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Enzyme-linked Immunosorbent Assay (ELISA) for Isotype-specific Quantitation of Antibodies to *Staphylococcus aureus* in Patients with Atopic Dermatitis

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An enzyme-linked immunosorbent assay (ELISA) was developed to study the serum antibody response against *Staphylococcus aureus* within four immunoglobulin classes (IgG, IgA, IgM and IgE) in patients with atopic dermatitis (AD) and in normal controls. Soluble antigens released from *S. aureus* Wood 46 (protein A deficient) were partially purified by gel filtration of supernatant culture fluid. Median ELISA activity against *S. aureus* antigens within the IgG and IgE classes was significantly higher in patients than in controls (IgG, $p<0.005$; IgE, $p<0.05$). Patients with severe disease had significantly higher ($p<0.05$) IgG antibody levels than those with mild AD. No such clinical association was found for IgE activity. The antibody levels showed no relation to the serum concentrations of total IgE. *Key words: Staphylococcus aureus; Atopic dermatitis; Serum antibodies; ELISA; IgG; IgE.*

Staphylococcus aureus does not regularly belong to the indigenous microflora of normal skin but is more frequently found in patients with various dermatoses. Patients with atopic dermatitis (AD) show a very high dermal carriage rate for *S. aureus*. Impetigenization occurs frequently in these patients, and the bacterial colonization seems to aggravate the disease or prevent its remission. The role *S. aureus* plays in the pathogenesis of AD is, however, still unclear. Its pathogenetic effect may result simply from direct irritation caused by the bacteria themselves or their products, or immunological hypersensitivity reactions may be elicited against bacterial antigens.

Schopfer et al. (1, 2) used a solid phase radioimmunoassay to show that patients with hyperimmunoglobulinemia E-staphylococcal abscess syndrome (HESA) have high levels of IgE antibodies to *S. aureus* and suggested that these antibodies react with the peptidoglycan interpeptide bridge or an unknown antigenic structure within the bacterial cell wall. They were unable to detect such antibodies in patients with AD. More recently, Walsh et al. (3) reported elevated IgE antibody levels to whole staphylococcal cells in some patients with AD and staphylococcal skin infection, and this finding has been confirmed by others (4). Abramson et al. (5) reported high levels of anti-staphylococcal IgE even in patients with AD without clinical signs of superinfection at the time of serum sampling. Friedman et al. (6), however, subsequently claimed that the use of purified cell walls from *S. aureus* was required for specific quantitation of IgE antibodies by immunoassay as non-specific binding to whole organisms could occur and produce false-positive results.

Elevation of the total serum IgE concentrations seems to represent the most striking immunological abnormality in AD; hypersensitivity reactions of type I may therefore be expected to be involved in the pathogenesis. However, it cannot be excluded that antibodies of other isotypes than IgE participate in bacterial hypersensitivity mechanisms. The latter possibility was examined in the present study by including IgG, IgA and IgM together with IgE in an isotype-specific immunoassay of antibodies to *S. aureus*.

MATERIALS AND METHODS

Patient material

Venous blood was drawn from 45 hospitalized patients (19 men and 26 women) with AD, with or without concurrent rhinitis and asthma (median age 25 years, range 9–75 years). The clinical degree of AD was evaluated by the criteria given by Rajka (7).

Volunteers of hospital staff personnel served as healthy controls. In addition, blood samples were obtained from non-atopic children (4 boys and 3 girls) with the informed consent of their parents. The median age of all controls was 26 years (range 12–78 years). The separated serum samples were stored at −20°C for periods up to 2 years until tested.

Total serum IgE levels were measured by use of PRIST kits (Pharmacia).

Test antigens

Bacterial antigens were purified according to Tolo et al. (8). *S. aureus* Wood 46 (protein A deficient) was kindly provided by Dr P. Oeding, Department of Microbiology, University of Bergen. The bacteria were cultivated in Todd-Hewitt medium for 24 h at 37°C. The supernatant fluid was then collected by centrifugation at 10000 g for 30 min and thereafter dialyzed against running tap water for 3 days and lyophilized. The powder was stored at −20°C, dissolved in deionized water (200 g/l), filtered at 4°C through a Sephadex G-75 column (100 cm×2.5 cm) and eluted with deionised water containing sodium azide (0.2 g/l). Optical density (OD) of the eluate was monitored at 280 nm and the fractions (10 ml) corresponding to the void volume peak were pooled, lyophilized and stored at −70°.

Antisera

Antiserum to purified staphylococcal antigens was raised in three rabbits by repeated subcutaneous and intracutaneous injections. Each injection of 1 ml consisted of 0.5 mg *S. aureus* antigen in phosphate-buffered isotonic saline (PBS), pH 7.6, emulsified with 0.5 ml Freund's incomplete adjuvant and was given every third week for 3 months, whereafter booster injections were given every third month for one year before bleeding. Sera from all rabbits were represented in the serum pool, which was stored at −20°C.

A goat antiserum to IgE was obtained from Nordic (Lot No. 22–980) and its IgG fraction was isolated as described by Brandtzaeg (9). After dilution (1 : 2) in PBS, the IgG fraction was precipitated with ammonium sulphate at 50% saturation at 4°C. Further purification was achieved by stepwise elution on a column of DE52 Whatman cellulose.

Enzyme-linked immunosorbent assay (ELISA)

IgE antibodies were detected by means of an indirect five-layer method. Dynatech M-29 polyvinyl microplates (Lot No. 76) were coated with goat IgG specific for human IgE at 8.6 mg/l in 0.1 M carbonate buffer, pH 9.6. Each well received 100 µl and incubation took place for 5 h at 37°C followed by at least 24 h at 4°C. The wells were then rinsed (0.15 M NaCl containing 0.05% Tween and NaN_3, 0.2 g/l) in an automatic washer (Microwash, Scatron, Norway) which was also used between each of the following steps. All subsequent incubations took place for 1.5 h at 37°C. After the first washing, the wells were filled with 100 µl PBS containing bovine serum albumin (BSA, 5 g/l) and NaN_3 (0.2 g/l). The third incubation step included human serum (100 µl) added at a dilution of 1 : 50 in PBS with BSA, 5 g/l, 0.05% Tween 20 and NaN_3, 0.2 g/l (hereafter called PBS-BT). Purified *S. aureus* antigen at 10 µg/ml in PBS-BT (100 µl) was added in the next step. Then 100 µl of rabbit antiserum to the *S. aureus* antigen (1 : 200 in PBS-BT) was applied. The fifth layer consisted of swine IgG alkaline-phosphatase conjugate specific for rabbit IgG (Orion, Finland) of which 100 µl was added at 1 : 200 in PBS-BT. Finally, each well received 200 µl of a solution (2 g/l) of p-nitro-phenyl disodium phosphate (Sigma) in 0.1 M diethanolamine buffer, pH 9.8. The colour reaction was recorded at 405 nm in a Titertek multiscan spectrophotometer (Flow Laboratories) after incubation at 37°C for 15–20 min.

IgG, IgM and IgA antibodies were detected by means of a direct four-layer method. Costar EIA microplates (No. 3490) were coated with purified *S. aureus* antigen at 10 µg/ml in PBS by incubation for 5 h at 37°C followed by at least 24 h at 4°C. All subsequent incubations and washings were performed as described above. After PBS-BSA incubation, human sera were added at a dilution of 1 : 200 in PBS-BT followed by isotype-specific rabbit antiserum produced in our laboratory and diluted 1 : 8000 in PBS-BT (7). The fourth step included swine IgG alkaline-phosphatase conjugate specific for rabbit IgG. Addition of substrate and reading of OD were likewise performed as above.

All test results were transformed to standard OD units by a computor attachment with reference to readings obtained for twelve control wells, six without addition of human serum (background) and six exposed to a standard dilution of a pool of positive AD sera. Each plate included eighteen test wells for each of three patients. Individual results were based on the mean reading.

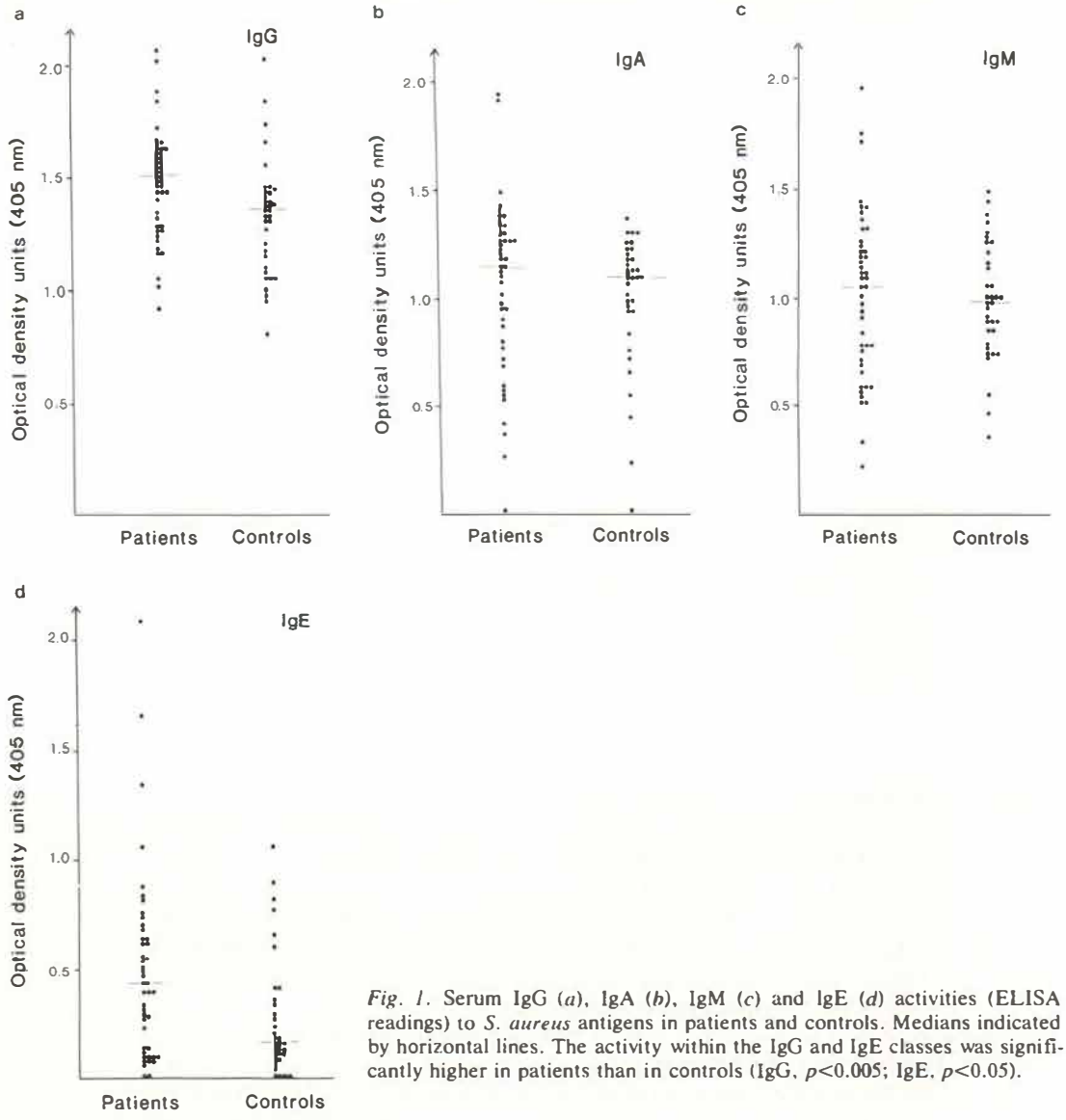

Fig. 1. Serum IgG (*a*), IgA (*b*), IgM (*c*) and IgE (*d*) activities (ELISA readings) to *S. aureus* antigens in patients and controls. Medians indicated by horizontal lines. The activity within the IgG and IgE classes was significantly higher in patients than in controls (IgG, $p<0.005$; IgE, $p<0.05$).

Statistical analyses

The median OD values obtained for the various subject groups were compared by means of Wilcoxon's test for unpaired samples (two-tailed).

RESULTS

Compared with normal controls, the patients showed signficantly increased serum levels of both IgG and IgE antibodies to *S. aureus* antigens (Fig. 1 *a*, *d*). IgA and IgM activities were similar in both groups (Fig. 1 *b*, *c*). No correlation was found between total serum IgE concentrations and IgE antibody activities (data not shown).

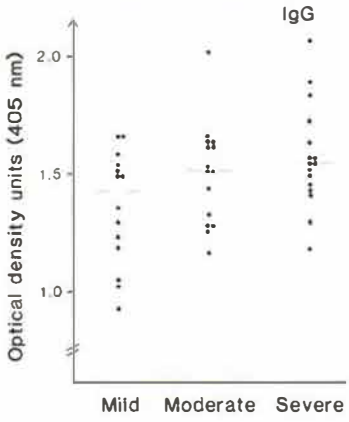

Fig. 2. Relation between serum IgG activities (ELISA readings) to *S. aureus* antigens and clinical severity of disease in patients with atopic dermatitis. Medians indicated by horizontal lines. The difference between patients with mild and severe disease was statistically significant ($p<0.05$).

We were unable to demonstrate any relation between IgE activity and clinical severity of AD or sign of infection at the time of blood sampling. However, patients with severe degree of AD showed significantly increased IgG activity to *S. aureus* antigens compared with patients with mild disease (Fig. 2). There was no correlation between IgG activity and the state of impetigenization, but this clinical variable was difficult to evaluate because most patients had excoriations and thus some degree of infection. *S. aureus* was cultured from 90% of the patients.

DISCUSSION

The notion that patients with AD may show hypersensitivity to antigens of bacteria on the skin has been widely discussed and subjected to several investigations. Skin testing with intradermally injected *S. aureus* antigens has not provided conclusive results, however (10). The recent reports on increased IgE activity to *S. aureus* in patients with HESA (1) and also in patients with AD (3, 4, 5) have revived the interest in bacterial hypersensitivity. It may be visualized that bacterial antigens penetrate the skin and reach the dermis where specific IgE attached to mast cells mediate the liberation of histamine and other mediator products which produce pruritus and worsening of the disease.

The initial reports of increased IgE activity to *S. aureus* (3, 4, 5) were based on assays with whole bacterial cells. According to some authors (6) such assays may produce false-positive results. We therefore developed an indirect ELISA method for quantitation of IgE antibodies based on partially purified soluble antigens from culture fluid. Our results confirmed that most AD patients have raised serum IgE activity to *S. aureus*. However, we were unable to show any correlation between this activity and clinical severity of disease or degree of impetigenization.

An increased production of IgE antibodies to *S. aureus* in AD may therefore be without pathogenetic significance. Individual patients with AD may indeed produce IgE to a large number of different antigens which seem to be without clinical significance (11). The fact remains, however, that a high degree of *S. aureus* colonization on the skin seems to intensify AD. It is as yet not possible to exclude that direct irritation by bacterial products explains this clinical observation.

It has previously been observed in patients with AD that a high carriage rate of *S. aureus* on the skin and a disrupted epidermal barrier may lead to increased levels of staphylococcal antibodies of classes other than IgE (12, 13). In our study IgG activity to *S.*

aureus antigens was significantly increased in AD; and this activity was higher in patients with severe and widespread disease than in the mildly affected ones. Our results could merely reflect stimulation of a second line of immunological defense following a deteriorated epidermal barrier function. IgG seems to be the most important antibody class for opsonization of staphylococci (14).

Persistent production of specific IgG may, in addition, be of pathogenetic importance as antibodies of this class can cause hypersensitivity reactions of both type 2 and type 3. The possibility of bacterial type 3 hypersensitivity (Arthus reaction) in eczema was suggested by Welbourn et al. (15) as early as 1976, but no immunological evidence has been provided for this theory and their investigations did not include patients with AD. The clinical impression of AD is not in favour of such a mechanism. Nevertheless, the possibility remains that a combination of type 1 and type 3 hypersensitivity is involved in the pathogenesis of AD. An IgE-mediated reaction to *S. aureus* antigens may initiate the lesion and explain the dominating itching symptom; subsequently a type 3 reaction may be more important for chronicity and relapse of the disease. Indeed, complement (C) activating IgG antibodies to *S. aureus* antigens may be involved even in the primary symptoms as split products of C3 and C5 are able to produce histamine release from mast cells (16).

There is evidence that immediate-type hypersensitivity may be mediated also by IgG (17), perhaps mainly the IgG_4 subclass of such antibodies (18). Specific IgG_4 antibodies have been demonstrated to several allergens (19) and Shakib et al. (20) reported elevated serum levels of IgG_4 in AD patients. Wutrich et al. (21), however, were unable to confirm this finding in children who suffered exclusively from AD. With the present availability of monoclonal antibodies to the IgG subclasses it will be possible to characterize further the IgG antibody response to *S. aureus* in AD.

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