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SENSITIVE FLUOROGENIC ENZYME LINKED IMMUNOSORBENT ASSAY: STAPHYLOCOCCAL ENTEROTOXIN B

by

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ABSTRACT

A highly sensitive four layer fluorogenic enzyme-linked immunosorbent assay (FELISA) has been developed for the detection and identification of staphylococcal enterotoxin B (SEB). The sandwich FELISA exhibited maximum sensitivity and detected 0.1 ag or 100 fg ml^{-1} quantities of purified SEB of the test samples. The monoclonal antibodies raised against SEB and used in FELISA reacted very specifically with SEB only. The FELISA is simple to perform and results can be obtained in approximately 3 hr.

RÉSUMÉ

Un test d'immunosorption enzymatique fluorogène (FELISA) en quatre couches hautement sensible a été mis au point pour la détection et l'identification de l'entérotoxine B staphylococcique (EBS). Le FELISA type sandwich a présenté la sensibilité maximale et a détecté des quantités de 0,1 ag ou 100 fg mL^{-1} d'EBS purifiée dan les échantillons testés. Les anticorps monoclonaux levés contre l'EBS et utilisés dans le test FELISA ont réagi de manière très spécifique contre l'EBS seulement. Le test est facile à exécuter et il faut environ trois heures pour obtenir les résultats.

INTRODUCTION

Among food poisoning, staphylococcal food poisoning is one of the most common foodborne illnesses. It is caused by a closely related group of enterotoxins, which are water soluble proteins and are released into the medium during growth by certain strains of gram-positive bacterium, *Staphylococcus aureus*. These toxins are of small molecular weight (26-30 Kilodalton), single, unbranched polypeptide chains and have been differentiated by their immunological specificity into seven distinct types designated as staphylococcal enterotoxin SEA, SEB, [1], SEC_1 [2], SEC_2 [3], SEC_3 [4], SED [5] and SEE [6].

These SEs have very potent and debilitating as well as lethal effect on humans. Therefore, remain both a national and international threat to civilian as well as military populations. From a defence and preventive point of view, their rapid detection and identification is of prime interest.

Large numbers of assay systems [7-10] have been developed for the detection of SEs, only a few are convenient or sensitive enough for routine use for diagnostic purposes. Currently, detection of these SEs is by sensitive immunological methods such as enzyme-linked immunosorbent assay (ELISA) [11,12] and latex agglutination tests [13,14] have been reported. As compared to colourimetric, fluorometric methods have been found more sensitive [15]. One can easily get 10^2 to 10^6 fold increase in sensitivity by using fluorometric assay methods [16,17].

In this communication, we describe a highly sensitive fluorogenic enzyme linked immunosorbent assay (FELISA) for the detection of SEB, which could detect down to attogram quantities of SEB.

MATERIALS AND METHODS

Reagents and Equipment

Staphylococcal enterotoxins, SEA, SEB, SEC_1, SEC_2, SEC_3, SED, SEE and toxic shock syndrome toxin (TSST1) were purchased from Toxin Technology Inc. (Madison, Wisconsin); diethanolamine (DEA), 4-methyl umbelliferyl phosphate (4-MUMP), alkaline phosphatase-labelled goat, anti-mouse IgG antibody, pristane (2,6,10,14-tetramethylo-pentadecane) and crystaline bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO); phosphate buffered saline (PBS) tablets were purchased from Oxoid Canada Ltd (Ottawa, ON) polyoyoxyethylene sorbitol monolaurate (Tween 20) was purchased from Bio-Rad Laboratories; Dulbecco Modified Eagle Medium (DMEM) and Freund's complete and incomplete adjuvants were purchased from Gibco/BRL (Burlington, ON); bicinchoninic acid (BCA™) protein assay kit was purchased from Pierce Chemical Co., (Rockford, Ilinois). Micro-FLOUR reader, Millitier™-HA immunoassay plates and Millititer filtration system, and tissue culture plates (Limbro 76-003-05) were purchased from Dynatech Laboratories (Alexandria, Virginia) Millipore Corporation, (Bedford, Massachusettes) and Flow Laboratories Inc. (McLean, Virginia) respectively. Protein A-sepharose CL-4B was obtained from Pharmacia (Pharmacia Canada, Dorval, PQ).

Analytical Methods

Protein Estimation

Protein concentrations were determined according to Meyer et al, [18] method. Crystalline BSA was used as standard.

Enzyme Assay

Alkaline phosphatase activity was assayed as described [19].

Buffers

PBS, pH 7.4 was prepared according to the manufacturers instruction. Blocking buffer was prepared by adding 3% BSA and 0.1% Tween-20 to PBS. Coating buffer was comprised of 50 mM Carbonate-bicarbonate pH 9.6 with 0.01% sodium azide, 1M DEA was prepared by dissolving 97 ml of DEA in water containing 20 mM $MgCl_2$ and pH was adjusted to 9.8 with 1N HCl. Enzyme substrate 4-MUP, 0.1 mM was prepared in DEA buffer immediately prior to use. Triple distilled de-ionized water was used in all the buffer preparations.

Preparation of Polyclonal Anti-SEB Antibodies

Six months old white New Zealand rabbits were injected subcutaneously with 10 μg of pure SEB in Freund Complete Adjuvant. Fourteen days later rabbits were injected subcutaneously with 25 μg of SEB in Incomplete Freunds Adjuvant. After one month rabbits were given third injections with 100 μg of SEB Incomplete Freunds Adjuvant. One week after the last injection rabbits were bled. Rabbit anti-SEB IgG from the blood serum was purified by affinity column chromatographically. SpA-Sepharose column and serum was equilibrated using 0.1M phosphate buffer pH 8.0. Rabbit anti-SEB IgG fraction was eluted by using 0.1M acetate buffer, pH 4.0.Fractions containing IgG were pooled and immediately neutralized to pH 7.2 with 2M Tris and stored at -20°C until used.

Preparation of Monoclonal anti-SEB Antibodies

Immunization of Mice. Balb/c mice (Jackson Laboratory, Bar Harbor, ME) 2-3 months old, were immunized intraperitoneally, at 1 week intervals, with 5, 10 and

20 μg of purified SEB emulsified in an equal volume of Freund's complete adjuvant. A volume of 0.2 ml was given to each mouse. After the first immunization protocol, the mice were allowed to "rest" for one month then boosted with 25 μg of SEB in Freund's incomplete adjuvant. After an additional month of "rest" the mice were given intravenously 30 μg of SEB in 0.2 ml sterile PBS. Three days later, the mice were killed and spleens removed aseptically.

Fusion Protocol

The basic procedure of Galfré et al., [20] and Kennet [21] was used. The spleens (usually from 2 mice) were teased apart and the resultant cell suspension was passed through a stainless steel mesh. Red blood cells were lysed by osmotic shock and washed with DMEM.

The fusion was carried out with a 10:1 ratio of splenic lymphocytes to NS-1 (ATCC-T1B13) non secretor mouse tumor line. After centrifugation, the supernatant liquid was removed and 0.8 ml of 50% polyethylene glycol 1500 pre-warmed at 40°C, was added to the cells dropwise over a period of 1 min with gentle stirring. The cells suspension was diluted with DMEM over a period of about 10 min. The cells were again pelleted, washed 3X with DMEM and resuspended in 40 ml of DMEM containing 20% fetal bovine serum. This suspension was distributed in 96-well tissue culture plates Linbro 76-003-05, containing 10^5 feeder cells (spleen cells) per well.

The next day, and at intervals thereafter, the culture medium was changed and supplemented with HAT-Selective Medium (Hypoxantine-Aminopterin-Thymidine). Hybridomas formed after 12-14 days of incubation at 39°C under 10% $C0_2$ and 95% humidity.

The content of each well was tested by ELISA for reaction with SEB. Cells from positive wells were further cloned in soft agarose by the method of Coffino et

al., [22]. Hybridomas were palted over a layer of rat hepatocytes as feeder cells. After about 8-10 days, the clones were picked with a micropipette and placed in DMEM containing 20% fetal bovine serum in 96-well culture plates. After 3-5 days, the culture fluid was tested for antibody content.

Class and Subclass Determination of Monoclonals Anti-SEB

Four clones were selected for production of ascites fluid (E3B9A7, H1C2H9, H1C3C5 and H1B9B4). Previously to ascites production the monoclonals were characterized for their class and subclass immunoglobulin type, using double gel immunodiffusion with specific antisera to mouse immunoglobulins. All selected hybridomas produced immunmoglobulins of IgG1, lambda subclass.

Production of Monoclonal Anti-SEB in Ascites Fluid

Three month old Balb/c mice were injected intraperitoneally three times with 0.5 ml pristane at weekly intervals. After a rest period of 10 days, 0.5 x 10^6 log-phase hybridoma cells were injected into each mouse. Ascites fluid was collected from the abdominal cavity between 7 and 10 days following injection of hybridoma. The frequency and volumes of collected ascites fluid varied from mouse to mouse.

The ascites fluid from each mouse was pooled individually, centrifuged at 10,000 x g and precipitated twice with 50% saturated $(NH_4)_2S0_4$. The precipitate was dissolved in distilled water at 25% of initial ascites fluid volume, and dialyzed for 72 h against 0.1 M phosphate buffer, pH 7.2. After dialysis, the samples were again centrifuged at 5000 g to remove any precipitate. The supernatants were aliquoted in 5 ml volumes and kept frozen at -20°C.

Standardization of Immunoreagents

Immunochemicals used in the fluorogenic ELISA were titrated against each other to determine the optimum concentration to be used in routine assays [23]. The antibody, immobilized on nitrocellulose membrane to capture the antigen, was titrated with the recommended dilution (1:1000) of enzyme-labelled antibody. The concentration of MCA-α-SEB used as a third layer to amplify the sensitivity was determined by checkerboard titration. Blocking steps were evaluated thoroughly and the experimental conditions that give the highest ratio between the test and background values were used in this study. Each of the sensitivity and cross-reactivity experiments were repeated several times for their reproducibility.

Immunoassay Procedure

Four layer "sandwich" FELISA method [17] was used with modification as follows. Rabbit polyclonal SEB-antibody (PCA-α-SEB) diluted appropriately (40 μg/ml) in coating buffer was immobilized on Millititer™ immunoassay plates (the bottom of these plates is made of nitrocellulose membrane of 0.45 μm pore size). The plates were incubated over night at 4°C. Wells were washed three times with PBS using the Millititer™ filtration system to remove excess antibody not attached to the solid phase and the remaining binding sites on nitrocellulose membranes were blocked with 200 μl of blocking buffer. Plates were then washed 3 times with PBS and incubated with SEB dilutions made in blocking buffer for 1 h. Plates were washed 3 times and MCA-α-SEB (1 μg/ml) was added to the wells to amplify the detection of antigen. After incubation for 30 min and washing with PBS, goat-α-mouse IgG labelled with alkaline phosphatase was added and the plates were reincubated for another 30 min. Plates were finally washed 6 times with PBS containing 0.05% Tween-20 to remove all unbound enzyme conjugate. After blotting the plate bottom dry, enzyme substrate in a volume of 200 μL was added. Immunoassay plates were incubated at room temperature in the dark and the relative

fluorescence due to the release of 4-methylumbelliferone (4 MUM) was measured in a MicroFLOUR® reader at excitation wave length of 365 nm and emission at 450 nm after 15 min.

Data Analysis

Samples of SEB were tested in six replicate wells and the fluorescence counts from each of the 6 values were averaged and standard deviation calculated. At least 4 separate samples were tested. Background (negative control) values were also averaged and two standard deviations were added to determine the cut-off value. Fluorescent counts equal to or above this cut-off value defined the lower limit of a positive result.

RESULTS AND DISCUSSION

Sensitivity of FELISA

The sensitivity of the sandwich FELISA was determined by titrating varying concentrations of SEB. Results depicted in Fig. 1 show that by using MCA-α-SEB as a third layer the procedure was made highly sensitive and this method detect down to 100 fg ml^{-1} or 0.1 ag ml^{-1} quantities of SEB in the sample. Recently, using ELISA method, Dupont et al., [24] and Windeman et al., [25] have demonstrated minimum detection limits for SEB as 5 μg ml^{-1} and 0.1 ng ml^{-1} respectively.

Specificity and Cross-reactivity

Specificity and cross-reactivity of MCA-α-SEB and PCA-α-SEB against SEB and other SEs were tested using "indirect" FELISA. Results presented in Fig. 2 show that MCA-α-SEB specifically reacted against SEB and no significant cross-reactivity was observed with other SEs. PCA-α-SEB reacted with SEB but also gave some

cross reaction against SEC and SED. Neither antibody gave any detectable cross reaction against SEE nor TSST1. A certain degree of cross-reaction, mainly observed with PCA-α-SEB can be explained by reported amino acids sequence conservation among SEs. By using a program designed by Lipman and Pearson [26], we found that SEB shares approximately 32, 62,61,39, 29% amino acid sequence conservation with SEA, SEC_1, SEC_2, SED and SEE (Micusan et al., unpublished results). It is also possible that rabbits used to obtain the PCA-α-SEB antibody contained some small contamination of endogenous antibodies to other SEs. The presented method is easy to perform and can be completed in less than 2.5 h provided the membranes are coated with antibodies in advance. The high sensitivity in detection limit of SEB compared to a classical ELISAs [24,25] was achieved by using a fluorogenic substrate such as 4-MUP which upon hydrolysis yields 4-MU which is detectable at at least 100 fold lower concentration than nitrophenol, the usual substrate for alkaline phosphatase. Shalev et al., [27] detected antigen at a concentration of 5×10^{-15} mg ml^{-1} which represents about 25000 molecules. Thus, fluorogenic substrates may allow detection limits of femtomolar (10^{-15}) or even attomolar (10^{-18}) levels. Other advantages of this system are much shorter reaction time (since the substrate is not a limiting factor), the savings in the cost of substrate and the wide range of detection now offered by modern spectrofluorometers. It has also been reported that if alkaline phosphatase can be substituted in antibody conjugates with beta-galactosidase reagent blanks can be lowered about 50-fold [28] thus expending even more the detection limit for a given antigen. One problem encountered in SEs detection by either radioimmunoassays or enzyme-linked immunoassays is the interference of Protein A, often secreted by *S. aureus* strains together with SEs. However, protein A interference could be prevented by adding normal rabbit serum to the sample [24] and consequently the described FELISA is very specific.

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LEGENDS

Fig. 1: Sensitivity profile of SEB detection by sandwich FELISA. Varying concentrations of SEB (1 μg to 100 fg per ml) were titrated by FELISA. Fluorescence counts were determined. Data are presented as the mean of six determinations on a single plate. Error bars represent SD of the mean. Dotted line shows the negative control plus two SD.

Fig. 2. Specificity and cross-reactivity of MCA-α-SEB and PCA-α-SEB using FELISA. Plates were coated with 50 μl solution containing 5 μg per ml of SEA, SEB, SEC_1, SEC_2, SEC_3, SED, SED and TSST1. After blocking the plates SEs were challenged with MCA-α-SEB and PCA-α-SEB at concentrations of 1 μg ml^{-1} respectively. Blocking buffer was used as a control. Data points are the means of six determinations on a single plate and error bars represent the SD of the mean.

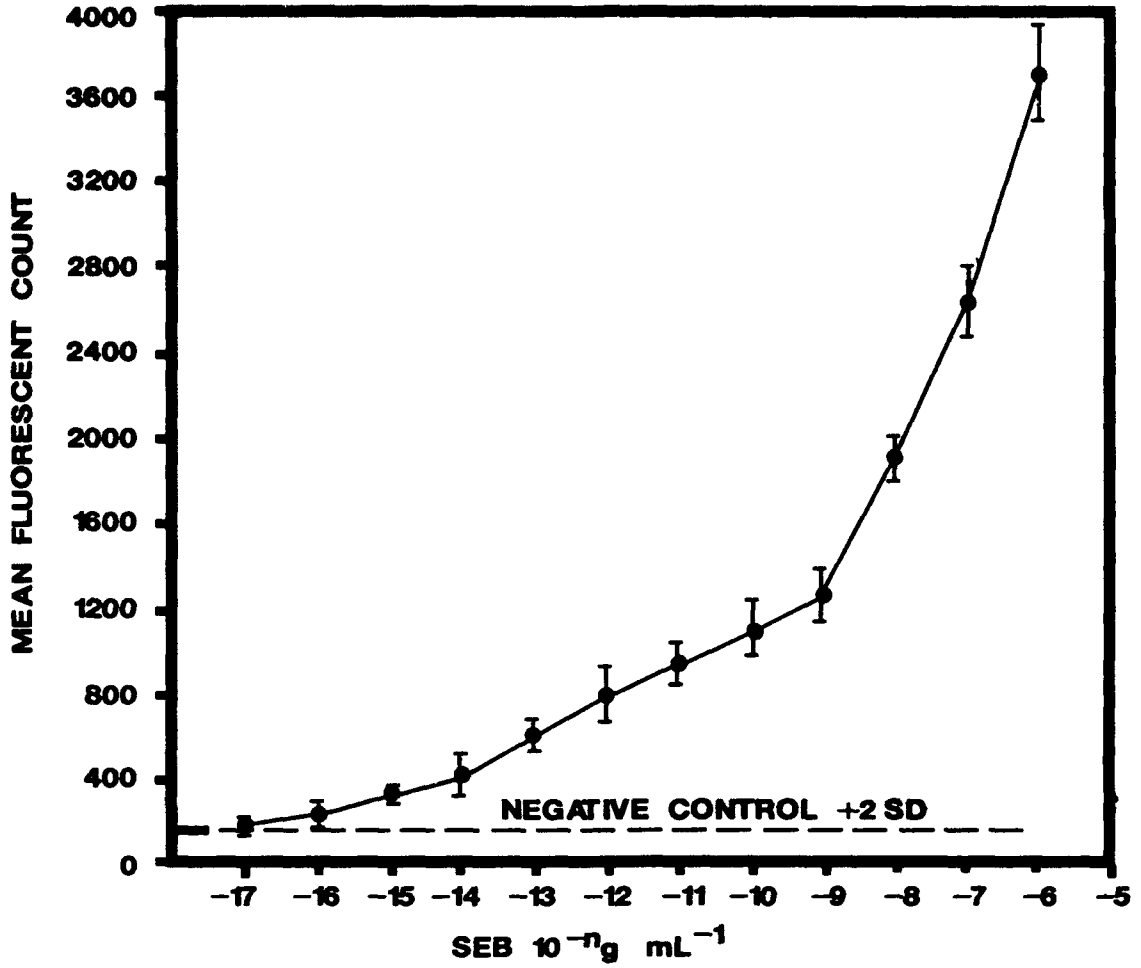

Figure 1

Sensitivity profile of SEb detection by sandwich FELISA. Varying concentrations of SEB (1 μg to 100 fg per ml) were titrated by FELISA. Fluorescence counts were determined. Data are presented as the mean of six determinations on a single plate. Error bars represent SD of the mean. Dotted line shows the negative control plus two SD.

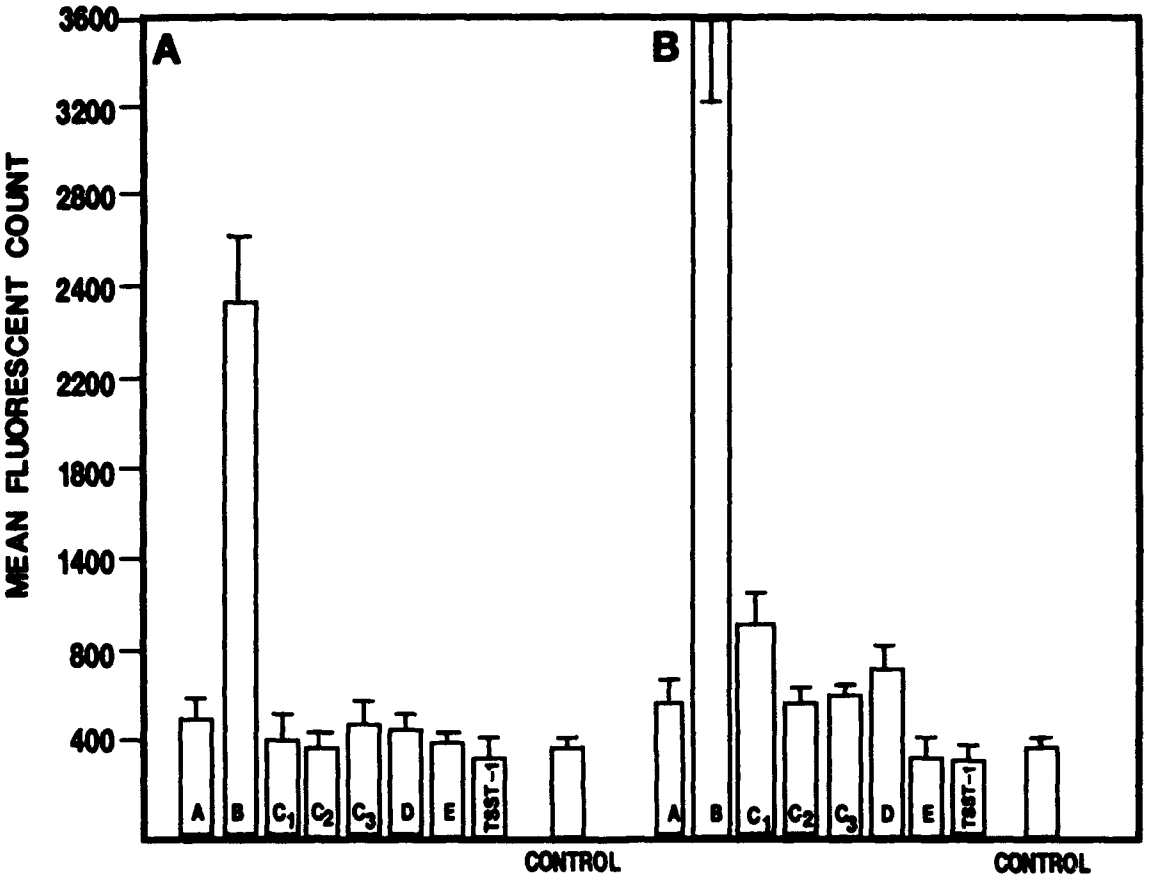

Figure 2

Specificity and cross-reactivity of MCA-α-SEB and PCA-α-SEB using FELISA. Plates were coated with 50 µl solution containing 5 µg per ml of SEA, SEB, SEC $_1$, SEC $_2$, SEC $_3$, SED, SED and TSST-1. After blocking the plates SEs were challenged with MCA-α-SEB and PCA-α-SEB at concentrations of 1 ug ml $^{-1}$ respectively. Blocking buffer was used as a control. Data points are the means of six determinations on a single plate and error bars represent the SD of the mean.

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A highly sensitive four layer fluorogenic enzyme-linked immunosorbent assay (FELISA) has been described for the detection and identification of staphylococcal enterotoxin B (SEB). The sandwich FELISA exhibited maximum sensitivity and detected 100 fg or 0.1 ag ml^{-1} quantities of purified SEB of the test samples. The FELISA is simple to perform and results can be obtained in approximately 3 hr.

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