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A 66-Kilodalton Heat Shock Protein of *Salmonella typhimurium* Is Responsible for Binding of the Bacterium to Intestinal Mucus

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***Salmonella typhimurium* infections have increased during the last few years. However, the interplay of virulence factors in *S. typhimurium* pathogenesis is still poorly understood, particularly with regard to the mechanisms and components of the bacterium which are involved in its interaction with the intestinal mucus. We have observed that *S. typhimurium* is aggregated by incubation with colonic mucus (guinea pig model). To quantify this phenomenon, an aggregation assay was established. By using this assay, it was found that the aggregation profile of *S. typhimurium* strains freshly isolated from patients (age 9 and older) with salmonellosis correlated with the severity of the disease. An isolate with high aggregation behavior was chosen for characterization of the bacterial component involved in binding to colonic mucus material. The component of *S. typhimurium* responsible for aggregation was purified and characterized as a 66-kDa protein which was able to completely inhibit mucus-mediated bacterial aggregation. This protein was recognized by monoclonal antibodies against the 65-kDa heat shock protein (HSP) of *Mycobacterium leprae*. The 66-kDa protein of *S. typhimurium* was inducible by incubating the bacteria at 50°C and was secreted into the supernatant, from which it could be isolated in both dimeric and polymeric forms. The monoclonal anti-HSP 65, as well as a polyclonal antibody against the 66-kDa protein of *S. typhimurium*, caused dose-dependent inhibition of the aggregation of *S. typhimurium* by crude mucus preparations. This is the first report showing that a bacterial HSP is involved in mucus-mediated interaction of pathogens with the host.**

Electron-microscopic studies have shown that *Salmonella typhimurium* is associated with and invades the mucosa of the gut (7, 18, 24, 25). Although mucus, a mantle of varying thickness covering brush border epithelial cells of the mammalian large and small intestine, was identified many years ago (1, 11, 17, 22), little attention has been focused on the interaction of *S. typhimurium* with the mucus layer. Literature concerning the bacterial component(s) of *S. typhimurium* which is involved in the binding to mucus is scant. Tannock et al. (25) indicated that the O-antigen, flagella, and pili of *S. typhimurium* do not appear to be essential for association with the mucosal surface of mouse ileum.

Recently, we observed that incubation of guinea pig colon with *S. typhimurium* resulted in binding of the bacteria to the intestinal mucus layer. Furthermore, *S. typhimurium* was found to be aggregated by isolated crude mucus. The agglutinin for *S. typhimurium* obtained from a crude mucus preparation from guinea pig colon was characterized as a 15-kDa glycoprotein (5). Here, we report the isolation and characterization of the component of *S. typhimurium* responsible for such aggregation. Under reduced conditions it is a 66-kDa protein which is recognized by monoclonal antibodies against the 65-kDa heat shock protein (HSP) of *Mycobacterium leprae*. The 66-kDa HSP from *S. typhimurium* is inducible by incubating the bacteria at 50°C and is secreted into the supernatant, from which it can be isolated in both dimeric and polymeric forms.

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MATERIALS AND METHODS

Bacterial strains and medium. *S. typhimurium* strains from patient were freshly isolated from stool probes (12 isolates). The patient (age 32; hence strain P 32) had suffered from chronic diarrhea for more than 1 month. *S. typhimurium* 10303 (L1) and 10305 (L2) were obtained from the culture collection of the Institute of Medical Microbiology, University of Mainz, Germany. For experiments, all strains were grown in nutrient broth (Oxoid, Ltd., Wesel, Germany) at 37°C.

Crude mucus preparation. Colons were removed from male and female outbred guinea pigs (weight, 250 to 400 g). Each colon was slit open longitudinally and washed twice with 0.9% NaCl (prewarmed to 37°C). Mucus was collected in 0.9% NaCl by scraping and incubated for 30 min at 37°C. Cells were sedimented by centrifugation at 650 × *g* for 15 min, and the supernatant was collected and stored at 4°C. The incubation and centrifugation procedures were repeated twice. The supernatants were pooled and centrifuged at 25,000 × *g* for 1 h at 20°C to remove particles and cells. The supernatant was filtered (0.2-μm-pore-size cellulose acetate filter; Nalgene, Rochester, N.Y.), dialyzed overnight against Milli Q water (Millipore, Eschborn, Germany) at 4°C, and lyophilized. The soluble supernatant was classified as crude mucus.

Aggregation assay. Aliquots (50 μl) of bacterial suspension (2×10^9 bacteria per ml; 4.5-h culture, stirred at 37°C at 80 rpm by using an orbital shaker; Heraeus, Hanau, Germany) were sedimented in an Eppendorf tube (Eppendorf, Hamburg, Germany), the supernatants were removed, and the pellets were each resuspended in 50 μl of a mucus solution or in 0.9% NaCl (negative control) and incubated at 37°C for various periods. The sample was vortexed with a Vortex

Genie (Bender & Hobein, Zürich, Switzerland) at maximum speed before microscopic examination. Samples (5 μl) were taken from the tube with an Eppendorf pipette. The numbers of aggregated and nonaggregated bacteria were microscopically determined by using a Neubauer counting chamber. Four large chambers (each consisting of 16 smaller chambers) were assessed. The percentage of aggregated bacteria was calculated by the following formula: percent aggregation = [aggregated bacteria/aggregated + nonaggregated bacteria)] × 100. Nonaggregated bacteria were classified as single bacteria and two linearly connected bacteria. Bacterial aggregates were classified as clusters of three or more bacteria and also two bacteria that were connected but not linearly.

Inhibition assay. Crude mucus material was diluted (1:2) with various concentrations of the test solution and incubated for a given time. As in the aggregation assay, sedimented bacteria were resuspended in 50 μl of these solutions and after a given incubation period the degree of aggregation was determined.

Screening for aggregation-positive and aggregation-negative substrains from *S. typhimurium* P 32. A bacterial suspension (2×10^9 bacteria per ml in 0.9% NaCl) was prepared from an overnight culture of *S. typhimurium* P 32. As in the aggregation assay, bacteria were incubated with mucus for 1 h. The suspension was diluted, plated on Columbia 5% sheep blood agar plates (Becton Dickinson, Heidelberg, Germany), and incubated overnight at 37°C. Four 12-well culture plates (well diameter, 2.5 cm; Nunc, Wiesbaden, Germany) were filled with 2 ml of nutrient broth, and each well was inoculated with a colony from the sheep blood agar plates and incubated at 37°C for 24 h with stirring at 80 rpm; the probes were then assessed by the aggregation assay (10^9 bacteria per ml; 3.6 mg of mucus protein per ml; incubation time, 15 min; 37°C). Four substrains were chosen, of which two were aggregation positive (Sal 5, Sal 33) and two were aggregation negative (Sal 2, Sal 31).

Purification of the 66-kDa protein. An 800-ml portion of a 1-liter *S. typhimurium* P 32/Sal 33 culture (8 h, 170 rpm, 37°C) was harvested (bacterial net wet weight, 3.95 g) and resuspended in 50 ml of 100 mM Na_3PO_4 (pH 7.0). A 10-ml portion of this suspension was added to each of five flasks, containing 150 ml of 100 mM Na_3PO_4 (pH 7.0) prewarmed to 50°C. After the bacterial suspension had been added, the flasks were incubated for 1 h at 50°C in a water bath. Bacteria were sedimented (10,000 × *g* for 30 min), and the supernatant was collected, chilled to 4°C, and precipitated overnight with crystalline ammonium sulfate to a final saturation of 10%. The precipitated material was pelled by centrifugation at 23,000 × *g* for 20 min. The pellet was dissolved in 5 ml of Milli Q water and dialyzed overnight against 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.5) at 4°C. Purification was performed by fast protein liquid chromatography (FPLC; Pharmacia, Freiburg, Germany), using a Mono Q HR (5/5) ion-exchange column. No protein was detected in the effluent. Elution was performed by linear salt gradient (23 ml): 20 mM HEPES (pH 7.5) to 20m mM HEPES containing 1 M NaCl (pH 7.5). Fractions were dialyzed against Milli Q water and tested in the aggregation assay.

Sucrose density gradient ultracentrifugation. A purified sample of the 66-kDa protein dissolved in PBS (15 mM Na_2HPO_4, 15 mM NaH_2PO_4, 150 mM NaCl [pH 7.5]) was applied to a discontinuous sucrose gradient (10 to 40%) in PBS. The protein was sedimented through the gradient at 35,000 rpm for 18 h by using an SW 60 Ti rotor and an L8-80 ultracentrifuge (Beckman, Munich, Germany). Fractions were collected in 500-μl aliquots. The sucrose gradient was checked by measuring the refractory index with a refractometer (Zeiss, Oberkochen, Germany). Sucrose was removed from the fractions by dialysis against Milli Q water. A 150-μl portion from each fraction was removed for detection of the reference substances (bovine serum albumin, 4.5S; human immunoglobulin G [IgG], 7S; and human IgM, 19S) by using commercial enzyme-linked immunosorbent assay kits (Dianova, Hamburg, Germany). The fractions were concentrated to 100 μl and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis by (SDS-PAGE).

SDS-PAGE. SDS-PAGE (3 to 15% or 9% polyacrylamide) was carried out by the methods of Shapiro et al. (19) and Weber and Osborn (28). Protein samples containing 2% SDS with or without 0.1% 2-mercaptoethanol were heated for 5 and 1 min, respectively, in a boiling-water bath and electrophoresed at 15 mA per gel for 7 to 8 h. Gels were fixed in methanol-acetic acid and stained with silver by the method of Tsai and Frasch (27), omitting the periodic acid step. One lane on each gel contained molecular mass markers from 29 to 91.4 kDa (Sigma Ltd., Munich, Germany) as indicated.

Western immunoblot. Immunoblotting was carried out with 1-mm-thick SDS-containing gels. The gels were electroblotted onto an Immobilon-P/PVDF transfer membrane (Millipore) for 3 h at 0.8 mA/cm^2 by using a semidry blotting chamber (Keutz, Reiskirchen, Germany) with 300 mM Tris-HCl–20% methanol (pH 10.4) at the anode, 25 mM Tris-HCl–20% methanol (pH 10.4) for the transfer membrane and the gel, and 25 mM Tris-HCl–40 mM 6-aminohexanoic acid–20% methanol (pH 9.4) at the cathode. The blot membranes were blocked for 1 h in TBST (50 mM Tris, 200 mM NaCl [pH 7.5], 0.05% Tween 20) containing 1% milk powder. Blots were incubated with monoclonal antibodies (diluted 1:100 to 1:500) for 1 h at 37°C. The blots were then washed in two (10-min) changes of TBST, incubated for 1 h with alkaline phosphatase-conjugated anti-mouse IgG (Sigma Ltd.), and washed in TBS and then in substrate buffer (0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$). The reaction was initiated by using 3.2 mg of 4-nitrotetrazolium chloride blue and 6.6 mg of 5-bromo-4-chloro-3-indolyl phosphate (in 20 ml of substrate buffer) and stopped by washing in Milli Q water.

Preparation of polyclonal antibody against the 66-kDa molecule of *S. typhimurium*. A 400-μl portion of purified 66-kDa antigen (10 μg of protein in PBS) was emulsified 1:2 with Freund's complete adjuvant and injected intramuscularly in the back of a rabbit. The procedure was repeated three times at intervals of 14 days but with Freund's incomplete adjuvant. One week after the final injection, the rabbit was bled from the marginal ear vein. The IgG fraction of the serum was processed on an FPLC protein A Superose column (Pharmacia). Material was applied to the column (in 3 M NaCl–1.5 M glycine [pH 8.5]), and the IgG fraction was eluted from the column by applying a gradient of 0 to 100% 0.1 M citrate buffer (pH 4.5) dialyzed against TBS.

Monoclonal antibodies. Monoclonal antibodies specific for the 65-kDa HSP from *M. leprae* were obtained from the IMMLEP Monoclonal Antibody Bank of the World Health Organization. The antibodies were mc5205 IIH9, mc2009 ML30-A1, and mc4220 IIC8.

Protein estimation. The protein content was routinely determined by using a Micro BCA (bicinchoninic acid) protein assay test kit (Pierce, Oud Beijerland, The Netherlands). Bovine serum albumin was used as a protein standard.

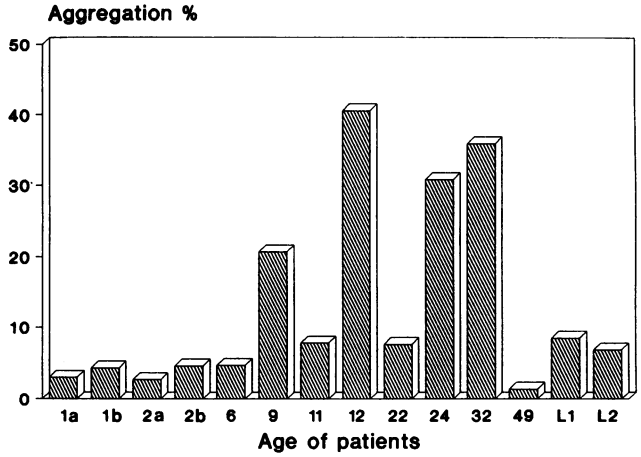

FIG. 1. Aggregation by guinea pig colonic mucus of *S. typhimurium* strains isolated from stools of different patients. The ages of the patients are indicated by the numbers along the *x* axis. A and b indicate two different patients of the same age. L1 and L2 represent two different laboratory strains. The aggregation assay was performed as described in Materials and Methods. The aggregation profile was very reproducible. For the standard deviations of the aggregation assay, see also the results in Tables 1 and 2.

RESULTS

Aggregation of *S. typhimurium* strains isolated from patient stool by colonic mucus. After storage, isolated colonic epithelial cells secreted a soluble substance which caused visible aggregation of *S. typhimurium*. To quantify this phenomenon, we established an aggregation assay as described in Materials and Methods. This assay was used to test whether all strains of *S. typhimurium* isolated from patient stool samples show the same aggregation behavior. *S. typhimurium* strains, isolated from 12 different patients suffering from salmonellosis, were incubated with guinea pig crude colonic mucus. The different strains of *S. typhimurium* exhibited various levels of aggregation (Fig. 1).

Comparing the age of the patients with the extent of aggregation of the *S. typhimurium* strains isolated, we found that aggregation of the strains isolated from patients aged up to 6 years was low (5%). The clinical diagnoses of these patients (the number represents the patient's age) so far available are as follows: 1a, no data available; 1b, gastroenteritis; 2a, enteritis; 2b, gastroenteritis; 6, diarrhea. All other strains, except one (from a 49-year-old patient), showed an aggregation of 7.6% or higher. It is interesting that the aggregation profile of *S. typhimurium* strains freshly isolated from the other patients with salmonellosis correlated with the severity of the disease. Patient 9 had suspected appendicitis. Patient 11 had stomach pains of unknown origin. Patient 12 suffered from massive enteritis with blood in the stool. Patient 24 had had diarrhea for more than 5 days. Patient 32 had suffered from chronic diarrhea for more than a month. During human immunodeficiency virus screening, stool samples of patient 49 were taken. Two strains (L1 and L2), originally isolated from patients' stool and maintained as so-called laboratory strains, showed aggregation of 8.5 and 6.8%, respectively. It was of interest to find whether all bacteria isolated from one patient exhibit the same aggregation behavior. Therefore, the strain (P 32) from one patient (aged 32) was screened for aggregation-positive and aggregation-negative bacteria as described in Materials and Methods. Of 48 colonies screened, 67% were positive and 33% were negative. It is worthwhile to note here that it was not possible to convert aggregation-negative colonies into aggregation-positive colonies; i.e., negative colonies remained negative, and positive colonies remained positive. Two *S. typhimurium* isotypes with high aggregation behavior (>80%, Sal 5 and Sal 33) and two aggregation-negative strains (0%, Sal 2 and Sal 31) were chosen for the following experiments to validate the reproducibility of the observed results.

TABLE 1. Influence of bacterial preparations from four isotypes of *S. typhimurium* (P 32) on the aggregation of Sal 5[a]

Strain	Protein concn (mg/ml)	Aggregation (%)[b]
P 32/Sal 2	0.11	44.1 ± 5.2
P 32/Sal 5	0.22	2.8 ± 0.1
P 32/Sal 31	0.14	33.2 ± 3.4
P 32/Sal 33	0.12	0 ± 0.0
Control	0	50.8 ± 6.9

[a] The heat (50°C)-eluted material from the bacterial isotypes (Sal 2 and Sal 31 [aggregation negative]; Sal 5 and Sal 33 [aggregation positive]) of *S. typhimurium* P 32 was tested as described for the inhibition assay in Materials and Methods. Mucus material contained 191.7 μg of protein per ml. As in the aggregation assay, percentages of aggregated bacteria were determined.
[b] Mean ± standard deviation of four different experiments.

Characterization of the bacterial component(s) involved in aggregation. Four isotypes of *S. typhimurium* P 32 were used to determine whether the bacterial component responsible for aggregation could be eluted by heat (50°C) treatment of the different strains. If it is assumed that the bacterial component involved in aggregation could be released into the supernatant by such treatment, it may be further assumed that the soluble fraction may prevent aggregation of *S. typhimurium* by crude mucus material.

The following experimental conditions were used for each isotype. The pellet of a 40-ml culture (incubated for 8 h at 170 rpm and 37°C) was resuspended in 80 ml of 100 mM Na_3PO_4 (pH 7.0) and incubated for 20 min at 50°C. After this treatment the supernatant was dialyzed against Milli Q water and concentrated to 10 ml. Then the eluates from the four strains were tested in the inhibition assay as described in Materials and Methods. The isotype P 32/Sal 5 was used as the test strain. Results of preincubation of the mucus preparation with the eluates of the different isotypes followed by incubation with strain P 32/Sal 5 are given in Table 1. From these experiments the following conclusions were drawn: (i) a component(s) from aggregation-positive bacteria is released into the supernatant at 50°C and abolish the aggregation of *S. typhimurium* by crude mucus material; and (ii) supernatants of aggregation-negative bacteria show only a slight or no effect on *S. typhimurium* aggregation.

Effect of temperature on the induction and release of the component(s) of *S. typhimurium* responsible for aggregation of the bacteria by crude colonic mucus. The experiment with the four isotypes of *S. typhimurium* revealed that bacteria grown at 37°C and incubated for 20 min at 50°C released into the supernatant a soluble material which inhibited the aggregation of the bacteria by colonic crude mucus. To further characterize the effect of temperature on the expression and release of this inhibitor of aggregation, we performed the following experiment. *S. typhimurium* P 32/Sal 33 was cultured in 500 ml of nutrient broth (170 rpm) for 4 h at 37°C. Afterwards the bacteria were harvested by centrifugation and resuspended in 80 ml of phosphate buffer (pH 7.0). Portions (20 ml) of this suspension were added to 300 ml of

TABLE 2. Effect of temperature on the induction and release of the component(s) of *S. typhimurium* (P 32/Sal 33) responsible for aggregation of the bacteria by crude colonic mucus

Temp (°C)	Aggregation (%) of heat-treated bacteria[a]	Amt of protein(s) released (mg/ml)[b]	Effect of released protein(s) on bacterial aggregation (%)[a]
21	15 ± 2.4	1.1	11 ± 1.1
30	15.7 ± 4.0	1.3	14.3 ± 1.3
42[c]	17 ± 2.2	1.3	13.2 ± 1.3
50[c]	38.5 ± 6.1	0.7	4.9 ± 0.7

[a] Mean ± standard deviation of four different experiments.
[b] Supernatants of bacteria were concentrated by ammonium sulfate precipitation (final saturation, 40%).
[c] In control experiments it was confirmed that incubation of the bacteria for 1 h at 42°C and at 50°C had no effect on bacterial viability.

prewarmed (21, 30, 42, and 50°C) phosphate buffer and incubated for 1 h. In control experiments it was confirmed that incubation of the bacteria at 42 and 50°C had no effect on bacterial viability. After incubation the bacteria were pelleted, resuspended to a final concentration of 2×10^9/ml, and immediately tested in the aggregation assay. The conditions of the aggregation assay were chosen so that aggregation of the bacteria incubated at 21°C was about 15% ± 2.4 (Table 2). The bacteria incubated at 30 and 42°C showed an aggregation of 15.7% ± 4.0 and 17% ± 2.2, respectively. However, the bacteria incubated at 50°C showed an aggregation of 38.5% ± 6.1. This indicated that incubation of *S. typhimurium* for 1 h at 50°C induced an increase in crude mucus-mediated aggregation of *S. typhimurium*.

Since in the previous experiments (Table 1) it was shown that at 50°C a bacterial component(s) which inhibits bacterial aggregation is released into the supernatant, the supernatants of *S. typhimurium* incubated at different temperatures were tested for their effect on bacterial aggregation. In additional experiments (data not shown) it was found that treatment of aggregation-negative colonies at 50°C did not convert these bacteria to aggregation-positive ones.

Ammonium sulfate was added (final saturation, 40%) to each supernatant to concentrate the component(s). The precipitates were collected by centrifugation, dissolved in Milli Q water, dialyzed against Milli Q water, and tested in the aggregation inhibition assay. The results shown in Table 2 indicate that only the supernatant of the 50°C-treated bacteria contained material showing any significant inhibitory activity. The material from the different supernatants was analyzed by SDS-PAGE under reducing conditions. Comparing the protein profile, it became apparent (Fig. 2) that levels of a 66-kDa protein were increased in the supernatant of the bacteria treated at 50°C.

These experiments show that aggregation of *S. typhimurium* by crude mucus material can be increased by preincubation of the bacteria at 50°C. This increase seems to be correlated with an increase in the level of a 66-kDa protein which, on the basis of the inhibitory capacity of the supernatant on *S. typhimurium* aggregation, may be involved in bacterial aggregation.

Purification of the 66-kDa component of *S. typhimurium*. The supernatant of *S. typhimurium* isotype P 32/Sal 33 was chosen for purification of the 66-kDa component. The culture conditions and the preparation of the supernatant material were as described in Materials and Methods. SDS-PAGE analysis of the concentrated protein fraction of the supernatant revealed, under reducing conditions, a major

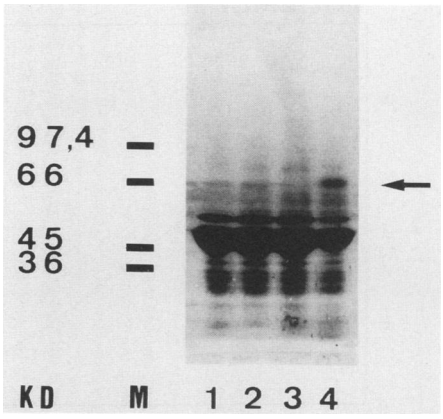

FIG. 2. SDS-PAGE analysis of bacterial preparations obtained by incubations of *S. typhimurium* P 32/Sal 33 at different temperatures. Lanes: M, marker; 1, 21°C; 2, 30°C; 3, 42°C; 4, 50°C.

protein band at 66 kDa (Fig. 3, lane 1). This protein was further purified by using a Mono Q column and FPLC. The fractions were tested in the inhibition assay and analyzed by SDS-PAGE. Fractions showing inhibitory capacity and found to be free of any other protein contamination were pooled and concentrated. The final fraction contained 5.4 μg of protein per ml and was silver stained after separation by SDS-PAGE analysis. The purified material was found to have a molecular mass of 66 kDa under reducing conditions (Fig. 3, lane 2) and 55 kDa under nonreducing conditions (Fig. 3, lane 3). The purified material completely abolished the aggregation of the *S. typhimurium* isotype P 32/Sal 33 by crude mucus material (the control value was 33% aggregation).

Sucrose density gradient ultracentrifugation. To determine whether the 66-kDa molecule is a 66-kDa monomer or whether it is composed of homopolymers, we analyzed the purified protein in a sucrose gradient (10 to 40%). Fractions (500 μl) were collected, dialyzed, concentrated to 100 μl, and analyzed by SDS-PAGE under reducing conditions. As shown in Fig. 4, two forms of the 66-kDa molecule were

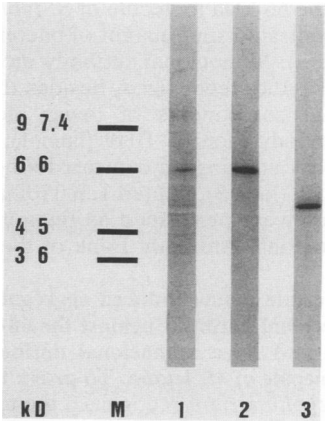

FIG. 3. SDS-PAGE (3 to 15% polyacrylamide) analysis of nonpurified and purified samples from the supernatant of *S. typhimurium* (P 32/Sal 33) separated under reducing and nonreducing conditions. Lanes: M, marker; 1, unpurified material, reducing conditions; 2, purified protein, reducing conditions; 3, purified protein, nonreducing conditions.

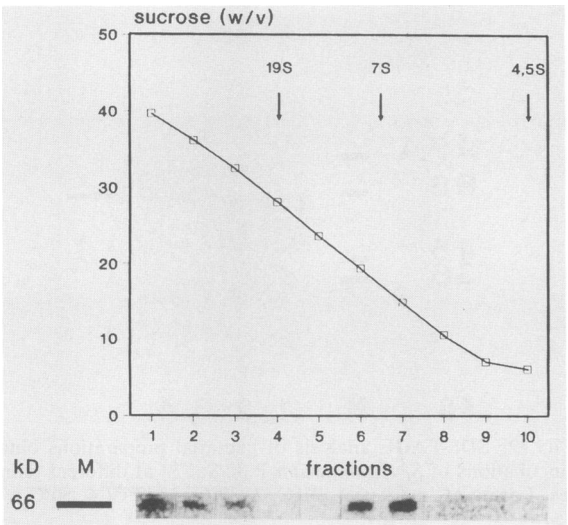

FIG. 4. Sucrose gradient (10 to 40%) of a purified sample containing the 66-kDa molecule from *S. typhimurium* (P 32/Sal 33). The sucrose gradient is shown (top) together with locations of the three reference substances: albumin (66 kDa; sedimentation coefficient, 4.5S), IgG (150 kDa, 7S), and IgM (900 kDa, 19S). The fractions obtained were analyzed by SDS-PAGE (9% polyacrylamide) (bottom) under reducing conditions (silver staining). M and the bar represent the marker protein albumin with a molecular mass of 66 kDa.

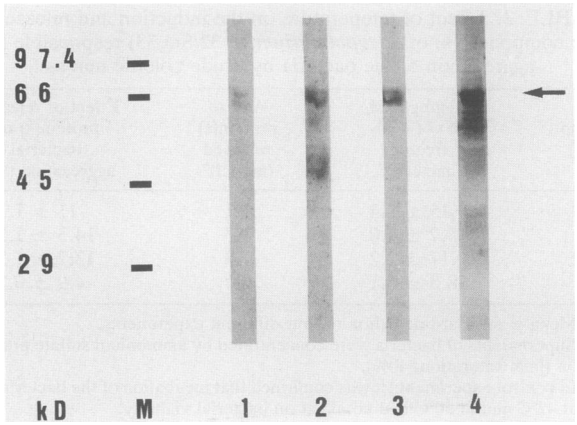

FIG. 5. Western blot analysis of a preparation of *S. typhimurium* (P 32/Sal 33). Binding of monoclonal antibodies against the 65-kDa HSP of *M. leprae* antibodies. Lanes: M, marker; 1, mc5205 IIH9 (1:100); 2, mc2009 ML30-A1 (1:100); 3, mc4220 IIC8 (1:500); 4, sample on SDS–9% polyacrylamide gel, silver staining. The arrow marks the position of the 66-kDa protein of *S. typhimurium* in the unpurified preparation.

detectable: a dimeric form of approximately 132 kDa (fractions 6 and 7) and a homomultimer form of >900 kDa (fractions 1, 2, and 3), also composed of 66-kDa subunits. A monomeric form of 66 kDa was not detectable.

Reactivity of the 66-kDa molecule with *Mycobacterium* monoclonal antibodies. Since the experiments presented so far have shown that the 66-kDa molecule can be induced by incubation of *S. typhimurium* at 50°C, it was of interest to find whether this 66-kDa molecule of *S. typhimurium* was related to the 65-kDa HSP from *M. leprae*. Immunological similarities of the 66-kDa molecule of *S. typhimurium* with the 65-kDa HSP of *M. leprae* were assessed by Western blotting, using three different monoclonal antibodies against the 65-kDa HSP of *M. leprae*. All three monoclonal antibodies recognized the 66-kDa molecule of *S. typhimurium* in an unpurified concentrated supernatant of bacteria exposed for 1 h at 50°C (Fig. 5). Monoclonal antibody mc2009 ML30-A1 (lane 2), diluted 1:100, recognized, besides the 66-kDa molecule, additional components of lower molecular mass. Monoclonal antibody mc5205 IIH9 (lane 1), diluted 1:100, showed only weak staining, in contrast to monoclonal antibody mc4220 IIC8 (lane 3), diluted 1:500 (dilutions of monoclonal antibodies were performed as recommended by the IMMLEP Monoclonal Antibody Bank of the World Health Organization).

Inhibition of crude mucus-induced aggregation of *S. typhimurium* by a polyclonal antibody against the 66-kD molecule of *S. typhimurium* and by a monoclonal antibody against the 65-kDa HSP molecule of *M. leprae*. To prove that the 66-kDa molecule of *S. typhimurium* recognizes the agglutinin in the crude colonic mucus, the bacteria were preincubated either with a monoclonal antibody against the 65-kDa HSP molecule of *M. leprae* (mc4220 IIC8) or with a polyclonal antibody against the 66-kDa molecule before exposure to crude colonic mucus. An unrelated polyclonal antibody (anti-human C1s) had no effect on mucus-mediated bacterial aggregation (Fig. 6). In contrast, the monoclonal antibody and, even more strongly, the polyclonal antibody inhibited, dose dependently, the aggregation of *S. typhimurium* by the colonic mucus preparation. This finding indicates the direct involvement of the 66-kDa HSP of *S. typhimurium* in the binding of the bacteria to the mucus.

DISCUSSION

One of the earliest events in *S. typhimurium* pathogenesis seems to be the interaction of the bacterium with the mucus of the gut. To find which components of the mucus layer and

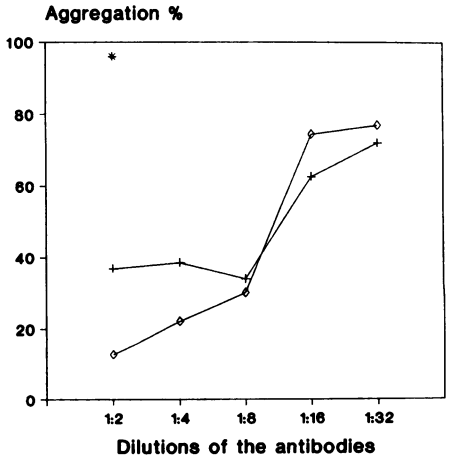

FIG. 6. Inhibition of crude mucus-induced aggregation of *S. typhimurium* by polyclonal anti-66-kDa antibody and monoclonal antibody mc4220 IIC8 (against the 65-kDa HSP of *M. leprae*). Equal volumes of crude mucus material (0.72 mg of protein/ml) and various concentrations of the antibodies were incubated for 20 min at 37°C. Anti-human C1s was used as an additional control besides the buffer control (i.e., 100%). Afterwards the aggregation assay was performed. Anti-poly 66-kDa antibody of *S. typhimurium* (◇), 0.62 mg of protein per ml; anti-mc4220 IIC8 antibody (+), 0.67 mg of protein/ml; anti-human C1s antibody (*), 0.65 mg of protein per ml.

the bacteria are involved in colonization of the bacteria in the gut, we incubated *S. typhimurium* with isolated guinea pig colon. We were able to demonstrate by electron microscopy that *S. typhimurium* bacteria were trapped on or in the mucus layer. Furthermore, crude mucus preparations aggregated the bacteria. The agglutinin for *S. typhimurium* cells that was isolated from guinea pig colonic crude mucus preparation has been identified as a 15-kDa glycoprotein (5).

In this report we have described and characterized the component of *S. typhimurium* that is involved in binding of the bacteria to guinea pig mucus. *S. typhimurium* cells freshly isolated from different patients' stools were found to be aggregated to differing degrees by the same mucus preparation. Comparison with the clinical course of the disease in patients aged 9 years and older revealed that aggregation of the bacteria by the mucus preparation correlated with the severity of the disease (Fig. 1).

Isotypes of *S. typhimurium* exhibiting a high degree of aggregation were isolated from the bacteria cultured from one patient (aged 32 years). These isotypes were used to purify the bacterial component recognized by the mucus agglutinin. Screening for this component was carried out in an aggregation inhibition assay. The observation that at 50°C a bacterial component which inhibited bacterial aggregation was released into the supernatant enabled us to isolate this component from the supernatant of *S. typhimurium* cells incubated at 50°C (Fig. 2). The purified component was characterized on SDS-PAGE, under reducing conditions, as a 66-kDa molecule (Fig. 3). Ultracentrifugation studies revealed that this 66-kDa molecule was present in dimeric and oligomeric forms in the supernatants of *S. typhimurium* cells incubated at 50°C (Fig. 4). The isolated component inhibited completely the aggregation of *S. typhimurium* by crude mucus preparations. Furthermore, a polyclonal antibody also prevented bacterial aggregation (Fig. 6). Since the expression and release of the 66-kDa molecule were temperature dependent, several monoclonal antibodies raised against the 65-kDa HSP of *M. leprae* were assessed for recognition of the 66-kDa *S. typhimurium* component. All three monoclonal antibodies against the 65-kDa HSP recognized the 66-kDa protein of *S. typhimurium* (Fig. 5). The monoclonal antibody mc4220 IIC8, the only one we tested for aggregation inhibition, was also found to abrogate aggregation of *S. typhimurium* by the crude mucus preparation (Fig. 6). Therefore, the bacterial component of *S. typhimurium* responsible for binding, i.e., localization of the bacteria to the gut mucus, appears to be a 66-kDa HSP. In further experiments we found that this HSP is not only released into the supernatant but is also detectable in the membrane of *S. typhimurium* cells incubated at 50°C (data not shown). To date, very little has been published on the induction of HSPs from *S. typhimurium* (9, 12, 15, 23), their regulation (4), and the correlation between their presence and thermotolerance (14). Furthermore, it has been shown that *S. typhimurium* HSPs are induced on infection of macrophages. Even in the absence of heat shock, HSPs are the most abundantly expressed proteins, indicating that uptake of the bacteria by macrophages may reflect a stress situation resulting in the expression of bacterial HSPs (3).

Cross-reactivity of mouse monoclonal antibodies directed against the 65-kDa antigen of *M. leprae* has, to date, been described for 23 different mycobacterial species (2). Some of these antibodies also reacted with antigen present in gram-negative bacteria (for example, ML II H9 with *Escherichia coli* [21]), gram-positive bacteria, spirochetes, and rickettsiae (2, 20, 26). The cross-reactive proteins correspond to a family of conserved bacterial proteins that have been called common antigens (10). The cross-reactive protein in *E. coli* was found to be the product of the *groEL* gene, an *E. coli* gene required for the growth of phage lamda (6). Both the *groEL* gene product and the *Mycobacterium* 65-kDa protein were found to be HSPs (16, 21).

In addition to shared epitopes, the 66-kDa *S. typhimurium* molecule and the 65-kDa protein of *M. leprae* display several common features. Neither of the components appear in a monomeric form: the 66-kDa molecule from *S. typhimurium* appears to be composed of dimers or homopolymers of over 900 kDa (Fig. 4), whereas the 65-kDa *Mycobacterium* protein exists as homomultimers of greater than 240 kDa (21).

HSPs have been intensively studied at the immunological level and are among the major targets of both antibody and T-cell immune responses. HSPs have been implicated in experimental and human autoimmune arthritis and in autoimmune insulin-dependent diabetes (13, 29).

As there is a positive correlation between the ability of a wide variety of microorganisms causing, e.g., gastroenteritis to adhere to epithelial cells in vitro and the infectivity of these bacteria in vivo, the possibility of bacterial adhesion to receptor substances in the mucus gel must certainly be considered as an initiating step in localization leading to colonization of bacteria within the gut. Therefore, the data presented here provide the first report that a 66-kDa HSP of *S. typhimurium* is involved in aggregation of the bacteria by crude mucus material and might therefore play an important role in the early step of localization. Since localization seems to be a prerequisite for colonization of the bacteria within the gut mucus, the 66-kDa HSP of *S. typhimurium* may be an important bacterial pathogenic factor in *S. typhimurium*-mediated salmonellosis of the gut. This implies that the 66-kDa molecule may be a good candidate for an *S. typhimurium* vaccine. Such a vaccine may prevent *S. typhimurium*-mediated salmonellosis in addition to the subsequent *S. typhimurium*-mediated reactive arthritis, in which 65-kDa HSP-reactive T cells play an important role (8).

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