Antibodies Raised against Rough Mutants of *Escherichia coli* and *Salmonella* Strains Are Opsonic Only in the Presence of Complement

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The opsonic capacity of antisera raised in rabbits against rough (R) mutants and smooth (S) parent strains of *Escherichia coli* and *Salmonella typhimurium* were studied. All specific antibodies in the antisera belonged to the immunoglobulin G (IgG) class. Radioactively labeled bacteria were preincubated in various dilutions of antisera, in which complement was inactivated. Fresh normal rabbit serum, as a standard complement source, was used in some experiments. After preincubation, washed bacteria were added to normal human neutrophils. Opsonization of R mutants for 5 min in 5% fresh normal rabbit serum resulted in effective phagocytosis; S strains needed at least a 30-min opsonization time or 20 to 50% serum. After incubation for 5 min in diluted, homologous antisera, phagocytosis of S strains was optimal, but preincubation of R mutants in diluted, homologous antisera did not lead to amelioration of phagocytosis compared with that of bacteria preincubated in buffer only. However, when fresh normal serum was added to homologous antisera, uptake of R mutants occurred at a faster rate than that of bacteria opsonized in fresh serum alone. Using six clinical isolates of members of the family *Enterobacteriaceae*, we found, that with or without complement, antisera raised against *E. coli* J5 or *S. typhimurium* Re had, with the exception of one strain, no opsonic activity for these strains. Thus, the protective effect of R antisera in gram-negative bacteremia, as shown by several investigators, is unlikely to be mediated through enhanced opsonization of invading bacteria by IgG antibodies directed against these R mutants.

Efficient phagocytosis of microorganisms by polymorphonuclear leukocytes (PMN) requires the participation of opsonins. C3b, which is activated via the classical or alternative pathway of complement, and antibodies to bacterial cell envelope components are important opsonins (4, 13). Gram-negative bacteria with capsular polysaccharides (K antigens) or certain O-antigenic side chains in their lipopolysaccharides (LPS) may resist opsonization in normal serum, and this resistance to opsonization is correlated with virulence (8, 9, 12, 14). Such strains need specific anti-K or anti-O antibodies for efficient uptake by PMN (14, 18, 19).

Antibodies raised against K antigens have been used successfully in protecting animals from infections with K antigen-positive strains (7, 15). These antibodies are usually type specific. Also, antibodies directed against O antigens are type specific in relation to protection (7, 23). Over the last 10 years, several investigators have shown that antisera obtained from animals or healthy human volunteers immunized with strains deficient in O antigens (rough [R] mutants) protect animals or humans, respectively, against the sequelae of serious infections by heterologous gram-negative rods (6, 10, 21–23). The mechanism of this protection is still unknown (20). One of the possibilities in this respect is an opsonic effect of such antisera.

Young et al. (21) showed that antisera raised against *Salmonella minnesota* Re595 do not effectively enhance opsonophagocytosis. In contrast, Crowley et al. (3) showed that phagocytosis of each of two *Escherichia coli* strains opsonized with fresh pooled serum and heated antisera raised against *S. minnesota* Re595 is significantly greater than that observed with fresh pooled serum alone. Van Dijk et al. (15) showed that J5 antiserum containing complement possesses a significantly better opsonic capacity for the homologous strain than fresh normal rabbit serum. However, the J5 antiserum of van Dijk et al. (15) had no opsonic activity for the parent strain and various other strains of *E. coli*.

Because of these controversies and the apparent lack of a study that specifically deals with the opsonic activity of antisera raised against R mutants and their smooth (S) parent strains, we investigated the opsonic capacity of such antisera and one standard source of complement in detail.

**MATERIALS AND METHODS**

**Bacteria.** *E. coli* O111 and its rough mutant J5, chemotype Rc, and *Salmonella typhimurium* M206 (O type 1.4.12) and its R mutants Ra (SF 1592, which lacks the O antigen), Rc (SF 1195, which possesses only lipid A, 2-keto-3-deoxyoctulosonic acid, and several sugars of the core region), and Re (SF 1398, which possesses only lipid A and 2-keto-3-deoxyoctulosonic acid) were used. *E. coli* J5 was a gift from M. P. Glauser (CHUV, Lausanne, Switzerland). This strain lacks both the enzyme uridine-5'-diphosphate-galactose 4-epimerase and the ability to incorporate exogenous galactose into its LPS (23). *E. coli* O111 and the *S. typhimurium* strains were kindly provided by P. A. M. Guinée (National Institute of Public Health and Environmental Hygiene, Bilthoven, The Netherlands). The *Salmonella* strains, of which the LPS structure has recently been described in more detail (5), were originally selected in the Max-Planck-Institut für Immunobiologie, Freiburg im Breisgau, Federal Republic of Germany, and provided by the Institut Pasteur, Paris, France. After conservation in 15% glycerol solution, these strains were kept at −70°C until use. Also, strains isolated from blood of patients with bacteremia

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hospitalized at the University Hospital of Utrecht, Utrecht, The Netherlands, were used. These strains were kept in a lyophilized state until use.

**Oposonins.** Normal rabbit serum (NRS) was collected from blood drawn through puncture of the ear vein of one rabbit (F; New Zealand 81 x Fleming giant 82) on several occasions and stored in 1.0-mL fractions at −70°C until use. This serum contained no specific antibodies against the strains mentioned above, as measured by a passive hemagglutination assay, as modified from the method of Neter et al. (11), and was used as the complement source.

The antisera raised against *E. coli* O111, *E. coli* J5, and *S. typhimurium* Re were prepared as follows. Bacteria were grown overnight in tryptic soy broth (Difco Laboratories, Detroit, Mich.), washed in phosphate-buffered saline (PBS, pH 7.4), boiled for 60 min, and adjusted to a concentration equivalent to 5 × 10⁵ CFU/mL of PBS. The vaccines were stored at −20°C and thawed prior to use. F; rabbits (4 to 6 kg) were subjected to a standard immunization schedule (15) and bled by heart puncture. The collected sera were stored in 1.0-mL volumes at −70°C and thawed prior to use. The rabbit antisera raised against *S. typhimurium* M206, Ra, and Rc were kindly provided by P. A. M. Guinée. Antisera were heated for 30 min at 56°C to inactivate complement.

**Enzyme-linked immunosorbent assay.** Bacteria were grown overnight at 37°C on Iso-Sensitest agar (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom), washed once, adjusted to a concentration of 2.5 × 10⁶ CFU/mL of PBS, and boiled for 1 h. In each well (polystyrene Microplate; Flow Laboratories, Amstelstad, Zwabenburg, The Netherlands), 0.1 mL of the bacterial suspension was allowed to adhere for 1 h at 37°C and subsequently overnight at 4°C. Plates were washed thoroughly with 0.5 mL of Tween 20 (E. Merck AG, Darmstadt, Federal Republic of Germany) per liter of tap water. Antisera were diluted 1:1,000 in PBS containing 0.05 mL of Tween 20 and 40 g of bovine serum albumin per liter. Serial twofold dilutions of antisera were made, and 0.05 mL of a dilution was added to each well. After incubation for 60 min at 37°C, plates were washed, and 0.05 mL of an appropriate dilution of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands), anti-rabbit immunoglobulin G (IgG; Litton Bionetics, Charleston, United Kingdom), or anti-rabbit IgM (Miles Laboratories, Inc., Elkhart, Ind.) per well was added. After incubation for 60 min at 37°C, plates were washed, and the absorbance was measured at 450 nm in a Titertek Multiskan spectrophotometer (Flow Laboratories). The titer of a serum sample was expressed as the reciprocal value of the dilution showing an optical density of 1.000.

**Immunofluorescence assay.** Bacterial suspension (0.01 mL), prepared as described above for the enzyme-linked immunosorbent assay (ELISA), was added to a well of a multistest slide (Flow Laboratories), dried, and fixed by heating. Antisera were diluted 1:40 in PBS. Serial twofold dilutions were made, and 0.01 mL of each dilution was added to a well. Slides were washed, dried, covered with a cover slip together with a few drops of DPX mounting fluid (BHD-Chemicals Ltd., Poole, United Kingdom), and examined under a fluorescence microscope. The immunofluorescence titer was defined as the reciprocal value of the highest dilution that still showed fluorescence.

**Opsonization procedure.** Normal serum and antisera, heat-inactivated if necessary, were diluted in Hank's balanced salt solution containing 0.1% gelatin (gHBSS; Gibco Bio-Cult Ltd., Paisley, Scotland). In polypropylene vials (Biovials; Beckman Instruments, Merthyr, Galway, Ireland), 0.2 mL of a prewarmed (37°C) bacterial suspension (2.5 × 10⁸ CFU/mL of gHBSS) was added to 0.8 mL of diluted serum. The vials were incubated at 37°C in a Marius shaking water bath (150 rpm), and opsonization was stopped by adding 2.0 mL of ice-cold PBS. After centrifugation for 15 min at 1,600 × g at 4°C, opsonized bacteria were suspended in 1.0 mL of gHBSS and kept at 0°C until use.**Leukocytes.** PMN were isolated by a method modified from that of Boyum (2), as described previously (16). In brief, venous blood samples from healthy adult donors were heparanized (10 U of heparin per mL of blood) and settled by gravity in 6% dextran (molecular weight, 70,000; Pharmacia Fine Chemicals, Upplala, Sweden) in normal saline (10 mL of blood, 3 mL of dextran solution). The leukocyte-rich plasma was withdrawn and centrifuged for 10 min at 160 × g. The pellet was suspended in gHBSS; 6 mL of cell suspension was carefully layered onto 3 mL of Ficoll-Paque (Pharmacia Fine Chemicals) and centrifuged for 35 min at 160 × g. Mononuclear cells of the interface were removed. Residual erythrocytes in the pellet were lysed with ice-cold 0.87% (H/O) NH₄Cl in sterile water. After centrifugation for 10 min at 160 × g, PMN were washed once in the NH₄Cl solution and once in gHBSS and adjusted to a concentration of 5 × 10⁶/mL of gHBSS.

**Phagocytosis assay.** Phagocytosis was measured by previously described methods (16, 17). After 18 h of growth at 37°C in 5 mL of Mueller-Hinton broth (Difco) containing 0.02 mCi of [³H]thymidine (specific activity, 5.0 Ci/mmol; Radiochemical Centre, Amersham, United Kingdom), bacteria were washed three times in PBS and adjusted to a concentration of 2.5 × 10⁸ CFU/mL of gHBSS. Opsonization was performed as described above.

Opsonized bacterial suspension (0.2 mL) was added to 0.2 mL of PMN suspension in four polypropylene vials (Beckman). The final bacterium-to-PMN ratio was 10:1. Vials were shaken (150 rpm) in a water bath at 37°C. After 2, 6, and 12 min, phagocytosis was stopped by adding ice-cold PBS to each of three vials. Leukocyte-associated bacteria, i.e., bacteria ingested by and attached to PMN, were washed free of non-leukocyte-associated bacteria by three cycles of differential centrifugation (160 × g at 4°C). The pellets were suspended in 2.5 mL of scintillation liquid (Aqua Luma Plus; Lumac/3M, Schaesberg, The Netherlands). Leukocyte-associated radioactivity was measured in a liquid scintillation counter (Philips, Eindhoven, The Netherlands) and expressed as a percentage of the total radioactivity added, as determined in the pellet of the fourth vial after centrifugation for 15 min at 1,600 × g and resuspension in scintillation liquid (Lumac/3M). Percent uptake at 12 min was taken as a measure of opsonization, because maximal phagocytosis had occurred at that time. For significance analysis Student's t test was performed.

**RESULTS**

**Antibody titers.** In ELISA all antisera contained high titers of antibodies directed against their homologous, heat-killed
strains (Table 1). By using anti-rabbit IgG or anti-rabbit IgM as conjugates in ELISA, it was shown that these antibodies belonged to the IgG class. Results of the immunofluorescence assay confirmed that heat-killed bacteria of R mutants were loaded with antibodies after incubation in homologous antisera (Table 1).

The antisera, mostly described in studies in which protection against heterologous gram-negative bacteria was investigated, are those raised against the J5 mutant or against Re chemotypes of Salmonella. Our anti-J5 and anti-Re antisera contained antibodies that cross-reacted with heterologous strains (Table 2).

Phagocytosis of E. coli and S. typhimurium S strains and their R mutants after opsonization in fresh normal serum and homologous antisera. Bacteria were incubated for different periods of time in various concentrations of fresh normal rabbit serum or heated homologous antisera, centrifuged, suspended, and added to PMN.

Opsonization for 5 min in 5% fresh NRS resulted in efficient phagocytosis of R mutants (Fig. 1). Controls showed that incubation in 5% serum for 5 min did not lyse the bacteria. Radioactive markers did not leak out the bacteria after opsonization before bacteria were added to PMN. Therefore, after incubation radioactivity associated with PMN could be used as a measure of uptake.

Incubation for 5 min in 5% fresh NRS was not sufficient for effective phagocytosis of S strains: S. typhimurium M206 needed an opsonization time of at least 30 min or incubation for 5 min in 20% NRS. For E. coli O111 opsonization even in 50% NRS did not lead to more than 40 to 50% uptake by PMN (data not shown).

Heated antisera raised against the S parent strains were highly opsonic for the corresponding bacteria (Fig. 2). After 5 min of opsonization in 1% homologous antisera, over 70% of E. coli O111 or S. typhimurium M206 was ingested. Uptake of these opsonized bacteria proceeded rapidly; most of the bacteria were ingested after 2 min of incubation with PMN. However, none of the heated antisera raised against R mutants showed any direct opsonic activity for the corresponding bacteria when incubated for 10 min in 10% dilutions of heated antisera; uptake was not higher than that of bacteria preincubated in buffer only (Fig. 3). Even preincubation of E. coli J5 in 50% homologous antisera resulted in less than 30% phagocytosis.

It could be that antibodies raised against R mutants contribute indirectly to opsonization by activating complement, and this activation could then lead to rapid deposition of C3b on the cell surface of the bacteria, thereby enabling phagocytosis. Therefore, bacteria were incubated for 2 min in 10% heated homologous antisera, together with 5% fresh NRS as a standard complement source. Phagocytosis of these bacteria was compared with that of bacteria incubated in 10% heated normal rabbit serum together with 5% fresh NRS. Again, when R mutants were preincubated in heated homologous antisera, virtually no uptake by PMN was observed (Fig. 3). However, after the addition of fresh NRS as a complement source, uptake was greatly enhanced. Over 80% of those bacteria were ingested after 12 min of incubation with PMN. Phagocytosis of bacteria opsonized in heated normal rabbit serum, together with fresh NRS, was significantly lower (P < 0.05).

Phagocytosis of heterologous strains after opsonization in antisera raised against S and R mutants of E. coli and S. typhimurium. Bacteria were incubated for 10 min in 10% heated antisera, centrifuged, suspended, and added to PMN. Virtually no uptake of bacteria was observed when heterologous mutants, parent strains, or clinical isolates of E. coli, Proteus mirabilis, and Enterobacter spp. were preincubated in heated antisera raised against E. coli O111 or J5 or S. typhimurium M206, Ra, Rc, or Re (data not shown). The only exception was that antisera raised against S. typhimurium Rc showed opsonic activity for the parent strain (61.5 ± 5% uptake). After preincubation in gHBSS only, uptake of the parent strain by PMN was minimal (2%).

Although our antisera raised against E. coli J5 and S.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Titer of homologous antisera by:</th>
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<tbody>
<tr>
<td></td>
<td>ELISA*</td>
</tr>
<tr>
<td>E. coli O111</td>
<td>15,200</td>
</tr>
<tr>
<td>E. coli J5</td>
<td>77,000</td>
</tr>
<tr>
<td>S. typhimurium M206</td>
<td>6,500</td>
</tr>
<tr>
<td>S. typhimurium Ra</td>
<td>14,000</td>
</tr>
<tr>
<td>S. typhimurium Rc</td>
<td>6,000</td>
</tr>
<tr>
<td>S. typhimurium Re</td>
<td>&gt;128,000</td>
</tr>
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</table>

* IF, Immunofluorescence. In both assays conjugated anti-total rabbit antisera was used.

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**TABLE 2.** ELISA titers of NRS, Re antiserum, and J5 antiserum incubated with heat-killed bacteria of each of six clinical isolates

<table>
<thead>
<tr>
<th>Strain</th>
<th>Titer when incubated with:</th>
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<tbody>
<tr>
<td></td>
<td>NRS</td>
</tr>
<tr>
<td>Escherichia coli O18</td>
<td>—</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>—</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>—</td>
</tr>
<tr>
<td>Klebsiella oxytoca</td>
<td>—</td>
</tr>
<tr>
<td>Enterobacter aerogenes</td>
<td>—</td>
</tr>
<tr>
<td>Enterobacter cloacae</td>
<td>—</td>
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</tbody>
</table>

* Anti-total rabbit immunoglobulins were used as the conjugate.

* — No detectable titer.
typhimurium Re did not exert direct heat-stable opsonic activity for heterologous strains, it is possible that anti-J5 or anti-Re antibodies contributed indirectly to opsonization of such strains when functional complement was present. Therefore, each of six clinical isolates was preincubated for 5 min in unheated antiserum and as controls in fresh NRS. Complement-containing anti-Re antiserum exhibited no more opsonic capacity than did fresh NRS (Table 3). Anti-J5 antiserum containing complement rendered only one E. coli strain, O18, susceptible to efficient phagocytosis, which was significantly better than results obtained with NRS (P < 0.05). When P. mirabilis (two strains), Klebsiella oxytoca, Enterobacter aerogenes, and Enterobacter cloacae were tested, no differences in this respect were observed (Table 3).

DISCUSSION

Results of several studies have shown that antisera raised against R mutants with only lipid A and core in their LPS, especially E. coli J5 and Re chemotypes of Salmonella, protect animals and humans against sequelae of gram-negative bacteremia (8, 21–23). The mechanism through which these antisera exert their protective role is still unclear (20); both antienodotoxin and antibacterial, e.g., opsonic, activities, have been suggested (6).

Our study provides evidence that rabbit antisera raised against R mutants, in which complement was inactivated, did not exert direct, heat-stable (i.e., antibody-mediated) opsonic activity for homologous strains, although the bacteria were loaded with antibodies after incubation in homologous antisera, as shown by ELISA and immunofluorescence methods. Antiserum raised against E. coli J5, S. typhimurium Ra, and S. typhimurium Re containing specific antibodies of the IgG class also possessed no direct heat-stable opsonic activity for heterologous strains. Antiserum raised against S. typhimurium Re had opsonic activity for the parent strain M206 only.

Opsonization occurs not only via antibodies but also through activation of the classical or alternative pathway of the complement system. The former is usually activated by antibody-antigen complexes. Although by themselves antibodies in our antisera raised against R mutants were not opsonic, they enhanced opsonization of homologous bacteria in the presence of fresh normal rabbit serum as a standard complement source. Also, Van Dijk et al. (15) showed that antiserum raised against their E. coli J5 strain had a significantly better opsonic effect on the homologous strain than did fresh normal serum. Thus, antibodies raised against R mutants only contribute to phagocytosis of homologous strains when complement is present. Here, complement presumably was activated rapidly via the classical pathway, leading to deposition of C3b onto the bacterial surface, consequently leading to enhanced recognition by neutrophils.

Complement-containing antiserum raised against S. typhimurium Re had no opsonic activity for clinical isolates, whereas that raised against E. coli J5 had opsonic activity for one E. coli isolate only. We conclude that the protective effect of anti-R antisera in gram-negative bacteremia, as shown by several investigators, is probably not mediated via enhanced opsonization of invading bacteria by IgG antibodies raised against such mutants.

Our findings are partly in agreement with those of Young and co-workers (21). They showed that immunization with
TABLE 3. Phagocytosis of six clinical isolates preincubated in fresh NRS, Re antisemum containing complement, or J5 antisemum containing complement\(^a\)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phagocytosis (%) when preincubated with:</th>
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<tbody>
<tr>
<td></td>
<td>NRS</td>
</tr>
<tr>
<td>Escherichia coli O18</td>
<td>48 $\pm$ 2.5(^b)</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>15</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>1</td>
</tr>
<tr>
<td>Klebsiella oxytoca</td>
<td>25</td>
</tr>
<tr>
<td>Enterobacter aerogenes</td>
<td>37</td>
</tr>
<tr>
<td>Enterobacter cloacae</td>
<td>40</td>
</tr>
</tbody>
</table>

\(^a\) Uptake was measured after 12 min of incubation of opsonized bacteria and PMN.
\(^b\) Values are the mean of three independent observations $\pm$ the standard deviation.

S. minnesota Re595 protects against the hemodynamic sequelae of bacteremia, augments intravascular clearance of a serum-sensitive strain, and abrogates the pyrogenic response to enteric bacilli; but it does not enhance clearance of serum-resistant bacteria. Although their canine and lapine antiserum, directed against LPS extracted from the mutant, protected mice against a heterologous challenge with E. coli 085:H9 (serum resistant), opsonic activity for serum-resistant S. marcescens O3 is at least 100-fold less than that of type-specific antiserum (21).

In contrast to the antiserum raised against R mutants, in our study antiserum raised against the parent strains had excellent direct heat-stable opsonic activity for corresponding strains, much better than that of fresh NRS.

Although data in the literature are conflicting, from our results and those of others some basis for the following hypothesis can be found. Members of the family Enterobacteriaceae may be divided into three groups according to the way they are recognized by PMN. Some bacteria (e.g., capsule-possessing E. coli) have an absolute requirement for high titer of opsonic antibodies (14); others can be opsonized by antibody or complement (most S strains), and in a third group strains have an absolute requirement for complement (e.g., R mutants). Antibodies alone have no effect on phagocytosis of the latter bacteria, but uptake is enhanced when antibodies and complement are present.

This study raises several new questions. Why are R strains, loaded with antibodies, as was shown in ELISA and immunofluorescence assays, not recognized by PMN? Do antiserum raised against certain R mutants exert their protective role against challenge with heterologous strains mainly through neutralization of endotoxins? Do they contribute to killing of heterologous, S bacteria? More studies are needed to find possible answers to these questions.

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LITERATURE CITED