversed passive hemagglutination or latexagglutination assay, radioimmunoassay, enzyme-linked immunosorbent assay, and skin test assay. However, such studies have not distinguished between staphylococcal enterotoxins and other toxins leading to false-positive results. For example, some common assays are nonspecific as they detect in addition to SEB other substances produced by microorganisms other than *Staphylococcus aureus*, such as *Enterobacter agglomerans*, *Enterobacter cloacae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, and *Serratia marcescens* (16). In contrast our assay is designed to specifically capture SEB to its receptor followed by immunodetection. Our method is fast, simple, and inexpensive immunodot assay to detect specifically SEB in biological fluids. This analysis does not require special equipment and the results can be obtained within few hours with the naked eye. The minimal sensitivity of the assay for SEB (1 ng/ml) is comparable to that of existing tests (12).

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TABLE 1. Structure of glycosphingolipids used in this study.

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Glycosphingolipid	Structure
Glucosylceramide	Glc $\beta$ 1-1Cer
Galactosylceramide	Gal $\beta$ 1-1Cer
Lactosylceramide	Gal $\beta$ 1-4Glc $\beta$ 1-1Cer
Digalactosylceramide	Gal $\alpha$ 1-4Gal $\beta$ 1-1Cer

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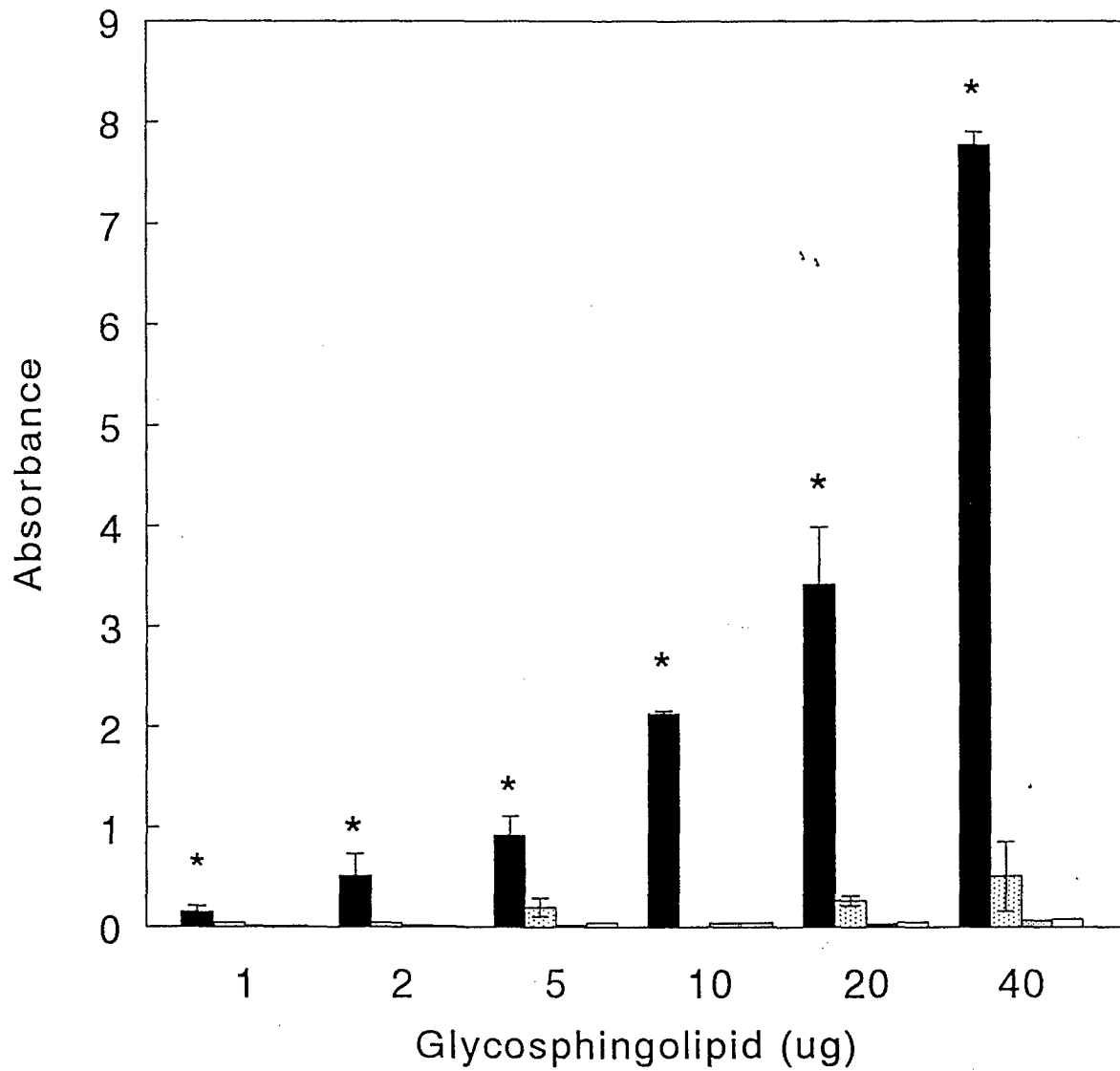


FIGURE 1. Specificity of binding of SEB to various glycosphingolipids. ■, diGalCer; ▨, GlcCer; ▩, GalCer; □, LacCer. Values are the mean of three experiments  $\pm$  SD.

\*, Significant difference from control,  $P < 0.05$  (Student's t-test).

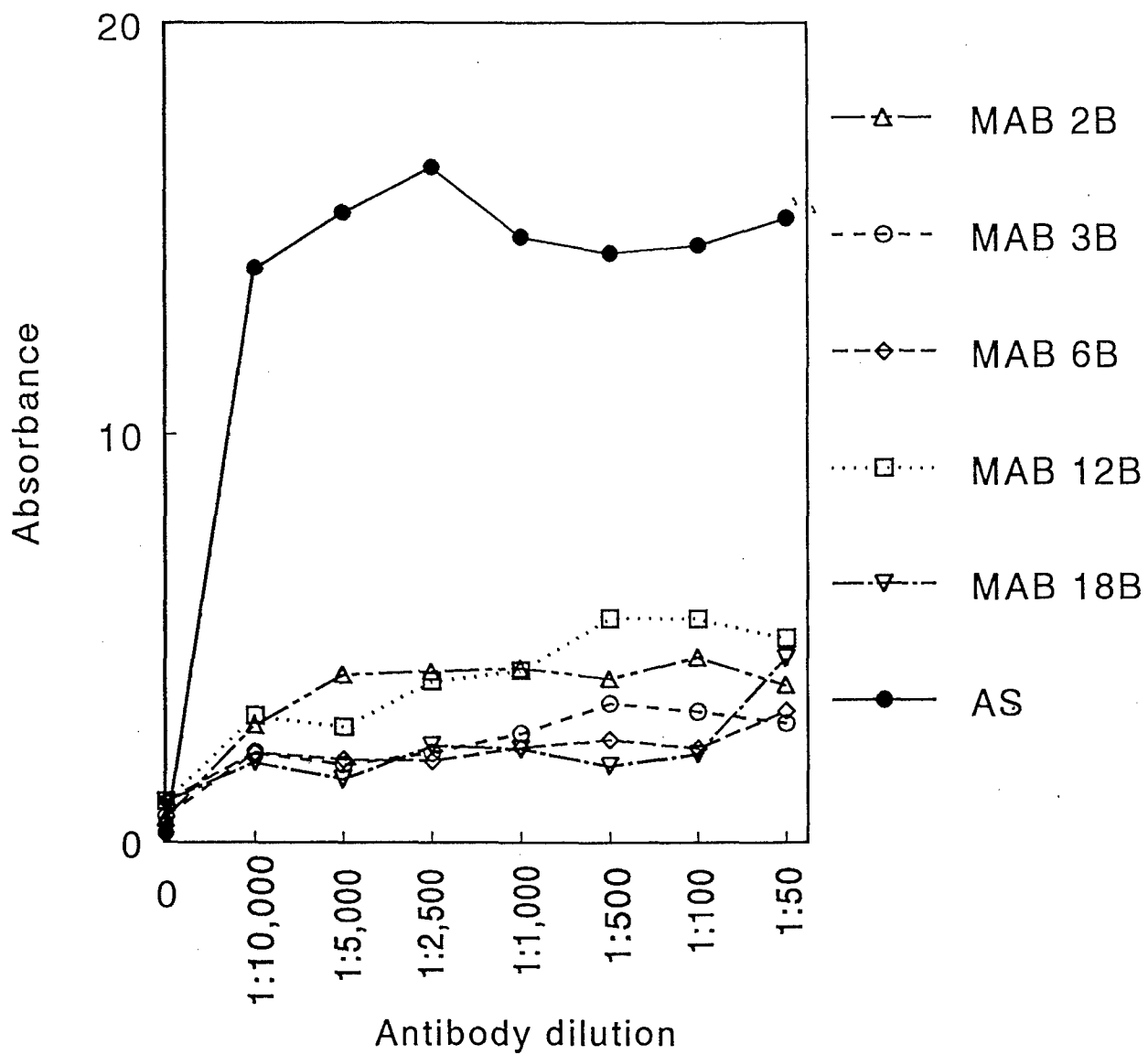


FIGURE 2. Effect of antibody dilution on the immunodetection of SEB.

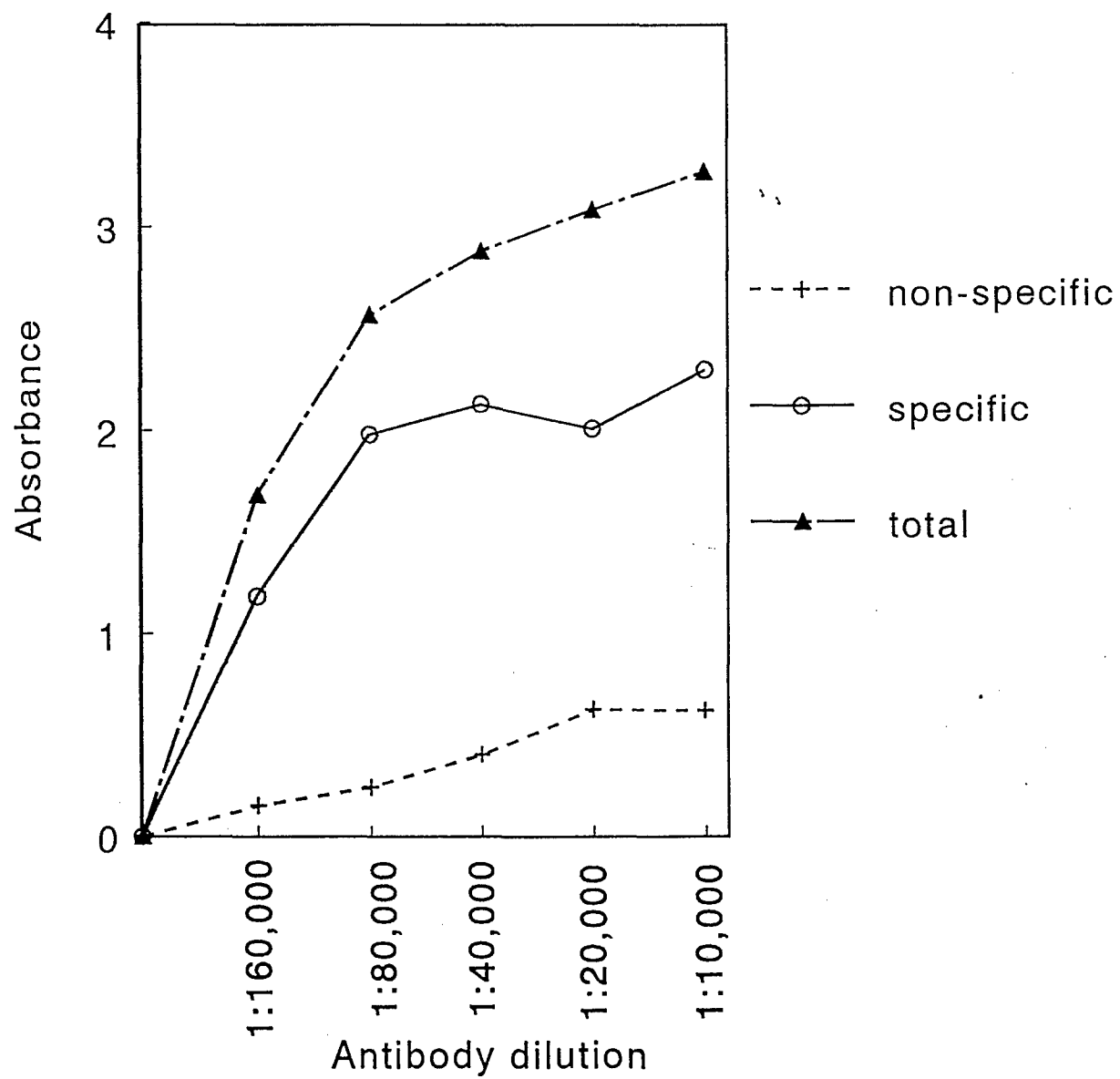


FIGURE 3. Binding of different dilutions of anti-SEB AS to the complex diGalCer-SEB (specific) versus diGalCer alone (non-specific).

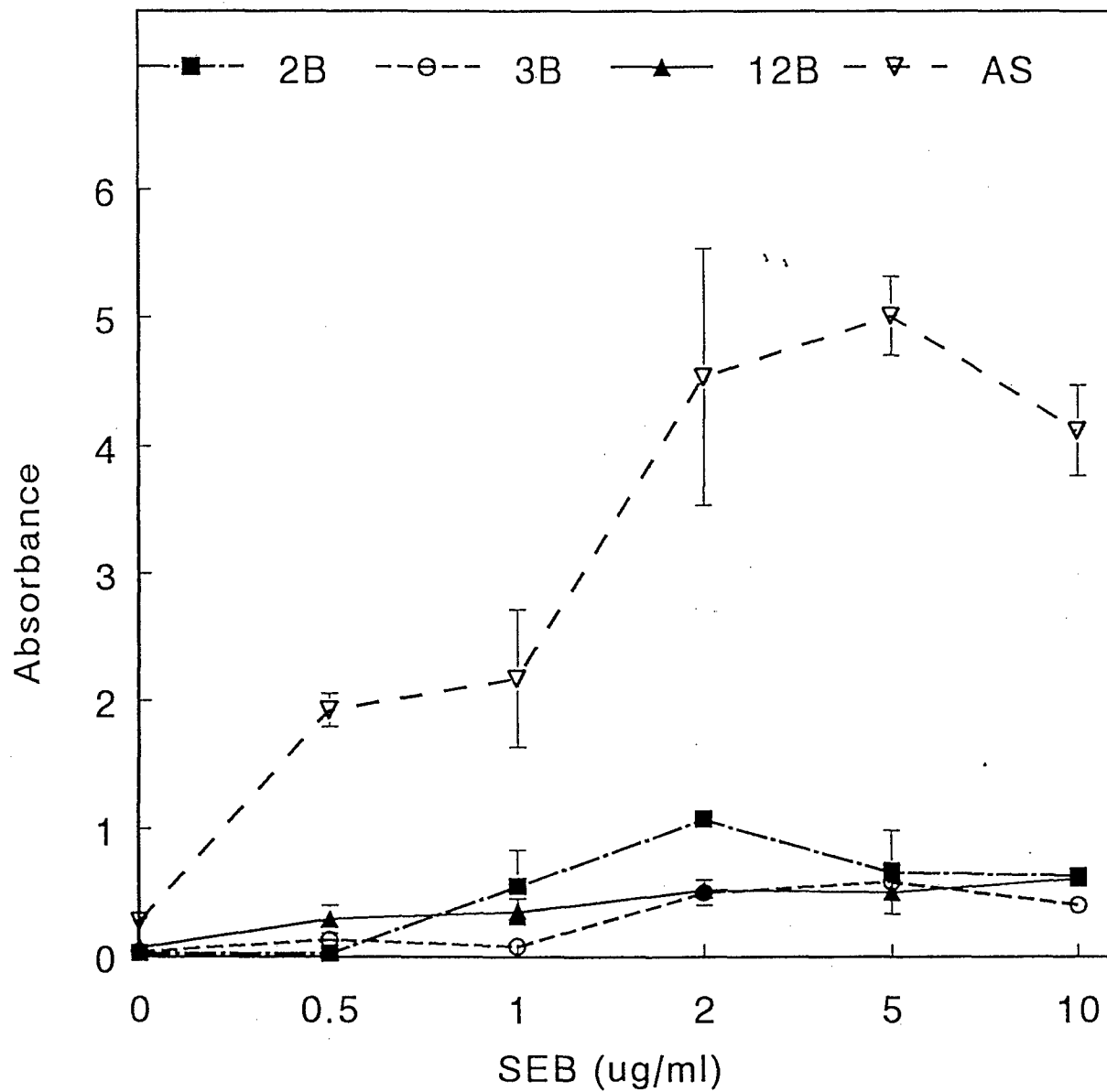


FIGURE 4. Immunodetection of different amounts of SEB with monoclonal antibodies 2B, 3B, 12B, and antiserum. Values are the mean of three experiments  $\pm$  SD.

\*, Significant difference from control,  $P < 0.05$  (Student's t-test).



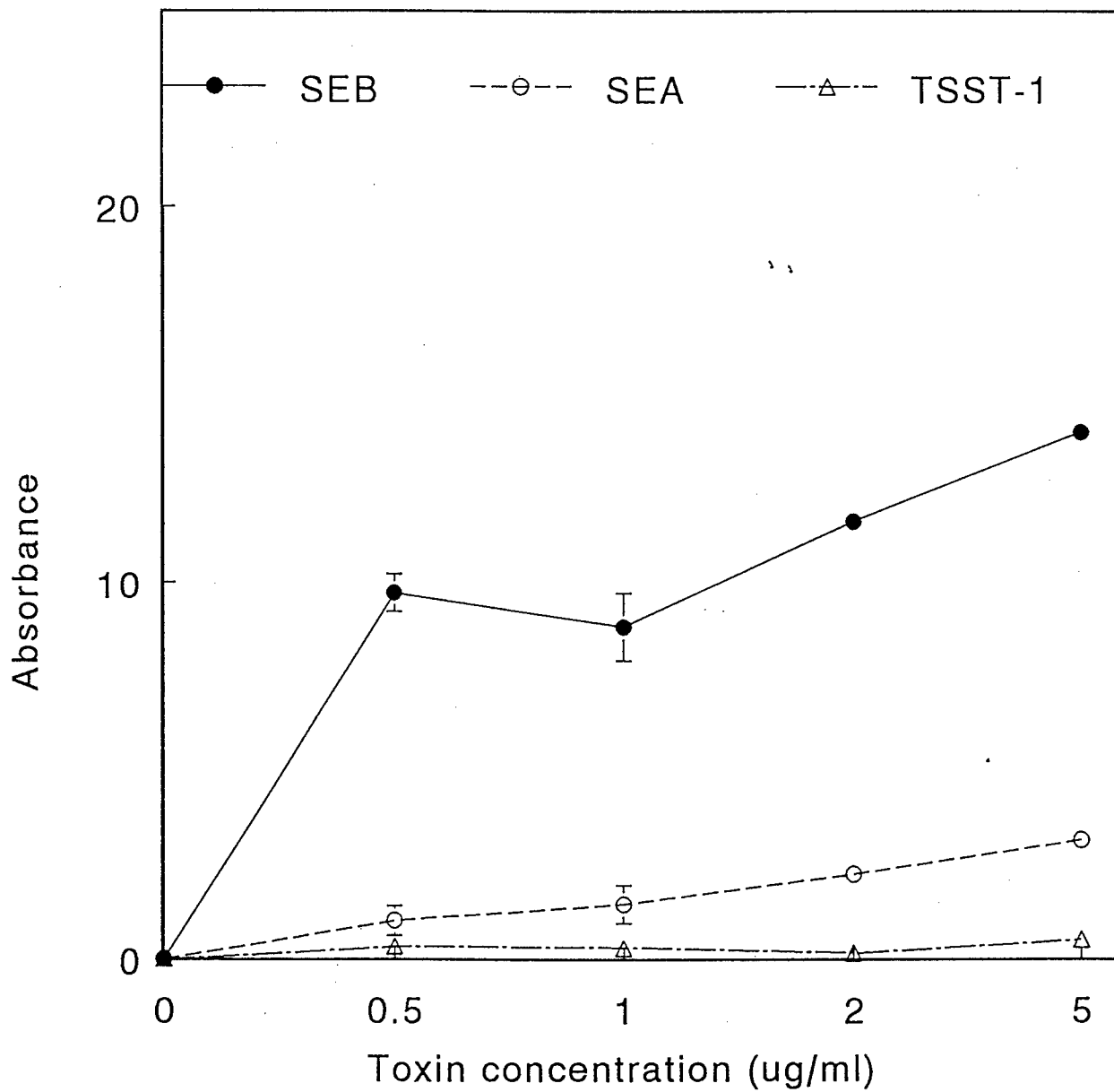


FIGURE 5. Immunodetection of structurally related toxins with anti-SEB AS after binding to diGalCer. Values are the mean of three experiments  $\pm$  SD. \*, Significant difference from control,  $P < 0.05$  (Student's t-test).

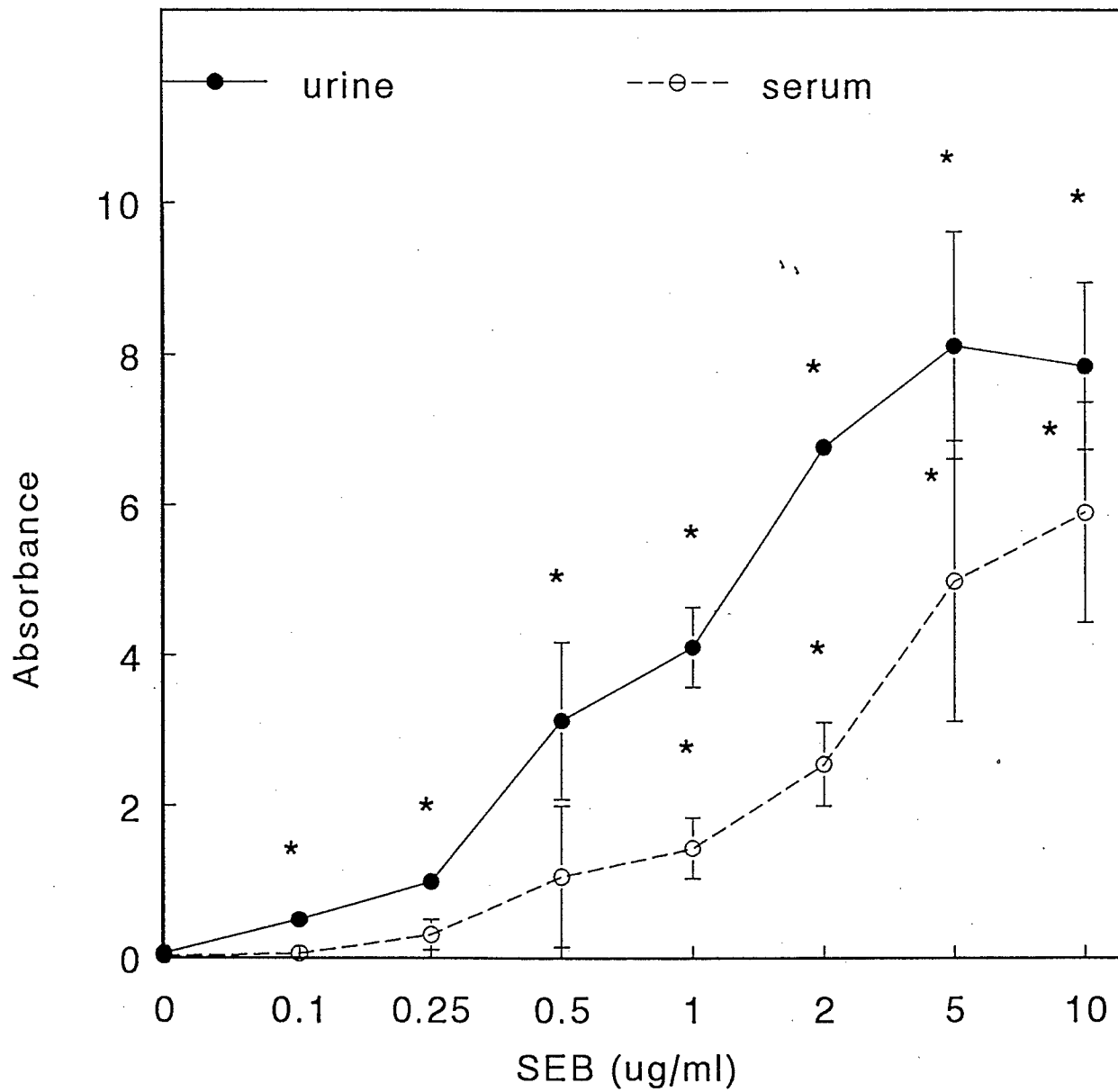


FIGURE 6. Immunodetection of SEB either in urine or in serum with anti-SEB AS.

Values are the mean of three experiments  $\pm$  SD.

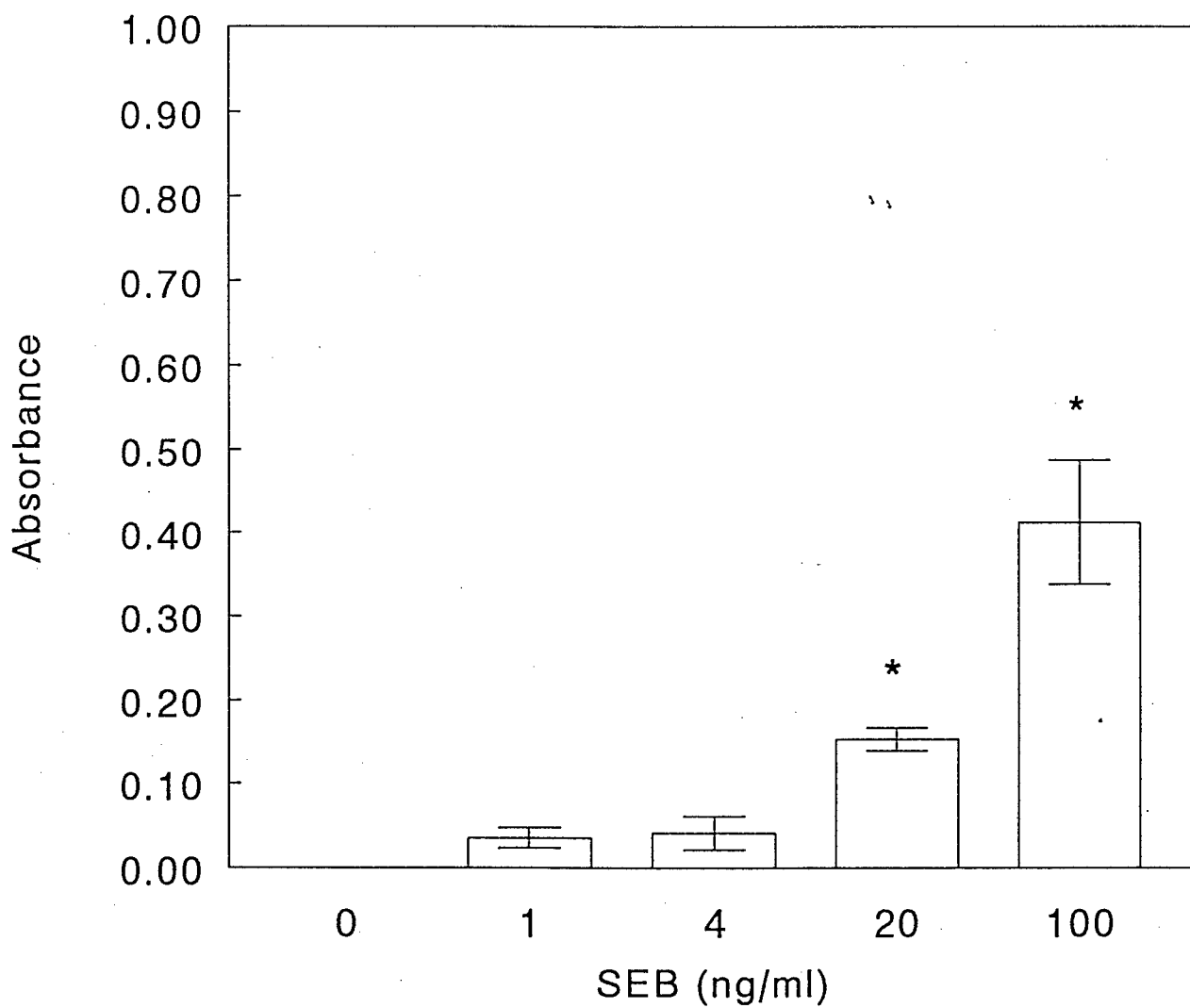


FIGURE 7. Immunodetection of SEB in urine with anti-SEB AS. Values are the mean of three experiments  $\pm$  SD. \*, Significant difference from control,  $P < 0.05$  (Student's t-test).