INFECTION AND IMMUNITY, Sept. 1991, p. 2999–3003
0019-9567/91/092999-05$02.00/0

Vol. 59, No. 9

Human Immune Response to an Iron-Repressible Outer Membrane Protein of *Bacteroides fragilis*

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Received 26 October 1990/Accepted 31 May 1991

Under conditions of iron starvation, *Bacteroides fragilis* expresses various iron-repressible outer membrane proteins (IROMPs). A 44-kDa protein appears to be one of the major outer membrane proteins (OMPs) in *B. fragilis* under iron stress and plays a role in heme uptake by this bacterium. To determine whether the 44-kDa IROMP of *B. fragilis* is expressed in vivo and whether this protein is immunogenic, we used Western immunoblotting to examine serum samples from patients with an infection caused by *Bacteroides* species. All the serum samples from patients and from normal controls showed reactivity with several proteins of *B. fragilis*. Only serum samples from patients infected with *B. fragilis* showed immunoreactivity with the 44-kDa protein. We also used a rat infection model to study the immune response against this protein during the process of an intra-abdominal infection in these animals. During the first 8 days of infection a gradual increase of antibodies to the 44-kDa protein in the rat was detected. These results suggest that the 44-kDa IROMP is expressed in vivo, since it induces an antibody response in patients and animals. We also analyzed 85 strains of the *B. fragilis* group for the presence of proteins antigenically related to the *B. fragilis* 44-kDa protein. The data indicate that this protein was conserved in *B. fragilis* strains and was absent in the other bacterial strains tested.

Bacteroides fragilis is a clinically important, gram-negative, nonsporeforming, anaerobic bacterium (3, 4, 10), which is often associated with mixed infections in combination with aerobic bacteria, especially *Escherichia coli* (5, 17). Moreover, *B. fragilis* is the most important anaerobic pathogen in intra-abdominal infections (5, 13), and it plays a crucial role in anaerobic bacteremia in the elderly, for whom an overall mortality of 66.1% as a consequence of these infections has been reported (20). By using animal infection models, several authors (12, 18, 23) have established that *B. fragilis* is more virulent than the more common member of the intestinal flora, *Bacteroides vulgatus*.

In our department, we studied the growth properties of *Bacteroides* species in relation to iron uptake. Only virulent *B. fragilis* strains could grow in iron-restricted media such as serum and plasma (24, 25). Several iron-repressible outer membrane proteins (IROMPs) were found in *B. fragilis* and *B. vulgatus* (16). A 44-kDa protein appears to be one of the major outer membrane proteins (OMPs) in *B. fragilis* under iron stress. In *B. vulgatus* cells this protein was absent under comparable conditions of iron limitation. These results suggest a relationship between the virulence of *B. fragilis*, expression of a 44-kDa protein, and ability to grow in serum. In many pathogens IROMPs are found both in vitro and in vivo during infection (8), and there is increasing evidence that these proteins behave as virulence determinants (6, 7).

Consequently, we were interested in whether the 44-kDa IROMP of *B. fragilis* is expressed in vivo and whether this protein is immunogenic. Therefore, we used Western immunoblotting to examine serum samples from patients with an infection involving *Bacteroides* species for evidence of an immune response to the 44-kDa protein of *B. fragilis*. We also used a rat model of intra-abdominal infection to study the immune response of the animals to this protein. Furthermore, we screened 85 *Bacteroides* strains for their ability to express a 44-kDa protein under iron-limited growth conditions to determine a possible antigenic conservation of this protein in *B. fragilis*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *Bacteroides* strains, *Prevotella ruminicola*, *Porphyromonas asaccharolytica*, and *Escherichia coli* used in this study were all clinical isolates obtained from the University Hospital of Vrije Universiteit. The anaerobic strains were identified with a BBL Minitek Numerical Identification System (Becton Dickinson). *E. coli* EB1 and EB4 (serotypes O8K43 and O21K$^-$, respectively) were identified by the API 20E (API System S.A., Montalieu Vercieu, France). Upon isolation, each strain was passaged only once on 5% horse blood agar plates (no. 2 agar; Oxoid) supplemented with hemin (5 μg/ml; BDH) and menadione (2 μg/ml; Merck); the organisms were then frozen (−70°C) in 50% glycerol until further use. The bacteria were cultured in a tryptone-yeast extract medium (BMP medium) as described before (23). Cysteine and glucose were omitted from this medium. Iron-limited cultures were grown in BMP medium supplemented with 2,2-bipyridyl (80 μM; BDH). In all experiments, the bacterial cells were incubated at 37°C in an atmospheric glove box (McCoy) under an atmosphere of 85% N_2–10% H_2–5% CO_2.

Serum samples. Serum samples were collected from patients with an infection in which species from multiple genera, including *Bacteroides*, were involved. Blood was collected by venipuncture when cultures confirmed the presence of a *Bacteroides* species. All these patients had a suspected intra-abdominal infection originating from the

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gastrointestinal tract (13). In most infections, mixed aerobic and anaerobic microorganisms were found. Normal human serum was obtained from a pool of individuals without a history of *B. fragilis* infection.

Serum samples were also obtained from rats experimentally infected with *B. fragilis*. For this purpose, we used a rat fibrin clot model to initiate intra-abdominal infections in the animals, as described by Verweij et al. (22). Fibrin clots infected with both *B. fragilis* BE1 and *E. coli* EB1 were implanted intraperitoneally in the rats. After 5 days this bacterial inoculum resulted in persistent abscesses containing large numbers of viable *B. fragilis* and *E. coli* organisms. Serum samples were collected daily. The rats were killed 8 days after the inoculation.

Polyclonal 44-kDa protein antiserum was prepared as previously described (14). Briefly, a rabbit was immunized with the 44-kDa protein of *B. fragilis* BE1. For this purpose, proteins of a cell extract obtained from an iron-restricted *B. fragilis* culture (16) were separated by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The 44-kDa protein band was excised from the gel after staining with Coomassie brilliant blue. The stained protein was electrophoretically eluted from the minced gel and dialyzed against buffered saline. The rabbit was bled after immunization with this purified protein. Subsequently, the collected serum was first adsorbed with BE1 cells, which were cultured in BMP medium under iron-replete conditions, and thereafter adsorbed with *B. fragilis* cell extract from which the 44-kDa protein was removed. This cell extract was prepared from bacterial cells grown under iron-limited conditions and blotted onto nitrocellulose paper. The 44-kDa band was excised from the nitrocellulose filter after staining with Ponceau S, and the rest of the proteins were used for the second adsorption. The second adsorption was needed to remove antibodies which cross-reacted with the other IROMPs of *B. fragilis*. The specificity of the antiserum was tested by immunoblotting (see below).

SDS-PAGE and Western blotting. Cell extracts of the various bacterial strains, cultured under iron restriction, were prepared as described previously (16). The proteins (10 μg per lane) were separated by SDS-PAGE (11% [wt/vol] polyacrylamide) (11) and subsequently electroblotted onto nitrocellulose paper by the method of Towbin et al. (21). The presence of a 44-kDa protein was identified by probing the filters first with the 44-kDa protein antiserum and then with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) (14).

B. fragilis BE1 cell extract, cultured under iron stress, was electroblotted on nitrocellulose paper after SDS-PAGE. The blots were used for testing the specificity of the adsorbed rabbit antiserum. Equivalent dilutions (1:8,000) of the nonadsorbed and adsorbed serum samples were used in these tests (14). Another application of these blots was the detection of antibodies against the 44-kDa protein in human and rat serum samples. We also used purified 44-kDa protein for testing the immunoreactivity of the human serum samples. The protein was purified by means of 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS)-PAGE (15), subjected to SDS-PAGE, and subsequently electroblotted onto nitrocellulose paper. The blots were probed with the patient serum, normal human serum, or rat serum, diluted in antibody buffer (20 mM Tris, 500 mM NaCl, 1% [wt/vol gelatin [pH 7.5]). Resulting antigen-antibody complexes were identified by goat anti-human immunoglobulins (IgG, IgM, and IgA; American Qualex) or by goat anti-rat IgG (H plus L chains; Sigma Chemical Co.). Both antisera were horseradish peroxidase conjugated. The procedures for the binding of the primary and secondary antibodies and for staining were the same as described earlier (14).

TABLE 1. Survey of different bacterial species for the expression of an antigenically related 44-kDa portein[a]

Species	No. positive/ no. tested[b]
B. fragilis	32/32[b]
B. vulgatus	0/10
B. distasonis	0/20
B. ovatus	0/14
B. thetaiotaomicron	0/5
B. capillosus	0/3
B. bivius	0/1
P. ruminicola	0/2
P. asaccharolytica	0/2
E. coli	0/2

[a] Among these bacterial strains tested are the isolates from patients from whom serum was collected for testing the immune response to the 44-kDa protein.

[b] Number of strains that were positive/number of strains tested. A positive reactivity indicates the presence of a band for the strain tested with an apparent molecular mass of 44 kDa on Western blots, probed with rabbit antiserum specific for the 44-kDa protein of *B. fragilis*.

RESULTS AND DISCUSSION

Expression of a protein antigenically related to the 44-kDa protein in *Bacteroides* species. Strains of various bacterial species were cultured in an iron-restricted medium. Cell extracts of these cultures were subjected to SDS-PAGE. The slab gels were screened by means of immunoblotting with a polyclonal 44-kDa protein antiserum to test the bacteria for their ability to express this protein under iron limitated conditions.

The polyclonal antiserum was obtained by immunizing a rabbit with purified *B. fragilis* 44-kDa protein. However, this antiserum showed cross-reactivity with other proteins of *B. fragilis* (data not shown). The cross-reacting antibodies were removed by absorption. The specific antiserum thus acquired not only reacted with denatured 44-kDa protein but also recognized the purified native protein and the protein in intact bacterial cells (14). This antiserum did not, however, react with any of the *E. coli* proteins (data not shown).

All 32 *B. fragilis* strains tested were positive for the 44-kDa protein on the immunoblot. This protein was absent in the 59 other bacterial strains tested (Table 1). Moreover, the protein profiles of these strains on SDS-PAGE did not reveal a protein with an approximate molecular mass of 44 kDa (data not shown). These results indicate a conservation of this protein within *B. fragilis* strains.

Human immune response to the 44-kDa IROMP of *B. fragilis*. We were interested whether the 44-kDa IROMP of *B. fragilis* is expressed in vivo and whether this protein is immunogenic. Therefore, we used Western blotting to study serum samples from patients with an infection involving *B. fragilis* for evidence of an immune response to this IROMP. We also tested serum samples from patients infected with other *Bacteroides* species. The involvement of *B. fragilis* was confirmed by positive culture results.

All the serum samples from patients and from a control group of 13 healthy persons reacted with several proteins of *B. fragilis* (Fig. 1). However, the reaction pattern of each serum sample on the blots was highly variable (data not shown). The overall reactivity of the normal serum samples

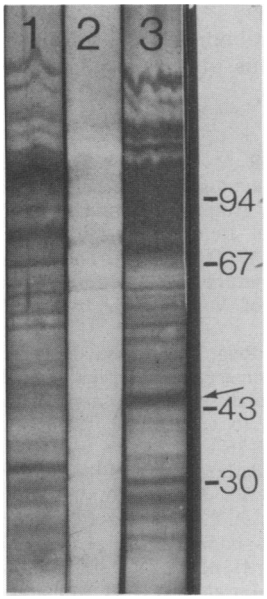

FIG. 1. Western blot of *B. fragilis* cell extract, grown under iron-limited conditions, reacted with patient serum. The patients had an infection involving *Bacteroides* species. Lanes: 1, serum from a patient infected with *B. distasonis*; 2, normal serum; 3, serum from a patient infected with *B. fragilis*. Serum samples were diluted 1:20. The arrow indicates the location of the 44-kDa polypeptide. Molecular masses are indicated in kilodaltons. This picture is representative of all serum samples tested. See Table 2 for the rest of the data.

TABLE 2. Immune response of patients to the 44-kDa protein of *B. fragilis* BE1, as determined by Western blotting

Patient[a]	Site of infection or clinical symptoms	Source of isolate	Isolate[b]	Response
1, 2, 3*–6*	Abdominal abscess	Pus	*B. fragilis*	+
7	Colonic fistula	Pus	*B. fragilis*	+
8	Gall bladder	Pus	*B. fragilis*	+
9	Surgical wound	Pus	*B. fragilis*	+
10	Ovarian abscess	Pus	*B. fragilis*	+
11*	Perforation of sigmoid	?[c]	*B. fragilis*	+
12*	Diverticulitis	?	*B. fragilis*	+
13*	Abdominal abscess	Pus	*B. fragilis*	±
14*	Perforation of sigmoid	?	*B. fragilis*	±
15*, 16	Abdominal abscess	Pus	*B. fragilis*	−
17, 18	Peritoneum	Blood	*B. fragilis*	−
19	Peritoneum	Ascites	*B. fragilis*	−
20*	Peritoneum	?	*B. fragilis*	−
21	Fever	Blood	*B. fragilis*	−
22	Diverticulitis	Blood	*B. fragilis*	−
23	Appendicular abscess	Ascites	*B. ovatus*	−
24	Perianal abscess	Pus	*B. ovatus*	−
25	?	Blood	*B. ovatus*	−
26	Sinus maxillaris	Fluid	*B. vulgatus*	−
27	Perforation of sigmoid	Ascites	*B. vulgatus*	−
28	Diverticulitis	Pus	*B. distasonis*	−
29	Perforation of colon	Ascites	*B. distasonis*	−
30	Liver	Pus	*B. distasonis*	−
31	Gallbladder	Gall	*B. distasonis*	−
32	Peritoneum	Pus	*B. capillosus*	−
33	Appendix	Blood	*B. capillosus*	−

[a] *, Serum samples from patients in Sweden.
[b] Besides *Bacteroides* species, other aerobic and anaerobic microorganisms were isolated in most infections.
[c] ?, not known.

with several proteins of *B. fragilis* was, in general, weaker than that of the serum samples from the patients (Fig. 1). These results show that antibodies directed against various proteins of *B. fragilis* are present in low titers in normal human serum.

Only serum samples from patients infected with *B. fragilis* showed immunoreactivity with the 44-kDa protein. Of the 22 samples from patients infected with *B. fragilis*, 14 recognized this protein in a Western blot assay (Table 2). The other eight serum samples from patients infected with *B. fragilis* (patients 15 to 22) and from patients infected with other *Bacteroides* species (patients 23 to 33) lacked immunoreactivity with the 44-kDa protein (Table 2). Antibodies to this protein were also absent in normal serum, as determined by Western blotting (data not shown). To exclude the possibility that the patient serum samples strongly reacted with a protein of a similar molecular mass to the 44-kDa IROMP, we also tested the reactivity of these samples with the purified protein (Fig. 2). Only samples from patients with a *B. fragilis* infection showed immunoreactivity with the purified protein. Some patient serum samples reacted both with the 44-kDa polypeptide and with degradation products of this protein in SDS-PAGE (lanes 2 and 3). The presence of an antibody response to the 44-kDa protein suggests that this protein is expressed in vivo.

Some patients with a *B. fragilis* infection (patients 13 and 14) showed a weak immune response to the 44-kDa protein (Table 2), whereas other patients showed no antibody response to this protein (patients 15 to 22). The *B. fragilis* strains isolated from these negatively responding patients all expressed a 44-kDa protein in vitro (Table 1). This phenomenon could be explained by the presence of small numbers of *B. fragilis* cells in the patient and hence the minor role of this bacterium in the infection. Another explanation could be the fact that the serum samples were not all obtained at similar stages of infection. In all cases the time interval between the isolation of bacteria from patients and the removal of a blood sample for antibody testing was at least 10 days. However, we cannot exclude the possibility that in some patients the infection was present for a longer period and that higher titers therefore developed.

The results of this study suggest that the 44-kDa protein of *B. fragilis* is both expressed and antigenic in vivo. In vivo expression of IROMPs has been described in the literature for *Vibrio cholerae* (19) and *Pseudomonas aeruginosa* (1). In addition, antibodies to the IROMPs of *E. coli* have been detected in the serum of healthy persons (9), and in patients with meningococcal or gonococcal disease, antibodies to the major IROMP of these bacteria have also been found (2).

Rat immune response to the 44-kDa IROMP of *B. fragilis*. Rats infected with both *B. fragilis* and *E. coli* were regularly bled during a period of 8 days. The serum samples were tested to monitor the appearance of antibodies to the OMPs of *B. fragilis*, especially the 44-kDa IROMP, as determined by Western blotting. Figure 3 shows the results of this experiment. Antibodies to proteins of *B. fragilis* were absent in the rat serum samples during the first 6 days of infection. After that period, an increasing immune response to the 44-kDa IROMP was observed. Beside this major immune response, other proteins of *B. fragilis* were recognized by the rat serum. Three proteins with molecular masses less than 44 kDa and a vague band in the higher-molecular-mass

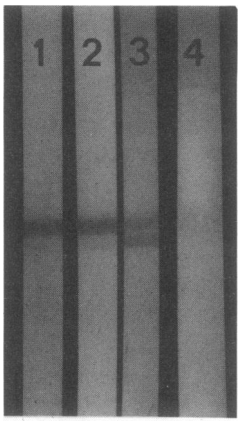

FIG. 2. Western blot of the *B. fragilis* 44-kDa IROMP reacted with patient serum samples. The protein was purified by means of CHAPS-PAGE (see Materials and Methods). The patients had an infection involving *Bacteroides* species. Lanes: 1 to 3, serum samples from patients infected with *B. fragilis*; 4, serum sample from a patient infected with *B. vulgatus*. Serum samples were diluted 1:20. The arrowhead indicates the position of the 44-kDa polypeptide. This picture is representative of all serum samples tested.

region were also detected (Fig. 3). Antibodies to proteins of *B. fragilis* were absent in the preinfection serum samples (data not shown).

Results from earlier studies suggest a relationship between the virulence of *B. fragilis*, expression of a 44-kDa protein, and the ability to grow in serum in which heme was the growth-restrictive factor (16, 24, 25). Recently, we have demonstrated that the 44-kDa IROMP is involved in the uptake of heme by *B. fragilis* (14). The ability to take up heme very efficiently from its environment could make an important contribution to the virulence of this microorganism. The conservation of this protein within *B. fragilis* strains may suggest that this function is indeed essential for the pathogenesis of *B. fragilis* infections. Analysis of serum samples from patients and animals with a *B. fragilis* infection indicated that antibodies specific for the 44-kDa IROMP were present. This suggests that the 44-kDa protein is expressed in vivo.

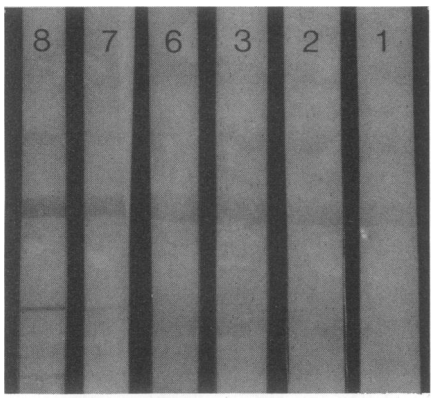

FIG. 3. Western blot of *B. fragilis* cell extract, grown under iron-limited conditions, reacted with rat serum. Serum samples were diluted 1:15. Fibrin clots infected both with *B. fragilis* and *E. coli* were implanted intraperitoneally in rats. The animals were killed 8 days after the inoculation. Serum was collected from the rats during that period. The numbers on the blot indicate the days after the inoculation when serum was collected. The arrowhead indicates the location of the 44-kDa polypeptide.

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