Antibody Response to Arcanobacterium haemolyticum Infection in Humans

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Arcanobacterium haemolyticum causes pharyngitis, exanthema, and other infections. The evidence of the pathogenicity of A. haemolyticum depends on clinical descriptions of culture-positive patients and a comparison of carrier rates of patients with pharyngitis and healthy, matched controls. In this investigation, the antibody response of the host was studied for the first time, using SDS-PAGE and Western blot analyses. Paired acute and convalescent sera showed development of antibodies to A. haemolyticum in 7 of 8 patients. The antibodies reacted primarily with four distinct cell wall–associated proteins with estimated molecular masses of 80, 60, 50, and 30 kDa. Moreover, the reactivity of convalescent sera from 19 patients was compared with that of sera from 19 controls. Antibodies to A. haemolyticum were found in sera from 16 patients and 6 controls (P < .005); the antibody response of the patients was strong compared with that of the controls. These results indicate that A. haemolyticum infection induces an antibody response in the host.

In the present study, we investigated the reactivity of acute and convalescent sera of patients with pharyngitis and of control persons, using the Western blot technique with cell wall–associated extracts of A. haemolyticum.

Materials and Methods

Patients and controls. Acute and convalescent sera were obtained from 9 patients (aged 15–40 years), 6 with tonsillitis, 1 with a peritonsillar abscess (yielding growth of A. haemolyticum), and 2 with pharyngitis. All of the patients, except for 1 with pharyngitis, also had a rash. Acute sera were sampled within 1 week from the isolation date of A. haemolyticum, whereas convalescent sera were sampled from 2 weeks to 3 months later. In addition, convalescent sera from 19 patients (aged 14–40 years) were compared with sera of age- and sex-matched control persons. Eleven of 19 patients had tonsillitis, 1 had a peritonsillar abscess (yielding growth of A. haemolyticum), 6 had pharyngitis, and 1 had rash without throat symptoms. In all, 13 of 19 patients had rash. The control persons were from the same population as the patients, and the sera were sampled during the same time period at an orthopedic emergency unit. The control persons had no signs of infection.
Bacterial strains and culture conditions. A strain of *A. haemolyticum* (designated as 49c, isolated from a pharyngitis patient in University Hospital MAS) was grown in Todd-Hewitt broth containing 10% heat-inactivated human serum for 48 h at 37°C. It was also grown in the same broth containing 0.5% human albumin instead of serum.

Preparation of bacterial cell wall–associated extracts. Bacteria were harvested by centrifugation (5300 g, 15 min) and washed three times in TRIS-buffered saline (TBS; 0.1 M TRIS-HCl, 0.15 M NaCl), pH 7.4, and suspended in 15 mL of the same buffer containing 1 mM phenylmethylsulfonyl fluoride. The bacterial suspension was sonicated on ice three times, 1 min each, and unbroken cells were removed by centrifugation (5300 g, 10°C, 15 min). The supernatant was ultracentrifuged (100,000 g, 15 min), and the cell wall–associated fraction was collected in the pellet. The pellet was then washed with TBS and with 1 mL of electrophoresis buffer (0.065 M TRIS-HCl, pH 6.8, containing 5% β-mercaptoethanol, 2% SDS, 0.03% bromophenol blue, and 10% glycerol).

**SDS-PAGE and immunoblotting.** The heated (100°C, 10 min) cell wall–associated extract was resolved by SDS-PAGE in a discontinuous buffer system [8]. The resolving gel contained 10% polyacrylamide, and electrophoresis was done in a Mini-protean II cell (Bio-Rad, Richmond, CA) at 30 mA until the tracking dye migrated to the bottom of the gel (~80 min).

After SDS-PAGE, gels were stained with Coomassie brilliant blue [9], or the proteins from the gel were electrophoretically transblotted onto a nitrocellulose membrane (Sartorius, Göttingen, Germany) using a Trans-Blot cell (Bio-Rad). The transfer buffer, pH 8.3, contained 0.025 M TRIS-HCl, 0.2% glycine, and 20% methanol. The blotted nitrocellulose membrane was cut into strips, and the free sites of the blot strips were blocked by incubating in TBS containing 2% bovine serum albumin (grade V; Sigma, St. Louis) at room temperature for 4 h. Thereafter, the blot strips were probed with appropriate serum diluted 1:200 in TBS containing 0.5% bovine serum albumin and incubated overnight at room temperature on a gentle rocker. After being washed three times with TBS containing 0.1% Tween 20 (Sigma) and then three times with TBS, the blots were incubated with peroxidase-conjugated rabbit anti-human IgG antibodies (γ-chain–specific) (Dako, Glostrup, Denmark) at a dilution of 1:2000 in TBS containing 0.5% bovine serum albumin. Two hours later, the blots were washed as described above and developed with chloronaphthol color reagent (Bio-Rad) according to the manufacturer’s instructions. After development for 5–10 min, the reagent was discarded and the blots were washed with distilled water.

**Results**

**SDS-PAGE.** The gels from the SDS-PAGE revealed several dense bands, indicating a good protein yield in the cell wall–associated extracts (figure 1). The gel from serum-grown bacteria showed several bands from 50 to <20 kDa that were weak or absent in the gel from albumin-grown bacteria. In particular, the 50-kDa band was much stronger in the gel from serum-grown bacteria.

**Immunoblotting with acute and convalescent sera from patients.** Acute and convalescent serum samples from 9 patients were tested with the extract from serum-grown *A. haemolyticum*, and 8 were evaluable. The samples from the ninth patient showed strong and uneven staining of the entire nitrocellulose strip with the acute serum. This made comparison with the convalescent serum sample impossible. The appearance of antibody activity in the convalescent sera against four different proteins with estimated molecular masses of 80, 60, 50, and 30 kDa was observed (figure 1). There was also antibody activity in some of the convalescent sera against smeared components of low molecular masses (from 20 kDa to the end of the lane) and against smeared components in the high-molecular-mass end of the lane. With all sera, as well as the second antibody control only, there was also weak reactivity against a 50-kDa protein.

Of 8 evaluable patients, 7 had antibodies in convalescent sera that were not apparent in acute sera; of the 7, samples from 6 showed reactivity with distinct bands. Of the samples from these 6 patients, 5 had antibodies against the 80-kDa protein, and the intensity of the reactivity varied (figure 1). Samples from 1 patient (no. 4) with a very strong antibody response to the 80-kDa protein also had activity against this protein in acute serum, but of much lower intensity. Samples from the patients with a strong antibody response to the 80-kDa protein also had a more diffuse band superimposed on the sharper band at 50 kDa (e.g., patients 1 and 4). Convalescent sera from 3 patients showed an antibody rise against the 60-kDa protein (e.g., patients 1 and 5), 1 (no. 5) having no other new antibodies. Sera from 2 patients (nos. 3 and 4) also had a rise of antibodies against a 30-kDa protein, while the convalescent and acute sera of 1 patient (no. 6) showed strong reactivity against this protein. In addition, this patient developed antibodies to smeared components of low and high molecular weights in convalescent serum. Convalescent sera of 4 patients had antibodies to smeared high-molecular-weight proteins (e.g., patients 1 and 4). One patient (no. 7) had no specific antibody activity in acute or convalescent sera. In sum, convalescent sera from 7 of 8 patients showed a rise of antibodies, and 6 of these reacted with clearly separated proteins of different molecular weights, while 1 showed no sign of antibody rise.

Acute and convalescent sera from 3 patients were also run with the extract from albumin-grown *A. haemolyticum*. The blots were similar to those obtained with serum-grown bacteria, except for the 50-kDa band, which was absent.

**Immunoblotting with sera from patients and control persons.** Of 19 patients, 16 showed an antibody reaction to one of the 80, 60, 30, or 22-kDa proteins, while 6 of the control persons had antibodies to these proteins (figure 2) (*P < .005, χ² test with Yates’s correction*). However, none of the sera from the controls showed strong reactivity (figure 2). Furthermore, 11 of the patients had very strong reactions (e.g., patients 11 and 12), mostly against the 80-kDa protein.

Eleven of the patients had antibodies against the 80-kDa protein, and 9 were very strong reactions (e.g., patients 11 and
Among the control sera, 5 displayed weak reactivity against the 80-kDa protein (e.g., patients 221 and 342). Six patients (e.g., no. 14), and 1 control person showed antibodies against the 60-kDa protein, while sera from 5 patients (e.g., no. 15) and 1 control person showed weak reactivity against the 30-kDa protein. One patient (no. 16) but no control person had antibodies to the 22-kDa protein. The reactions to the 80-kDa protein were regularly much stronger than the reactions to other proteins. Antibody activity against smeared proteins of low molecular masses was noted in 5 patients (e.g., no. 14) and 1 control person, while the activity against smeared proteins of high molecular masses in 5 patients (e.g., no. 13) and none of the control persons was also noted.

Analysis of the interaction of secondary antibody with A. haemolyticum. When A. haemolyticum was grown in serum-containing medium, a reactive band at the 50-kDa position was present in the second antibody control blot (figure 1) and in all the blots incubated with patient sera. This band was not present when bacteria were grown in the presence of human albumin instead of serum, giving a nonreactive second antibody control blot (data not shown). Reduced preparations of pure IgG and cell wall–associated proteins from serum-grown A. haemolyticum reacted with a γ-chain–specific antibody at the 50-kDa position.

Discussion

Seven of 8 patients formed antibodies against A. haemolyticum after an infection with this organism. Convalescent sera of 6 of these patients reacted with clearly separated cell wall–associated proteins of different molecular masses, while the seventh patient showed reactivity against smeared components with low molecular masses, a less distinct picture but still a sign of host reaction against an infecting organism. In addition, the acute and convalescent sera of this patient also reacted with the 30-kDa protein. This could have been due to late collection of the acute serum. The exact day of onset of the disease was not always known, so we had to refer to the isolation date of

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**Figure 1.** SDS-PAGE gel of cell wall–associated extract of *A. haemolyticum* grown in medium containing albumin (alb) or serum (ser). Immunoblots from 7 patients with *A. haemolyticum* infection tested against cell wall–associated extract of *A. haemolyticum* grown in serum-containing medium, with acute (a) and convalescent (c) sera and second antibody control. Nos. = patient identity. Closed arrowheads indicate specific reactions; open arrowheads indicate unspecific reactions.

**Figure 2.** Immunoblots from 6 patients with *A. haemolyticum* infection with convalescent sera (2 digits) compared with matched control persons (3 digits). Sera were reacted with cell wall–associated extract of *A. haemolyticum* grown in serum-containing medium. Closed arrowheads indicate specific reactions; open arrowheads indicate unspecific reactions.
the organism, which can be misleading if the patient has been waiting before consulting the physician. This kind of delay probably explains why patient 4 had antibodies against the 80-kDa protein in low concentration in the acute serum and in high concentration in the convalescent serum.

Compared with the sera of the control persons, the sera of the majority of the patients reacted very strongly with the bacterial proteins. The 80-kDa protein seems to be the dominant antigen. The most frequent picture among the patients, a strong reaction to the 80-kDa antigen, is not seen among the control persons. The occurrence of weak reactions among the control persons may be due to cross-reactions with antibodies to other organisms or may reflect remote previous infection with *A. haemolyticum*.

Of the five bacterial proteins inducing an antibody response, the 80-kDa protein produced the most strong and consistent band in the blots and thus can be considered the major antigen. For the same reasons, the 60- and 30-kDa proteins can be considered the second and third most important antigens, respectively.

The regularly appearing band at the 50-kDa position with the serum-grown *A. haemolyticum* extract was due to weak but consistent adsorption of human IgG to the bacteria during culturing. This conclusion is based on the molecular mass of the γ-chain of IgG (50 kDa) and the reactivity with γ-chain-specific antibodies. Finally, this band is eliminated when the bacteria are cultured in serum-free medium.

Previously, the evidence of the pathogenicity of *A. haemolyticum* depended on clinical descriptions of culture-positive patients and on comparison of carrier rates of patients with pharyngitis and healthy, matched controls. This study shows that patients with a throat infection or an exanthema, harboring *A. haemolyticum*, also produce antibodies to this organism during the acute infection.

References