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Immunological response to outer membrane vesicles of Haemophilus influenzae in patients with acute sinusitis

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SUMMARY

The immunological response of 30 patients with acute sinusitis was examined by using an enzyme-linked immunosorbent assay (ELISA) designed to detect antibodies against outer membrane vesicles of Haemophilus influenzae. Using this ELISA, we found that 15 patients had slight increases in specific antibody in their convalescent serum. Maxillary sinus secretions from 15 patients had specific antibodies. IgG and IgA antibodies were detected with equal frequency, but IgA antibody was often found in maxillary sinus secretions while it was absent from serum. Thus it appears that patients with acute sinusitis respond systemically and locally with the specific antibody to H. influenzae.

INTRODUCTION

Haemophilus influenzae is the leading cause of acute sinusitis (Evans et al., 1975; van Cauwenberge et al., 1976). It has been isolated in approximately one-third of all episodes of acute sinusitis and a retrospective study showed an increasing incidence of H. influenzae in this disease (van Cauwenberge et al., 1976). Although the capsular polysaccharide antigen of typable H. influenzae is accessible to host defense mechanisms, serological typing of H. influenzae isolated from maxillary sinus secretions revealed that less than 10% are type b and that the remainder are non-typable (Shapiro et al., 1980).

These clinical observations stimulated us to investigate the immunological response to the outer membrane vesicles of H. influenzae in patients with acute sinusitis using an enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Bacterial strains

Strains of H. influenzae serotypes a–f were supplied by the Institute of Medical

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Science, University of Tokyo, Tokyo. The strains were: type a (ATCC 9327), type b (ATCC 9334), type c (ATCC 9007), type d (ATCC 9332), type e (ATCC 8142), and type f (ATCC 9833).

Preparation of outer membrane vesicles

As described in detail previously (Harada, 1984), cells were grown to late logarithmic phase, collected, washed, and centrifuged. A modification of the LiCl extraction method of McDade and Johnston (McDade and Johnston, 1980) was used to prepare outer membrane vesicles from intact H. influenzae cells. A quantity (1.0 g, wet weight) of cells harvested in the late logarithmic phase of growth was suspended in 20 ml of LiCl extraction buffer (200 mM LiCl, 100 mM lithium acetate, pH 6.0) in a 50 ml flask containing about 30 glass beads (7 mm diameter). The beads and cells were vigorously agitated on a rotary shaker for 3 h at 45 °C. The cell suspension was decanted, and the beads were washed with three 5 ml volumes of LiCl extraction buffer. The washings were added to the original suspension and centrifuged 12.000 × g for 15 min. The supernatant was then centrifuged at 20,000 × g for 15 min to remove any remaining cells. The suspension was placed within a dialyzing membrane (Visking Co., Chicago, Ill.) and the membrane was covered with dry Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden). After the suspension was concentrated to 3 ml, it was passed through a Sepharone CL-4B column (Pharmacia) and the void volume fraction, which containes outer membrane vesicles, was collected, lyophilized and stored at − 70 °C.

Samples

Samples of maxillary sinus secretion (0.3–1.0 ml) were obtained from 30 patients with acute sinusitis ranging from 7 to 40 years of age. These samples were aspirated by a needle inserted into the maxillary antrum through the inferior meatus. None of the patients were taking antibiotic at the time of the puncture, and they had no disease other than sinusitis. Each sample aspirated from the maxillary sinus was incubated immediately in broth agar bottles, prepared by Clinical Supply Co., Hashima, and transported to the laboratory (Tokyo Clinical Bacteriological Laboratories, Tokyo) as quickly as possible for aerobic and anaerobic culture. Fresh samples of maxillary sinus secretions were centrifuged at 986 × g for 15 min after dilution (1 : 2) in phosphate-buffered saline (PBS). The supernatant were stored at − 70 °C until they were assayed. Pair sera were obtained. "Acute sera" were obtained at the same time of the puncture, and "convalescent sera" were taken about three weeks later. Normal control sera were also obtained from 15 healthy subjects (6 children and 9 adults). All sera were kept at − 70 °C until the assay.

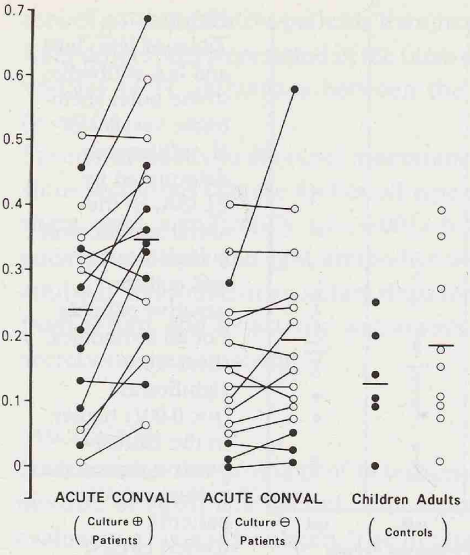

Figure 1

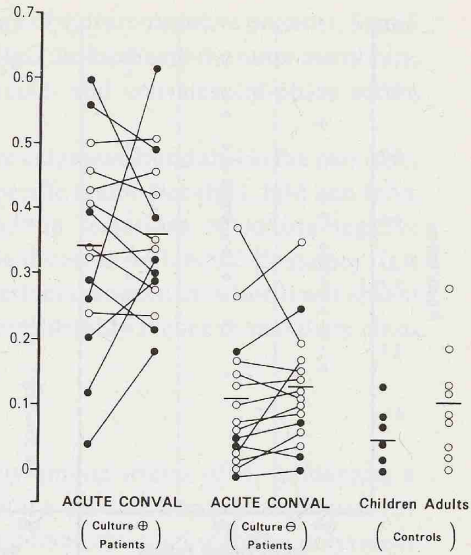

Figure 2

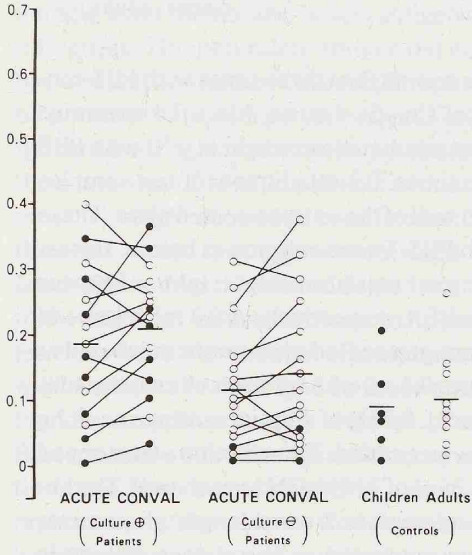

Figure 3

Figures 1-3.
Titers of IgG (Figure 1), IgM (Figure 2), and IgA antibodies (Figure 3) to the outer membrane vesicles of H.influenzae, determined by ELISA, in acute- (ACUTE) and convalescent-(CONVAL)phase sera from patients with acute sinusitis and sera from healthy children and adults. For both IgG and IgM antibodies, titers were significantly ($p < 0.01$) higher in sera of culture-positive patients than in sera of culture-negative patients. Closed circles: children; open circles: adults. The ordinate shows the absorbance ELISA values.

ELISA procedure

The antibodies against outer membrane vesicles were quantified by enzyme-linked immunosorbent assay (ELISA), as previously described (Harada, 1984). Briefly, the outer membrane vesicles of H. influenzae diluted with carbonate buffer (0.005 M, pH 9.6) were added to a new polystyrene microtiter plate (Nunc, Roskilde, Denmark) and adsorbed for 18 h at room temperature. The antigen

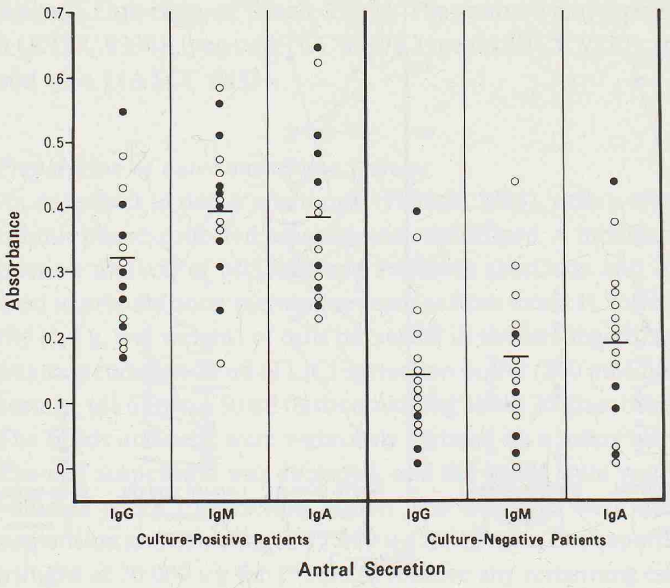

Figure 4.
Titers of IgG, IgM and IgA antibodies to the outer membrane vesicles of H. influenzae, determined by ELISA, in the antral secretions of culture-positive and culture-negative patients. For all antibodies, titers were significantly ($p < 0.001$) higher in the culture-positive than in the culture-negative patients. Closed circles: children; open circles: adults.

solution was aspirated and the microplate was washed three times with PBS containing 0.05% Tween-20 (Sigma Chemical Co., St. Louise, Mo.). To minimize nonspecific reactivity, the plates were next incubated overnight at 4 °C with PBS containing 1% BSA. After washing three times, 0.3 ml aliquots of test samples with PBS-1% BSA were transferred to each well of the antigen-coated plate. These plates were again washed three times with PBS-Tween solution as before. Horseradish peroxidase (HRP) conjugated with goat anti-human IgG, IgM or IgA was diluted 1 : 1000, 1 : 250 or 1 : 500 in PBS-1% BSA, respectively. A 0.3 ml volume of this solution was added to each, and the antigen-coated plates were incubated at 37 °C for 1 h. After the washing procedure, 0.3 ml of a solution of enzyme substrate (2 g of 5-amino-2-hydroxynenzoic acid, 0.03 g of 4-amino-antipyrine, 1.7 g of KH_2PO_4, 3.5 g of K_2HPO_4, H_2O_2 10 µl) was added. The reaction was stopped after incubation for 1 h at 37 °C by adding 25 µl of 1 N NaOH to each well. The absorbance was measured with a Corona microplate 2-wave length photometer ($\mu_1 = 450$ nm, $\mu_2 = 660$ nm). Results were expressed as absorbance of sample minus that of antigen-free control to correct for non-specific adherence.

RESULTS

In all, 15 patients had a positive culture for H. influenzae and none had previous acute sinusitis or treatment. The distribution of serological results for the culture-positive and culture-negative groups is presented in Figures 1–3.

For both IgG and IgM antibodies, titers were significantly ($p < 0.01$) higher in

sera of culture-positive patients than in sera of culture-negative patients. Significant differences were noted in the titers of IgG antibodies to the outer membrane vesicles of H. influenzae between the acute- and convalescent-phase serum ($p < 0.0.1$).

Specific antibody to the outer membrane vesicles was found also in the maxillary sinus secretions (Figure 4). For all type-specific antibodies (IgG, IgM and IgA), titers were significantly ($p < 0.001$) higher in secretions of culture-negative patients and IgG and IgM antibodies were detected with equal frequency. IgA antibody was found in maxillary sinus secretions on occasions when it was absent from serum, and its activity was always considerably higher in maxillary sinus secretions than in serum.

DISCUSSION

To overcome the problem of heterogeneity among strains of H. influenzae, a mixture of equal amounts of outer membrane vesicles from six serotypes was evaluated as coating antigen. The binding curves obtained with the polyvalent antigen were either superior or at least equal to the binding curves obtained with a single outer membrane vesicle antigen of both capsulated and uncapsulated H. influenzae. This polyvalent antigen did not cross-react with antisera against other bacteria, such as Escherichia coli, Streptococcus pneumoniae etc. These data will be presented elesewhere (in preparation).

IgM antibody against the outer membrane vesicles of H. influenzae was found in the acute sera of 14 of 15 patients with acute sinusitis due to H. influenzae. The presence of IgM antibodies correlated with the time after onset of illness at which the acute serum specimen was obtained. The presence of IgM antibodies correlated with neither the clinical degree of illness nor the amount of bacteria present in the maxillary sinus secretions. In future studies acute sera could be obtained at least one week after the start of symptoms to determine if more frequent sampling will increase the rate of IgM antibody positivity in infected patients. IgM was not found in sera from healthy children or adults with exception of two cases.

Bacteriocidal antibodies against noncapsular components of H. influenzae infection have been demonstrated in humans (Dahlberg-Langergård, 1983). In this report we provide evidence that patients with acute sinusitis respond systemically and locally with specific antibody to the outer membrane vesicles of H. influenzae. This antibody response usually developed in the early phase of illness. The mucosal immune response to the outer membrane vesicles appears to take place independent from the serum antibody response. The predominant immunoglobulin classes in the serum were IgG and IgM. The relative proportions of class-specific antibodies in the maxillary sinus secretions as compared with the serum supports our opinion that mucosal antibodies result from secretion rather than transudation.

RÉSUMÉ

La réponse immunologique de 30 malades souffrant de sinusite aigüe était étudié à l'aide d'ELISA (Enzyme Linked ImmunoSorbent Assay) dirigé vers les vésicules de la membrane externe d'Haemophilus influenzae.

Cet ELISA a montré que 15 malades avaient une légère augmentation d'anticorps spécifiques sériques après la phase aigüe. Chez 15 malades d'anticorps spécifiques étaient retrouvés dans les sécrétions du sinus maxillaire. Malgré que l'IgG et l'IgA étaient retrouvés avec la même fréquence, l'IgA était présent plus fréquemment sans les sécrétions du sinus maxillaire s'il était absent dans le sérum. Il paraît que des malades souffrant de sinusite aigüe présentent une réaction générale et locale avec des anticorps contre H. influenzae.

REFERENCES

1. Cauwenberge P van, Verschraegen G, Renterghem L van. Bacteriological findings in sinusitis. Scand J Infect Dis 1976; Suppl. 9:72–7.
2. Dahlberg-Langergård T. Bactericidal antibodies against noncapsular components of H. influenzae. J Clin Microbiol 1983; 17:428–31.
3. Evans FO Jr, Sydnor JB, Moore GR et al. Sinusitis of the maxillary antrum. New Engl J Med 1975; 293:735–9.
4. Harada T. Immunological response to outer membrane protein of H. influenzae in patients with acute sinusitis. Mie Med J 1984; 33:395–405.
5. McDade RL Jr, Johnstone KH. Characterization of serological dominant outer membrane proteins of Neisseria gonorrhoeae. J Bact 1980; 141:1183–91.
6. Shapiro ED, Milmoe GJ, Wald ER, Rodnan JB, Bowen A. Bacteriology of the maxillary sinuses in patients with cystic fibrosis. J Infect Dis 1980; 146:589–93.

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