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Naturally Occurring Antibodies in Human Sera that React with the Iron-Regulated Outer Membrane Proteins of *Escherichia coli*

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Sera from normal healthy human adults and infants, as well as sera from mice, rabbits, and guinea pigs, were examined by immunoblotting for naturally occurring antibodies reacting with outer membrane proteins of two *Escherichia coli* strains, O111 and O18. Some individuals had antibodies reacting very strongly with the iron-regulated outer membrane proteins, including the ferric-enterochelin receptor protein (M_r, 81,000), as well as with ompA. However, sera from infants contained predominantly antibodies to ompA; antibodies recognizing the iron-regulated outer membrane proteins were either absent or barely detectable. In human serum the antibodies were mainly of the immunoglobulin G class. No serotype-specific antibodies to the lipopolysaccharide of *E. coli* O111 or O18 were found in the sera tested.

The multiplication of bacteria in the largely undefined and changing environment of host tissues is an essential feature of any infection. In general, little is known about the effect of host factors on the properties of invading bacteria. Recently, however, our understanding of one feature of this environment and of the way in which pathogenic bacteria adapt to it has increased considerably. We now know that the amount of iron that might normally be readily available to bacteria in the tissue fluids of humans and animals is extremely small (3, 9, 10, 11, 25). This iron-restricted environment induces phenotypic changes in both the metabolism and in the composition of the outer membrane of bacteria growing in vivo (10, 11, 14).

Under iron-restricted conditions both in vitro and in vivo, pathogenic *Escherichia coli* produce iron-chelating compounds (siderophores) which sequester iron bound to host iron-binding proteins, and they also derepress several outer membrane proteins. Some of these proteins, with M_rs in the range 74,000 to 83,000, act as receptors for ferric siderophores and are essential for transporting iron into the bacterial cell; they are not synthesized in significant amounts by the iron-replete organisms (18). Recently, Brown et al. (2) showed that *Pseudomonas aeruginosa* obtained without subculture from the sputum of a patient with cystic fibrosis also expressed three iron-regulated proteins in its outer membrane, and Sciortino and Finkelstein (21) found that *Vibrio cholerae* expressed iron-regulated membrane proteins when grown in vivo in the intestines of infant rabbits. Similarly, some *E. coli* strains isolated without subculture from the urine of patients with urinary tract infection also express iron-regulated outer membrane proteins (C. Lam and F. Turnowsky, personal communication).

It has already been shown by ^{125}I labeling with lactoperoxidase that the siderophore receptors are exposed on the surface of at least some pathogenic strains of *E. coli* and can interact with fairly large proteins (14). The possibility that the iron-regulated outer membrane proteins can interact with antibodies and may be important protective antigens is therefore worth exploring. To investigate the ability of anti-siderophore receptor antibodies to block siderophore-mediated iron uptake, and to inhibit bacterial multiplication, we decided to immunize rabbits with membrane preparations of *E. coli* grown under iron-restricted conditions and to monitor the production of specific antibodies by immunoblotting. However, preliminary work showed that antibodies directed against the iron-regulated proteins were already present in preimmune serum from the rabbits used. In this paper, we report the presence of antibodies directed against the iron-regulated outer membrane proteins of *E. coli* in sera obtained from normal rabbits, mice, guinea pigs, and, in particular, humans.

(Parts of this work have already been reported in preliminary form [10]).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *E. coli* strains used in this study were: O111 K58 H2 (12); O18 K1H7, a pathogenic strain carrying a Col V plasmid (from H. Williams Smith); O149 K91 K88ac H10, a strain which produces diarrhea in piglets; and O2, a strain isolated from the urine of a patient with suspected pyelonephritis. Bacteria were stored in brain heart infusion medium (Difco Laboratories) containing glycerol (10% [vol/vol]) and grown in Trypticase soy broth (BBL Microbiology Systems) or in Trypticase soy broth containing ovotransferrin (0.5 mg/ml) as described previously (12); the gas phase was 5% CO_2–95% air. Cultures were grown for 6 h. The iron-regulated outer membrane proteins were also induced in *E. coli* by growing the bacteria in the following media: (i) Trypticase soy broth containing α,α' dipyridyl (200 μM; Sigma Chemical Co.), in which 200-ml cultures were grown at 37°C on an orbital shaker (180 rpm) overnight; or (ii) the Tris-buffered medium of Simon and Tessman (22), made up without added $FeCl_3$ and supplemented with sodium succinate (10 g/liter) instead of glucose (the pH was adjusted to 6.8 with HCl). Cultures were grown with aeration for 1 to 3 days. The rationale for using a Tris-succinate medium is discussed by Braun (1) and Neilands (18). In vivo-grown *E. coli* cells were obtained as described previously (13).

Outer membrane proteins and lipopolysaccharides. Outer membranes were isolated by the procedure described previously (14) or by the method of Filip et al. (8); similar outer membrane protein profiles were obtained by both procedures. ^{125}I-labeled proteins were obtained by radioiodinating membrane proteins exposed on the surface of intact *E. coli* by the method described by Griffiths et al. (14). Labeling was

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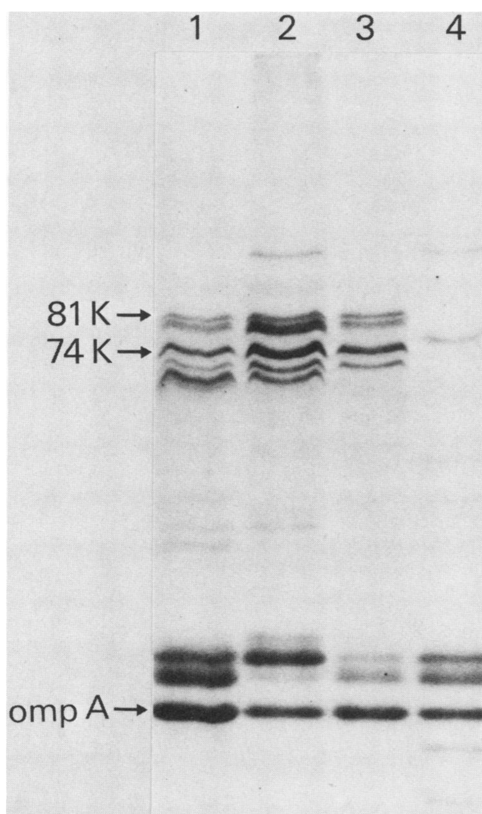

FIG. 1. SDS-PAGE of the outer membrane proteins of *E. coli* O18 grown in Trypticase soy broth containing α,α′-dipyridyl (lane 1), in vivo (lane 2), Trypticase soy broth containing ovotransferrin (lane 3), or Trypticase soy broth alone (lane 4). Each lane contained ca. 30 μg of protein. Electrophoresis was carried out for an extended time.

improved by increasing the lactoperoxidase concentration fourfold. Lipopolysaccharide (LPS) was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and detected by silver staining as described previously (5, 24).

Detection of antibodies by immunoblotting. Outer membrane proteins were solubilized and separated by SDS-PAGE as described before (14). A constant current of 50 mA was used, and electrophoresis was stopped when the bromophenol blue dye marker reached the bottom of the separating gel. For improved resolution of the iron-regulated outer membrane proteins, gels were sometimes run for a longer time. In such cases, the gels were run overnight with a constant current of 10 mA and then for a further 2 to 3 h at 20 mA the next morning, after the bromophenol blue marker had migrated off the bottom of the separating gel.

At the end of the electrophoresis, the gels were either stained with Coomassie blue or used for immunoblotting. An EC 420 Electroblot System (E-C Apparatus Corp., St. Petersburg, Fla.) was used for this purpose, and the proteins were transferred onto nitrocellulose sheets essentially as described by Towbin et al. (23) with the modifications described by Johnstone and Thorpe (16). Blotting was carried out for 1.5 h at a current of 400 to 500 mA in a buffer containing 0.025 M Tris, 0.192 M glycine, and 20% (vol/vol) methanol. Under these conditions, much of the protein was transferred, but gels contained enough residual protein to produce visible bands on staining with Coomassie blue (0.025% [wt/vol] Coomassie brilliant blue R250, 50% [vol/vol] methanol, 5% [vol/vol] acetic acid); gels were destained with 5% (vol/vol) methanol–7.5% (vol/vol) acetic acid.

After transfer of the proteins, the nitrocellulose sheet was immersed in 0.14 M NaCl–2.7 mM KCl–5.35 mM Na_2HPO_4–1.5 mM KH_2PO_4–0.02% (wt/vol) NaN_3 (phosphate-buffered saline [PBS]-azide) containing 3% (wt/vol) bovine hemoglobin (hemoglobin-PBS), and shaken for 1 to 2 h at room temperature on an orbital shaker. The hemoglobin-PBS was then discarded and replaced by a similar volume of hemoglobin-PBS containing 20 to 200 μl of the serum to be tested, and the gentle shaking was continued for a further 16 h; the nitrocellulose sheet was subsequently washed extensively with fresh hemoglobin-PBS. Hemoglobin-PBS containing the appropriate ^{125}I-labeled anti-immunoglobulin (ca. 10^6 cpm per lane) was added and incubation continued for 3 to 4 h. The nitrocellulose paper was finally washed extensively (six changes of 100 ml each) with PBS-azide over about 30 min and dried in an oven at 45°C for 20 to 30 min. The immunoblot was analyzed by autoradiography at −70°C as described by Laskey and Mills (17) with a cassette fitted with a fast tungstate intensifying screen. The optimum time of exposure was found by trial and error (up to 14 days).

Sera and anti-immunoglobulin antibodies for immunoblotting. Serum was obtained from normal mice (NIH 3 and BALB/c), guinea pigs (Hartley), rabbits (New Zealand White), and humans in the usual way and stored frozen at −30°C. Anti-human immunoglobulin G (IgG) [heterologous normal IgG, Fc, and $F(ab')_2$] and anti-guinea pig IgG immunoglobulin G were from Nordic Immunological Reagents Maidenhead, United Kingdom; anti-rabbit and anti-mouse $F(ab')_2$ were prepared at this institution. Anti-immunoglobulin antibodies were iodinated by the Chloramine T method, and residual unbound iodine was removed by Dowex 1-X8 (Cl) ion-exchange resin as described by Johnstone and Thorpe (16). Monoclonal antibodies specific for human IgG and IgM were kind gifts from M. Spitz and A. Gearing. Specific anti-*E. coli* antisera were raised in rabbits by using membrane proteins from bacteria grown in Trypticase soy broth containing ovotransferrin. Rabbits were immunized with a combination of intradermal and intramuscular injections of 150 to 200 μg outer membrane protein preparation suspended in PBS. The intramuscular booster injection was given 11 days after the intradermal injection and antiserum obtained 7 days later.

RESULTS

Analysis of iron-regulated outer membrane proteins. We have shown previously that pathogenic strains of *E. coli* produce new outer membrane proteins, with M_rs of 74,000 to 83,000, when growing in vitro in the presence of ovotransferrin (14). We have also shown that there is considerable variation in the relative abundance of each new protein expressed in different strains. Since *E. coli* O111 and O18 were two strains which showed quite different patterns of iron-regulated outer membrane proteins when grown in the presence of ovotransferrin, we decided to examine the responses of these two organisms to iron restriction more closely and to use them to search for serum antibodies directed against outer membrane proteins. Analysis of the outer membrane proteins by SDS-PAGE confirmed that the pattern of proteins expressed by *E. coli* O111 during growth in the presence of ovotransferrin differed from that produced by *E. coli* O18. The use of extended electrophoresis times (see above) to improve resolution showed that *E. coli* O111 expressed large amounts of the proteins with M_rs of 74,000

and 81,000 and a smaller amount of the protein with an M_r of 78,000. In contrast, *E. coli* O18 expressed four new proteins with M_rs of ca. 81,000, 78,000, 74,000, and 71,000 (Fig. 1, lane 3). The bands of iron-regulated outer membrane proteins from *E. coli* O18 were, however, often distorted (Fig. 1). This is likely to be due to the presence in the outer membrane protein preparation of high-molecular-weight lipopolysaccharide (LPS) which comigrates with the iron-regulated proteins (Fig. 2). The LPS may also interfere with the mobility of individual proteins, making the assessment of their molecular weights difficult. This is not a problem in the case of *E. coli* O111 since most of the high-molecular-weight *O*-polysaccharide chains are shorter and migrate faster than the iron-regulated outer membrane proteins during SDS-PAGE (Fig. 2). The same four iron-regulated proteins were present in the outer membrane of *E. coli* O18 isolated without subculture from the peritoneal cavities of infected guinea pigs. Unlike *E. coli* O111, however, which shows the same pattern of iron-regulated outer membrane proteins when grown in vivo during infection as when grown in vitro in the presence of ovotransferrin (14), *E. coli* O18 produced an additional new protein during growth in vivo: this protein had an M_r of about 68,000 (Fig. 1, lane 2; compare lanes 2 and 3). The extra protein could also be induced, along with

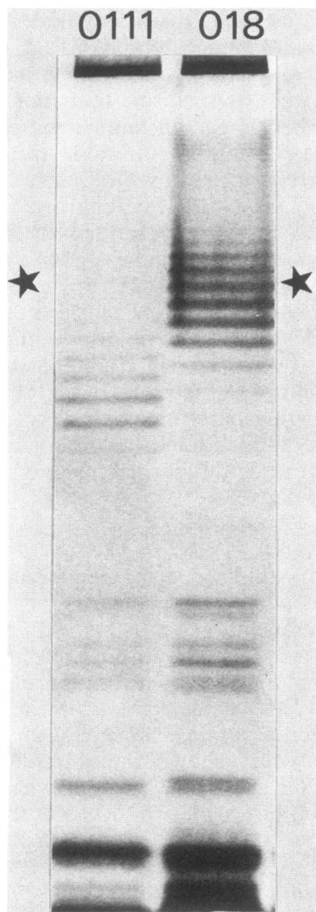

FIG. 2. SDS-PAGE of LPSs from *E. coli* O111 and O18. Whole cell lysates of bacteria were digested with proteinase K and subjected to electrophoresis (5). LPS was detected by silver staining (24). Identical profiles were obtained with purified LPS. ★ indicates the approximate position of the iron-regulated outer membrane proteins.

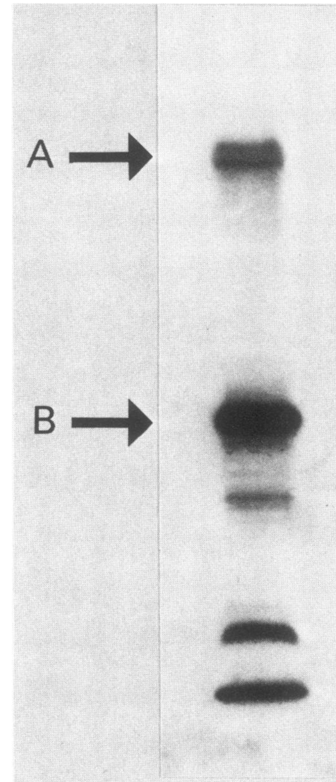

FIG. 3. Immunoblot of the separated outer membrane proteins of *E. coli* O111 (60 μg of total protein) reacted with normal guinea pig serum (200 μl). The bacteria were grown in Trypticase soy broth containing ovotransferrin. Approximate positions of the iron-regulated proteins (A) and the major outer membrane proteins (B) are indicated.

the other iron-regulated proteins, by growing *E. coli* O18 in Trypticase soy broth containing α,α′-dipyridyl (Fig. 1, lane 1) or in the Tris-succinate medium (data not shown). We do not know if the new protein is directly related to bacterial iron metabolism. However, its appearance is certainly not associated with the presence of the Col V plasmid since the Col V^- derivative of *E. coli* O18 behaved in exactly the same way.

Interesting differences in the pattern of the major outer membrane proteins, with M_rs of 30,000 to 40,000, were also seen when the outer membrane protein profile of *E. coli* O18 grown in vivo was compared with that from the bacteria grown in vitro with ovotransferrin (Fig. 1, lanes 2 and 3). However, although α,α′-dipyridyl induced a pattern of outer membrane proteins with M_rs of 68,000 to 80,000 similar to that found in bacteria grown in vivo, the pattern of the major proteins remained essentially the same as that in bacteria grown in broth, or in broth with ovotransferrin (Fig. 1, lanes 1, 3, and 4). Bacteria grown in the Tris-succinate medium, however, had a major outer membrane protein profile similar to that of the in vivo-grown organisms (not shown). As in the case of *E. coli* O111 (14), the iron-regulated outer membrane proteins of *E. coli* O18, including that with an apparent M_r of 68,000, are exposed on the surface of the cell and can be labeled extrinsically with ^{125}I by lactoperoxidase-catalyzed radioiodination of the intact bacteria (see Fig. 7, lane 1).

Detection of serum antibodies to outer membrane proteins. The outer membrane proteins of *E. coli* grown in broth containing ovotransferrin were separated by SDS-PAGE,

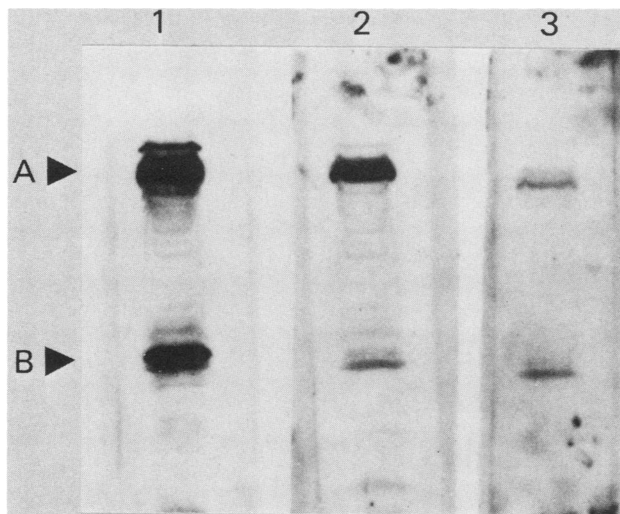

FIG. 4. Immunoblots of the separated outer membrane proteins of *E. coli* O111 (60 μg of protein per lane) reacted with normal human adult serum (100 μl per lane). Bacteria were grown in Trypticase soy broth containing ovotransferrin. Serum samples 1 through 3 were obtained from three different individuals. Approximate positions of the iron-regulated proteins (A) and the major outer membrane proteins (B) are indicated.

transferred electrophoretically onto nitrocellulose sheets and reacted with serum from normal mice, rabbits or guinea pigs. Antibodies bound by the antigens immobilized on the nitrocellulose sheets were detected using a second labeled antibody directed against the first antibody (^{125}I-labeled anti-mouse, anti-rabbit or anti-guinea pig IgG). All of the mice, rabbits and guinea pigs that we examined had antibodies directed against the iron-regulated outer membrane proteins of *E. coli* O111, as well as against one of the major outer membrane proteins (M_r 30,000 to 40,000). Although immunoblotting cannot be considered to be a strictly quantitative assay, it does give some indication of antibody levels or their avidity, and the relative amounts of antibodies present or their reactivity clearly varied considerably from individual to individual. Each serum tested, however, showed the presence of detectable amounts of anti-siderophore receptor antibodies. Antibodies to other outer membrane proteins were either not detected or were seen in lower amounts relative to the anti-siderophore receptor and anti-major outer membrane protein antibodies. Figure 3 shows the results obtained with guinea pig serum. Results for sera from mice and rabbits were similar and are not shown.

Figure 4 shows that human serum also contains antibodies to one of the major outer membrane proteins and to the iron-regulated proteins. Although we have not examined sera from a large number of people, all of the human adult sera we assayed with outer membrane proteins from *E. coli* O111 or O18 (total number of adults, 20) had detectable levels of anti-siderophore receptor antibodies. Furthermore, it was clear that sera from some individuals produced considerably stronger reactions than those from others (Fig. 4 and 5). Of possible interest are the extremely low levels of anti-receptor antibodies found in sera from 13 infants aged between 2 months and 3 years; in these sera the anti-receptor antibodies were absent or barely detectable. However, most of the infant sera examined produced substantial reactions with one of the major outer membrane proteins (Fig. 5).

Immunoblotting with normal sera from humans or other animals failed to detect antibodies directed against the LPSs, of the two *E. coli* strains used. This was not due to the inability of the immunoblotting procedure to detect such antibodies. Specific antiserum to *E. coli* O111 or O18 raised in rabbits clearly contained anti-LPS antibodies, the presence of which was readily shown by immunoblotting (Fig. 6, lanes 4 and 5). These immunoblots, which showed the typical ladder-like profile of LPS, were quite different from those produced with normal animal or human sera (compare Fig. 3, 4, and 5 with Fig. 6., lanes 4 and 5). The naturally occurring antibodies reacted mainly with the iron-regulated membrane proteins and with one of the major outer membrane proteins. However, immunoblots similar to those obtained with normal sera could be obtained with the specific antisera when they were reacted with outer membrane components from heterologous *E. coli* strains which produced non-cross-reacting *O*-polysaccharides (Fig. 6, lanes 1 through 3) or when the antisera were reacted with the homologous strain after preincubation with purified homologous LPS to adsorb anti-LPS antibodies (not shown). Figure 6 shows the results obtained when specific anti-*E. coli* O111 antiserum was reacted with membrane protein preparations from iron-restricted *E. coli* O2, O149, and O18.

Identification of antigen-antibody pairs. Although the immunoblotting experiments described above showed that antibodies to one or more iron-regulated membrane proteins and to certain other outer membrane proteins were present in the serum of animals and humans, poor resolution of the iron-regulated proteins on the gels together with shrinkage of the gels during drying made it impossible to align immunoblots and gels so that antibody-antigen pairs could be matched with certainty. This problem was overcome by extrinsically labeling the outer membrane proteins with ^{125}I and using labeled preparations as markers. The ^{125}I-labeled proteins were electrophoresed in gels adjacent to unlabeled preparations and then transferred on to the nitrocellulose sheet along with the unlabeled proteins. The sheet was then cut into two, between the two lanes, with pinking shears, which produce a serrated edge. The paper carrying the unlabeled proteins was then processed in the usual way to

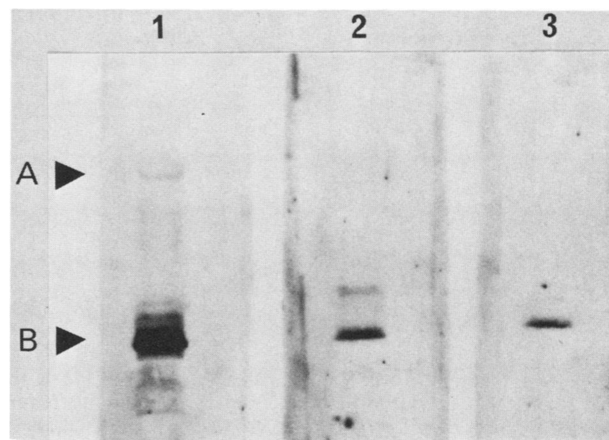

FIG. 5. Immunoblots of the separated outer membrane proteins of *E. coli* O111 (60 μg of total protein per lane) reacted with serum from human infants (100 μl per lane). Bacteria were grown in the presence of ovotransferrin. Serum samples were from infants aged 3 months (lane 1), 11 months (lane 2), and 18 months (lane 3). Approximate positions of the iron-regulated proteins (A) and the major outer membrane proteins (B) are indicated.

detect antibodies. Both the immunoblot and the nitrocellulose paper carrying the electrophoretically separated ^{125}I-labeled proteins were finally placed onto X-ray film and aligned exactly with their serrated edges. In this way, an outer membrane protein profile was produced on the film alongside and aligned with the antibody blot profile.

Using this technique, we found that human sera may contain antibodies to several of the iron-regulated outer membrane proteins, including the ferric-enterochelin receptor (M_r, 81,000); Fig. 7 shows the results obtained with proteins from *E. coli* O18. The use of the ^{125}I-labeled marker proteins also enabled us to show that the antibody that reacts with one of the major outer membrane proteins during immunoblotting in fact recognizes ompA (M_r, ca. 30,000) (Fig. 7). Anti-ompA antibodies were detected in all human sera examined, including the sera from babies.

The use of murine monoclonal antibodies specific for either IgG or IgM showed that the anti-siderophore receptor antibodies and the anti-ompA antibodies present in human sera were predominantly of the IgG class, although some IgM antibody was also detected.

DISCUSSION

It is already known that human sera may contain antibodies to various *E. coli* antigens such as LPS, lipoprotein, and α-hemolysin (4, 7, 15, 19, 20). It is also known that in humans, the level of these antibodies increases after *E. coli* infection. In this paper, we show that sera from healthy human adults, and also from animals, contain antibodies directed against the iron-regulated outer membrane proteins of *E. coli*, as well as against the major outer membrane protein ompA. In humans, these antibodies are mainly of the IgG class, although some IgM antibody can also be detected. Of course, it is possible that there are antibodies present in serum which react with ompC and ompF or other membrane proteins, but these were not readily detected by the immunoblotting method used here. Similarly, no antibodies to the O111 or O18 serotypes were found in the small number of normal animal and human sera examined, although they were easily detected in specific immune serum from rabbits. The possibility that the anti-LPS antibodies are mainly IgM and not detected by the anti-immunoglobulin antibodies used in immunoblotting is highly unlikely. Indeed, the use of murine monoclonal antibodies specific for human IgM also failed to detect antibodies recognizing *E. coli* O111 or O18 LPS in the sera tested. It is possible that further work will identify individuals who do have serum antibodies directed against the LPSs of these particular serotypes.

All of the human adults examined in this study had detectable levels of serum antibodies reactive with *E. coli* ompA protein and with one or more of the iron-regulated

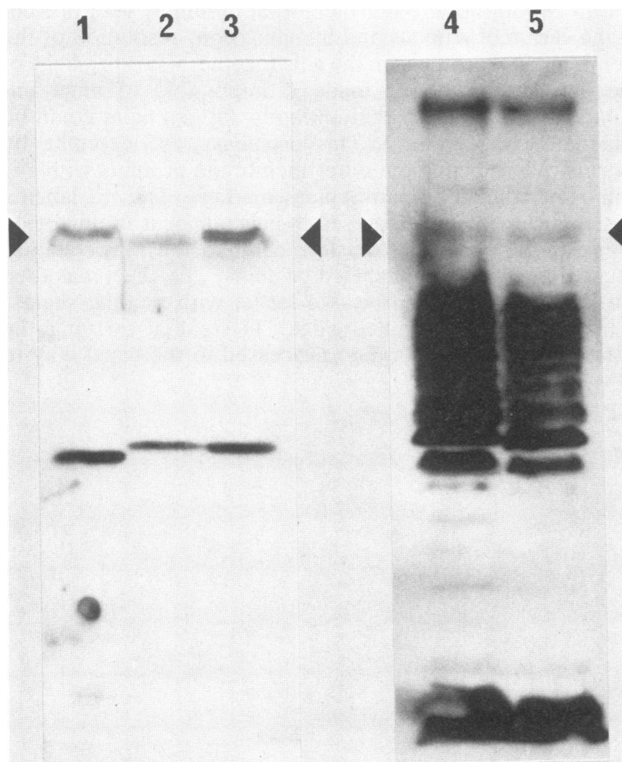

FIG. 6. Immunoblots showing the reaction of specific rabbit anti-*E. coli* O111 antiserum with the separated outer membrane components from *E. coli* strains of heterologous (lanes 1, 2, and 3) and homologous (lanes 4 and 5) serotypes. Lanes 1 through 3 were reacted together with 75 μl of antiserum; lanes 4 and 5 together were reacted with 20 μl of antiserum. Outer membrane preparations were from bacteria grown in broth containing ovotransferrin. Strains and quantities of protein used were *E. coli* O18, 60 μg (lane 1); *E. coli* O149, 60 μg (lane 2); *E. coli* O2, 60 μg (lane 3); *E. coli* O111, 30 μg (lane 4); and *E. coli* O111, 15 μg (lane 5). The position of the iron-regulated outer membrane proteins is indicated by arrows.

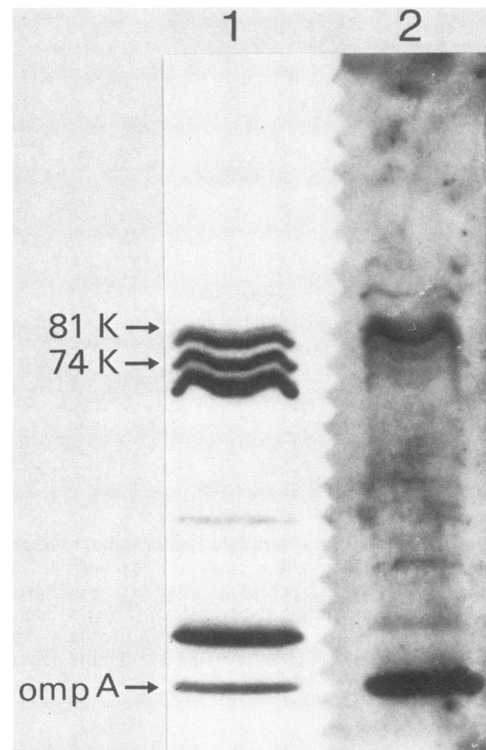

FIG. 7. Immunoblot of the separated outer membrane proteins of in vivo-grown *E. coli* O18 (60 μg of total protein) reacted with serum from a healthy human adult (100 μl) (lane 2). Lane 1 carried ^{125}I-labeled outer membrane proteins of in vivo-grown *E. coli* O18. The radiolabeled proteins were electrophoresed and transferred to the nitrocellulose paper simultaneously with the unlabeled proteins run in lane 2. The nitrocellulose sheet was cut in between the 2 lanes with pinking shears, and the unlabeled proteins were processed as described in the text. The immunoblot and the nitrocellulose paper carrying the ^{125}I-labeled proteins were aligned exactly with the serrated edges before being placed on the X-ray film.

proteins. Sera from some individuals produced very strong reactions with the siderophore receptors but it is not known if this was a reflection of the presence of more avid antibodies or simply the presence of larger amounts of antibodies. In contrast, infants either had no antibodies to the siderophore receptors or produced reactions which were barely detectable. However, most sera from these children had antibodies which produced a significant reaction with the ompA protein. Since the number of individuals examined in this initial study was very small, it remains to be seen if infants in general are deficient in antibodies directed against the iron-regulated outer membrane proteins. It also remains to be seen if anti-siderophore receptor antibodies play a role in protection. The possibility exists that antibodies directed against the iron-regulated outer membrane proteins will interfere with siderophore-mediated iron transport and thus inhibit bacterial multiplication under iron-restricted conditions. Coulton (6) has recently reported that specific antibodies raised in rabbits against the fhuA protein (M_r, 78,000) of *E. coli* K-12, which is the outer membrane receptor for ferrichrome, can partially inhibit ferrichrome-mediated iron uptake. Further studies on the specificities of the naturally occurring serum antibodies which react with the iron-regulated outer membrane proteins of *E. coli*, their origin, distribution, and levels in humans, and their ability to inhibit siderophore-mediated iron uptake processes are now in progress.

ACKNOWLEDGMENTS

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