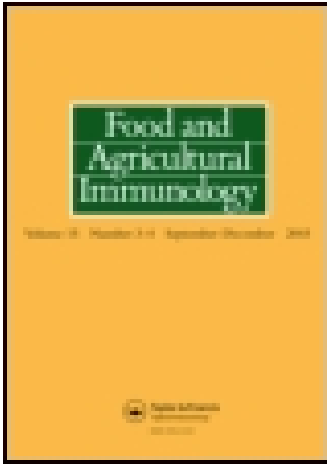




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## The Use of an Anti-idiotypic Monoclonal Antibody for Reliable Detection of Staphylococcal Enterotoxins

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*During the past decade several immunoassays for detection of staphylococcal enterotoxins (SE) have been developed. Of the assays developed the enzyme-linked immunosorbent assay (ELISA) has been proven to be the most appropriate method for detecting small quantities of SE in foods. In the course of time, however, it has become clear that in using the ELISA equivocal results may be obtained. Therefore, control experiments have to be introduced to check for both false negative and false positive results. In this study the use of anti-idiotypic monoclonal antibody to control false positive ELISA-reactions is described. Using a monoclonal antibody as coating antibody, it has been shown that the anti-idiotypic monoclonal antibody and its F(ab')<sub>2</sub> fragment prevents binding of SE. Addition of anti-idiotypic F(ab')<sub>2</sub> fragments to test samples changed a positive reaction into a negative only if SE is present. Samples with positive reactions without SE remained positive. Therefore F(ab')<sub>2</sub> fragments of anti-idiotypic monoclonal antibodies can be used to recognize false positive reactions.*

### INTRODUCTION

Staphylococcal food poisoning results from the ingestion of enterotoxins (SE) preformed in food by certain strains of *Staphylococcus aureus* (Bergdoll, 1970). Symptoms are usually observed 2-6 h following consumption of toxin containing food, and vomiting is the most common manifestation. Detection of SE in epidemiologically implicated food has a high evidential value to confirm an outbreak of *S. aureus* food poisoning. It has been observed that quantities of 0.5-5 µg of SE in a portion of consumed food can cause symptoms (Bergdoll, 1970). New information showed that a dose of 0.1-0.2 µg of SE is sufficient to result in staphylococcal food poisoning (Kokan and Bergdoll, 1987). Therefore, only sensitive detection methods of SE are of interest. The most appropriate method for detecting SE in food is the enzyme-linked immunosorbent assay (ELISA). This method allows detection of as low as 1-5 ng of SE per g of test sample.

However, in some cases analyses performed with the ELISA do not lead to reliable results. For example, staphylococcal protein A present in test samples is known to

cause false positive results (Koper *et al.*, 1973; Notermans *et al.*, 1982). It has also been demonstrated that lysozyme can cause unreliable results in sandwich ELISA procedures (Essink *et al.*, 1985). Besides the presence of interfering substances, false positive results may also be obtained if the assay is carried out incorrectly.

False negative results can easily be checked by addition of antigen to the test sample. For testing false positive results different possibilities exist. One of these possibilities is the specific blocking of the paratope of the monoclonal antibody bound to the solid phase. After such blocking, no antigen can be bound and the positive ELISA has to render a negative result. In this study we tested the use of an anti-idiotypic antibody as specific blocking agent in the ELISA for detection of staphylococcal enterotoxin B (SEB).

## MATERIALS AND METHODS

*S. aureus strains.* The *S. aureus* strains used included reference strains S6 and 14458 producing SEB and some selected wild type strains. Strains were tested for enterotoxin production using the sac-culture method of Donnelly *et al.* (1967). After inoculation the sac-culture was incubated at 37°C on a rotary shaker (120 rpm.) for 40 h, and then centrifuged at 10 000 g for 10 min. The supernatant was tested for the presence of enterotoxin using the optimal sensitive plate method (OSP-method) as described by Robbins *et al.* (1974). The OSP-method is a modified immuno-gel diffusion technique (Ouchterlony, 1958). Furthermore the presence of the gene encoding SEB was tested by DNA hybridization as described earlier by Notermans *et al.* (1988).

*SEB.* For SEB production, strain 14458 was used. Production and purification was carried out by the method described by Shinagawa *et al.* (1975).

*Polyclonal antibodies (Pabs).* Pabs anti-SEB were produced by immunizing sheep with SEB (Notermans and Dufrenne, 1982). The immunoglobulin G (IgG) fraction was isolated from the serum with the aid of caprylic acid (Steinbuch and Audran, 1969) and stored freeze dried.

*Monoclonal antibodies (Mabs).* Mab 17/17a anti-SEB and anti-idiotypic Mab 17/17a used in this study were earlier produced by Bamberger *et al.* (1986). F(ab')<sub>2</sub> were prepared by digesting anti-idiotypic Mab 17/17a with pepsin (twice crystallized porcine pepsin, Serva Feinbiochemica, Heidelberg, FRG) in 0.07 M-acetate buffer, pH 3.8, containing 0.05 M-NaCl at an enzyme: substrate ratio of 1:100 for 25 h at 37°C.

*ELISA procedures.* For determinations of SEB the sandwich ELISA as described by Notermans *et al.* (1983) was used. However, instead of Pab's now Mab 17/17a was used as coating antibody. The principle of the sandwich ELISA is schematically presented in Figure 1 (A). The concentration of IgG used for coating was 5 µg/ml. After sample incubation the amount of absorbed SEB was detected using sheep anti-SEB IgG conjugated to peroxidase. Conjugation of IgG with enzyme was carried out with periodate (Nakane and Kawaoi, 1974). The enzyme reaction was determined spectrophotometrically at 450 nm after addition of 5-amino salicylic acid and H<sub>2</sub>O<sub>2</sub>. The IgG peroxidase conjugate concentration was determined by checkerboard titration. An ELISA is considered to be positive if the extinction is equal or higher to the average extinction value from 10 blank samples + 2.81 × standard deviation of these blank values (confidence limit 99%).

*Inhibition experiments.* For inhibition of positive ELISA reactions the anti-idiotypic Mab 17/17a was added to the test sample at various concentrations. For this purpose the complete IgG was used as well as the F(ab')<sub>2</sub> fragment of the IgG. The principle of this inhibition test is schematically presented in Figure 1 (B1/B2).

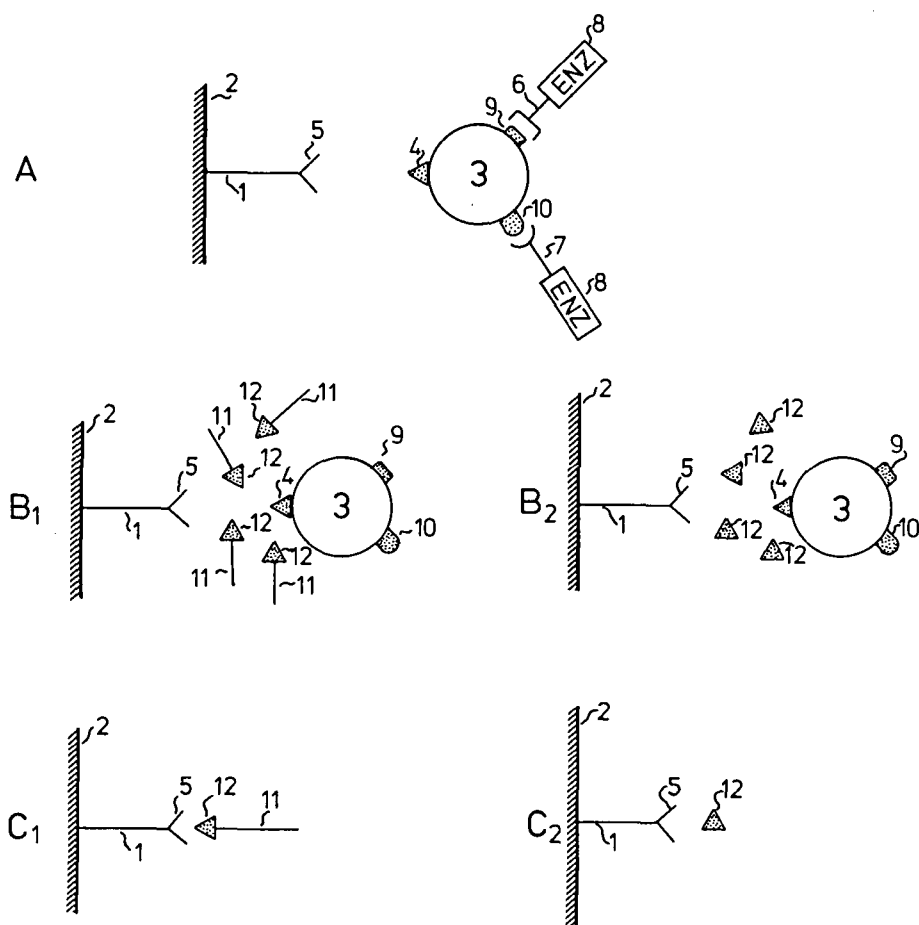


FIG. 1. Detection of staphylococcal enterotoxin by ELISA (A) and possibilities for controlling false positive reactions. (B1, B2 and C1, C2).

(A) Sandwich ELISA for the detection of staphylococcal enterotoxin B (SEB). The monoclonal antibody (1) is coupled to a solid surface (2). The SEB antigen (3) will be bound by its epitope (4) to the paratopes (5) of the antibody (1). Thereafter polyclonal antibodies (6, 7) are added. These antibodies, conjugated with an enzyme marker (8) will bind to the epitopes (9, 10) of the SEB antigen (3). The enzyme activity is related to the presence of SEB.

(B1) Specific inhibition by competition. Now to the test sample, containing the SEB antigen (3), a quantity of anti-idiotypic monoclonal antibody (11) is added. The paratope (12) of these antibodies are equal to the epitope (4) of the SEB molecule (3). Therefore the paratope (12) will compete with the epitope (4) for binding to the paratope (5) of the monoclonal antibody (1). If sufficient anti-idiotypic antibody (11) is present, no SEB antigen (3) will be coupled and it will be removed by subsequent washing. This results in a negative ELISA reaction.

(B2) Identical to B1, however instead of anti-idiotypic monoclonal antibody, only the  $F(ab')_2$  fragment of it are added (12).

(C1) Specific inhibition by blocking. After coupling of the monoclonal antibody (1) to the solid surface (2) anti-idiotypic monoclonal antibody (11) is added. This antibody will be coupled specifically by its paratope (12) to the paratope (5) of antibody (1). Now no binding of the SEB antigen is possible and the ELISA reaction has to be negative.

(C2) Identical to C1, however, instead of anti-idiotypic monoclonal antibody,  $F(ab')_2$  fragments (12) of it are added.

**Blocking experiments.** In the blocking experiments the anti-idiotypic Mab 17/17a IgG and its F(ab')<sub>2</sub> fragment were added at various concentrations immediately after coating with Mab 17/17a. The principle of this blocking test is presented in Figure 1 (C1/C2).

**Lysozyme.** Lysozyme used was Sigma grade 1 lysozyme. Lysozyme was tested in ELISA experiments only after heating the enzyme at 100°C for 5 min. (Essink *et al.*, 1985).

**Preparation of food extracts.** Foods were homogenized with an equal amount of distilled water in a Colworth stomacher. After centrifuging, the supernatant was adjusted to pH 7.2 using 1 M-NaOH and centrifuged again.

## RESULTS

**Inhibition and blocking capacities.** The inhibitory concentrations of anti-idiotypic Mab 17/17a and its F(ab')<sub>2</sub> fragment were tested in the sandwich ELISA. The 50% inhibitory concentration of anti-idiotypic Mab 17/17a and its F(ab')<sub>2</sub> fragment varied from 0.4 to 0.6 µg/ml (Table 1). An inhibition of 100% was obtained at a concentration of approximately 1.5–2.6 µg/ml. In all further experiments 5 µg/ml either anti-idiotypic Mab 17/17a or its F(ab')<sub>2</sub> fragment was used in the ELISA B1 and B2 (see Figure 1). In the blocking experiments, in which both anti-idiotypic Mab 17/17a and its F(ab')<sub>2</sub> fragment were used specifically to block the coated Mab 17/17a (ELISA C1 and C2, see Figure 1), a 100% blocking was obtained at a concentration of approximately 1.1 µg/ml (Table 1). In all further experiments carried out with ELISA C1 and C2 (see Figure 1) 2 µg/ml of each blocking reagents was used.

TABLE 1. Inhibition and blocking capacities of anti-idiotypic Mab 17/17a and its F(ab')<sub>2</sub> fragment in the sandwich ELISA for the detection of SEB.

| ELISA <sup>a</sup> | SEB<br>concentration<br>(ng/ml) | Inhibitory<br>concentration<br>(µg/ml) |      | Blocking<br>concentration<br>(µg/ml)<br>100% |
|--------------------|---------------------------------|----------------------------------------|------|----------------------------------------------|
|                    |                                 | 50%                                    | 100% |                                              |
| B1                 | 100                             | 0.5                                    | 2.6  | —                                            |
|                    | 500                             | 0.6                                    | 2.0  | —                                            |
| B2                 | 100                             | 0.4                                    | 1.5  | —                                            |
|                    | 500                             | 0.5                                    | 1.9  | —                                            |
| C1                 | 100                             | —                                      | —    | 0.9                                          |
|                    | 500                             | —                                      | —    | 1.0                                          |
|                    | 2500                            | —                                      | —    | 1.0                                          |
| C2                 | 100                             | —                                      | —    | 1.0                                          |
|                    | 500                             | —                                      | —    | 1.1                                          |
|                    | 2500                            | —                                      | —    | 1.1                                          |

<sup>a</sup>See for ELISA applied Figure 1.

**Reaction of culture fluids.** In the reference strains S6 and 14458 the SEB gene was found to be present (Table 2). In culture fluid of both strains SEB was present as tested with the OSP-method. With the ELISA A (see Figure 1), also positive reactions were observed. These reactions did not occur neither in the inhibition ELISA B1 and B2 nor in the blocking ELISA C1 and C2. These results clearly indicate that in the culture fluid tested, SEB was present. Additionally, *S. aureus* strains were selected which did not produce SEB as tested by the OSP-method. In the ELISA, however, culture fluids of these strains gave weak to strong reactions. Hybridization experiments carried out

with some of these strains did not show the presence of the gene encoding SEB (Table 2). Culture fluid of these SEB negative strains again showed false positive reaction with the ELISA A (see Figure 1). Addition of anti-idiotypic Mab 17/17a (ELISA B1) resulted for culture fluid of strain OB36 and strain VC in a negative ELISA reaction. With strain 913 a decreased reaction was observed, while with strain K801 an reaction identical to ELISA A was observed. If sheep anti-SEB IgG was added instead of anti-idiotypic Mab 17/17a, identical results were obtained. However, addition of F(ab')<sub>2</sub> fragments of anti-idiotypic Mab 17/17a (ELISA B2) did not result in a decrease of the ELISA reaction. Using the blocking ELISA C1 and C2, identical results were obtained as were found in the inhibition ELISA B1 and B2, respectively. As demonstrated in Table 2, lysozyme showed a positive ELISA reaction (ELISA A). This reaction was neither inhibited nor blocked by the F(ab')<sub>2</sub> fragment of the anti-idiotypic Mab 17/17a.

TABLE 2. Reactions of culture fluids of some selected *S. aureus* strains in the different ELISA systems.

| <i>S. aureus</i> strain | Presence of gene encoding SEB | type of SE produced | ELISA <sup>a</sup> applied |                |                |                |                |
|-------------------------|-------------------------------|---------------------|----------------------------|----------------|----------------|----------------|----------------|
|                         |                               |                     | A                          | B <sub>1</sub> | B <sub>2</sub> | C <sub>1</sub> | C <sub>2</sub> |
| S6 (ref. strain)        | +                             | SEB                 | >5000 <sup>b</sup>         | <5             | <5             | <5             | <5             |
| 14458 (ref. strain)     | +                             | SEB                 | 2500                       | <5             | <5             | <5             | <5             |
| OB36                    | —                             | —                   | 1000                       | <5             | 1000           | <5             | 1000           |
| VC                      | —                             | SEC                 | 100                        | <5             | 100            | <5             | 100            |
| K108                    | —                             | SED                 | 10                         | 10             | 10             | 10             | 10             |
| 913                     | —                             | SEA, SEC            | 1200                       | 50             | 1200           | 50             | 1200           |
| Lysozyme (1 mg/ml)      |                               |                     | 100                        | 80             | 100            | 90             | 100            |

<sup>a</sup> See for ELISA applied Figure 1.

<sup>b</sup> Titer is reciprocal of dilution of culture fluid and lysozyme solution respectively, giving a positive result in the ELISA.

*Testing of food extracts.* Different food extracts were prepared and different quantities of SEB were added. The different samples were tested in ELISA A and in the blocking ELISA C2. Cheese extract, meat extract and lettuce extract to which SEB was added showed positive reactions in ELISA A. These reactions did not appear in the blocking ELISA C2. However, if lettuce extract was supplemented with lysozyme, reactions in the blocking ELISA C2 were observed. With walnut extract without SEB, reaction in ELISA A were observed as well as in ELISA C.

## DISCUSSION

ELISA systems are widely applied for detection of, among others, bacterial metabolites such as toxins. In the past it has been demonstrated that by testing culture fluids, faecal material or food extracts by ELISA reliable results are not always obtained (Essink *et al.*, 1985; Dhand *et al.*, 1988). In this study anti-idiotypic antibodies were examined for their use in distinguishing false positive reactions. For this purpose monoclonal antibodies were used as coating antibody in the ELISA. Anti-idiotypic monoclonal antibodies were used to specifically block the paratopes of the coated antibodies. In this way binding of antigen is prevented. Subsequent addition of enzyme-linked polyclonal antibodies does not result in binding of enzyme and a negative ELISA reaction is obtained. A true positive reaction can only be confirmed when specific blocking results in a negative reaction in the ELISA. From the results obtained it became clear that both the anti-idiotypic monoclonal antibody and its F(ab')<sub>2</sub> fragment prevents binding of SEB to the coated anti-SEB-monoclonal anti-

TABLE 3. Testing of food extracts to which SEB was added in the ELISA.

| Type of food                                          | Quantity of SEB added (ng/ml) | ELISA <sup>a</sup> |    |
|-------------------------------------------------------|-------------------------------|--------------------|----|
|                                                       |                               | A                  | C2 |
| Cheese extract                                        | 10                            | + <sup>b</sup>     | —  |
|                                                       | 100                           | ++                 | —  |
| Lettuce extract                                       | 10                            | +                  | —  |
|                                                       | 100                           | ++                 | —  |
| Lettuce extract supplemented with 1 mg of lysozyme/ml | 0                             | ++                 | ++ |
|                                                       | 10                            | ++                 | ++ |
|                                                       | 100                           | ++                 | ++ |
| Meat extract                                          | 10                            | +                  | —  |
|                                                       | 100                           | ++                 | —  |
| Walnut extract                                        | 0                             | +                  | +  |
|                                                       | 10                            | ++                 | +  |
|                                                       | 100                           | ++                 | +  |

<sup>a</sup> See for ELISA's applied Figure 1.<sup>b</sup> — No reaction observed.

+ Reaction observed in undiluted extract.

++ Reaction observed in 1/10 diluted extract.

body. Prevention of binding of SEB was obtained both by addition of the anti-idiotypic antibody to the SEB containing test samples and by a previous blocking of the coated monoclonal antibody.

To distinguish false positive results, culture supernatant of some selected *S. aureus* strains was used. These strains showed positive ELISA-reactions. These reactions were false positive since it was shown by DNA-DNA hybridization that the gene encoding SEB was absent. Addition of the complete anti-idiotypic monoclonal antibody resulted in a negative ELISA reaction for some of the strains (strain OB36 and VC). Addition of the F(ab')<sub>2</sub> fragment did not change the original ELISA reaction. An explanation for this is that the cross-reacting substance present in the culture fluid, binds to the Fc part of the immunoglobulin used. Furthermore addition of the whole immunoglobulin (anti-idiotypic Mab) to the test sample binds the cross-reacting substance and avoids its cross-reactivity in the ELISA. Therefore addition of the F(ab')<sub>2</sub> fragment of the anti-idiotypic monoclonal antibody is of advantage in recognizing false positive results.

Identical results were found if the anti-idiotypic monoclonal antibody or its F(ab')<sub>2</sub> fragment were used to specifically block the coated monoclonal antibody (see Figure 1 (C1) and (C2)). After blocking with the complete anti-idiotypic monoclonal antibody (Figure 1 (C1) cross-reacting substances present in a sample may be coupled to the Fc part of the coated Mab and to the coupled anti-idiotypic Mab. Due to this binding, the cross-reacting substance is no longer able to couple the enzyme-linked immunoglobulin. This results in a negative ELISA reaction. However, if F(ab')<sub>2</sub> fragments were used, the false positive ELISA reaction was not inhibited! This is confirmed by the finding that sheep IgG showed an identical effect. In the past the specificity of ELISA results are confirmed by addition of antibodies to test samples (Kokan and Bergdoll, 1987). If neutralization occurs it is assumed that the reaction is due to the antigen present. Our results demonstrate that addition of IgG anti SEB to confirm positive ELISA results is not always sufficient. The IgG added does not only neutralize the antigen present but may also inactivate non-specific components.

The F(ab')<sub>2</sub> fragment of the anti-idiotypic Mab was also used to confirm the

presence of SEB in food samples. It was demonstrated that the use of  $F(ab')_2$  as specific blocking agent (Table 3) was indeed suitable for confirming the presence of low quantities of SEB in food. If samples were used containing cross-reacting substances (lettuce extract to which lysozyme was added and walnut extract) no blocking was observed. Such samples have to be considered to be unsuitable for testing in an ELISA, unless the cross-reacting substances have been removed.

In this study the use of an anti-idiotypic Mab to distinguish false positive ELISA-reactions has been described. Addition of anti-idiotypic  $F(ab')_2$  fragments to test samples changed a positive reaction into a negative only if SEB was present. Samples with positive reactions without SEB remained positive. Finally, it has to be mentioned that a specific inhibition as described here, can also be obtained by using a synthesized peptide which is reactive with the coated Mab. The simplest way to obtain such Mab is to synthesize a peptide according to the aminoacid sequence of SE, to couple it to a carrier and to use the conjugate for immunizing mice.

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