Bactericidal and Opsonizing Effects of Normal Serum on Mutant Strains of *Salmonella typhimurium*

MEN-FANG SHAIO AND HAK ROWLAND*

Department of Clinical Tropical Medicine, London School of Hygiene and Tropical Medicine, London WC1, England

Received 8 February 1985/Accepted 6 June 1985

The bactericidal and opsonizing effects of normal human serum on six strains of *Salmonella typhimurium* LT-2 having different lipopolysaccharide (LPS) composition were demonstrated through five indices. Complement activity in the presence of antibody was important for the opsonization of all six strains and for the bactericidal effect on rough mutants. Complement activity, either in the presence or absence of antibody, was involved in the ingestion strains of SL 901 (SR) and SL 1032 (Rd,) by human neutrophils. Strain SH 5014 (Rb,) was avidly ingested by neutrophils and totally dependent on complement activity in the presence of antibody. The ingestion of strain SH 2201 (S) was also mediated exclusively by complement activity in the presence of antibody but not as efficiently as were rough mutants. Antibody, as demonstrated by quantitative fluorescence, enhanced the complement activity on the ingestion of the S, SR, and Rb2 strains by neutrophils. The intracellular killing of six strains was enhanced significantly by complement activity in the presence of antibody. The overall survival in the presence of serum and neutrophils decreased as the LPS became shorter. Complement activity in the presence of antibody enhanced extracellular killing only for strains SL 901 (SR) and his S 515 (Ra). It was shown that there was no difference between SR and Ra strains in all five indices, suggesting that the one additional O-antigen side chain does not make the SR strain more resistant than the Ra strain. Although resistance by *S. typhimurium* to host defense mechanisms increases as the LPS chain length increases, the specific LPS structure appears to be of greater importance, especially with respect to opsonization.

Much time and work has been devoted to the investigation of serum-mediated killing and phagocytosis of gram-negative bacteria (16, 19). There are three possible mechanisms for clearance of bacteria by serum or neutrophils or both in vitro: (i) complement activation in the presence of antibody, (ii) complement activation in the absence of antibody, and (iii) antibody alone. The effect of these mechanisms on gram-negative bacteria is determined mainly by the structure of the outer membrane and the surface properties (4, 7, 23, 24).

*Salmonella typhimurium*, having the typical outer membrane structure, and its mutants with progressively shorter lipopolysaccharide (LPS) chains, is one of the most frequently studied gram-negative organisms. Smooth (S) strains contain intact core structure and repeating O-antigen side chains. Rough (R) mutants are defective in various stages of LPS biosynthesis; all of them with the exception of semirough (SR) strains lack O-antigen side chains. It was shown that the sensitivity to the bactericidal–permeability-increasing protein of neutrophils increased with decreasing LPS chain length (2). Thus it was believed that the resistance of *S. typhimurium* might be directly related to the LPS chain length of the outer membrane (16). However, recent reports have shown that small changes in LPS influence complement activation and phagocytosis simultaneously (7, 8). Furthermore, complement activation by LPS was sensitive to small variations in chemical structure but not to changes in side-chain length (5).

Despite extensive immunochemical studies on the outer membrane during the past decades, the role of LPS in serum-mediated lysis and phagocytosis by human defense mechanisms is still not well understood. In the present study, the bactericidal and opsonizing effects of normal human serum (NHS) on mutant strains of *S. typhimurium* have been measured. By means of five indices, human defense mechanisms against six strains of *S. typhimurium* with different LPS compositions have been examined.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** *S. typhimurium* LT-2 SH 2201 (S) and LPS-deficient mutants SL 901 (SR), his S 515 (Ra), SH 5014 (Rb2), SL 1032 (Rd,), and SL 1102 (Re) (Table 1) were obtained through the courtesy of P. H. Makelä (National Public Health Institute, Helsinki, Finland). Each strain was preserved in 12% glycerol in small aliquots at −70°C and was not refrozen after thawing. In studies of phagocytosis, bacteria were grown at 37°C in Mueller-Hinton broth (Oxoid Ltd., London, England) for 15 h. Washed organisms were suspended in Hanks balanced salt solution (HBSS) with 0.1% gelatin at 10⁹ cells per ml. Total counts were made in a chamber, and live organisms were estimated by the standard colony counting method.

**Radioactive labeling of bacteria.** In the immunofluorescence assay for serum antibodies, organisms were grown in 10 ml of Mueller-Hinton broth containing 0.02 mCi of [methyl-³H]thymidine (specific activity, 5 Ci/mmol; The Radiochemical Centre, Amersham, England) as previously described (21). After 15 h of cultivation at 37°C, the bacteria were washed three times with sterile isotonic phosphate-buffered saline (pH 7.4) and adjusted to a concentration of 10⁹ cells per ml.

**Sera.** Venous blood from seven healthy donors was allowed to clot, and the pooled NHS was stored at −70°C in 0.5-ml aliquots. Heat inactivation to abolish complement activity and produce heat-inactivated serum (HIS) was carried out at 56°C for 30 min, and absorption to remove antibody was carried out by reaction with homologous organisms at 10¹⁰ cells per ml of serum at 4°C for 30 min to produce absorbed serum (ABS). NHS was heated and then absorbed (HABS) by both procedures.

* Corresponding author.
Opsonization of bacteria. To measure serum antibodies, bacteria were opsonized by incubation at 37°C for 15 min at 2 × 10^8 cells per ml in the presence of different concentrations (0.5, 1, 2, 4, 8, and 10%) of NHS and ABS; HBSS acted as a control. Because of the total lysis of strain SL 1102 (Re) in the presence of higher concentrations of NHS and ABS, HIS, and HABS were used to measure the serum antibody for the Re strain. Opsonized bacteria were washed twice and resuspended at 2 × 10^8 cells per ml in phosphate-buffered saline.

Indirect fluorescence antibody assay. A fluorescein-conjugated sheep anti-human immunoglobulin G (Wellcome, Beckenham, England) was used for the indirect fluorescence antibody assay. In brief, each milliliter of opsonized bacteria (2 × 10^8) was reacted with 30 μl of undiluted fluorescein-conjugated sheep anti-human immunoglobulin G for 15 min at room temperature. To remove nonreacted conjugate, the bacteria were then washed three times with phosphate-buffered saline and finally suspended in 1 ml of phosphate-buffered saline. Each of two 50-μl aliquots of this suspension in 4 ml of scintillation fluid (Opti-fluor; Packard, Caversham, England) was measured for radioactivity in a liquid scintillation spectrophotometer (LS 9800; Beckman Instruments, Inc., Fullerton, Calif.). The amount of antibody attached to the bacterial surface was quantitatively measured in a fluorimeter (LS-2 filter; Perkin-Elmer, Beaconsfield, England). Fluorescence was measured with excitation and analyzing wavelengths of 485 and 525 nm, respectively. Results were corrected for radioactive counts.

Separation of cells. Venous blood from healthy donors was drawn into plastic syringes containing 10 IU of heparin per ml; 4.5% dextran (wt/vol) was added in a ratio of 1:5, and the syringe was incubated nozzle upward at 37°C for 30 min. The upper fraction was then layered onto Lymphoprep (Nygaard, Oslo, Norway) at a ratio of 2:1 and centrifuged at 1,500 × g for 10 min. The pellet was washed, contaminating erythrocytes were lysed with 0.83% (wt/vol) ammonium chloride, and the neutrophils were suspended in HBSS at 10^7 cells per ml. Both purity and viability were consistently greater than 95% by this method.

Reaction mixtures. Reaction mixtures were set up containing (i) 1% serum, either untreated (NHS), treated (HIS, ABS, or HABS), or absent (HBSS); (ii) 10^8 bacteria; (iii) 5 × 10^8 neutrophils, present or absent; (iv) HBSS to 1 ml. The mixtures were sampled at time zero (t0) for colony counting (C0), then incubated at 37°C and rotated end over end for 20 min. At t20, samples were taken for colony counts (C20) and for cyt centrifuge counts (C20). From the remaining mixture neutrophils were separated by centrifugation at 100 × g for 10 min. Colony counts were made on neutrophils suspended in distilled water in the first dilution tube to lyse the cells (C).

Colony counting. Colony counting was carried out by the standard dilution method after overnight incubation on agar plates (Oxoid).

Cytocentrifuge preparations. Portions (75 μl) of reaction mixture were pipetted into the chambers of the cytocentrifuge (Cytospin 2; Shandon, Runcorn, England) and centrifuged at 165 × g for 10 min. The preparations were stained with Giemsa stain, and the number of bacteria in 200 neutrophils was counted. Organisms outside but in contact with the neutrophils were not counted.

Indices. From the results several indices were calculated: (i) VIS = (C20/C0) × 100, for mixtures containing no neutrophils, = viability index for bacteria in serum alone; (ii) PI = (C1/C0) × 100, where C1 is the total number of bacteria added to the mixture, = phagocytic index; (iii) VIC = (C2/C0) × 100, = viability index for ingested bacteria; (iv) VIO = (C20/C0) × 100, for mixtures containing neutrophils, = viability index in the presence of neutrophils and serum; (v) VEC = [VIS/C0] × 100, where VIS(C) = VIO − (PI × VIC)/(1.0 − PI) = extracellular viability in the presence of neutrophils. VEC is the factor by which extracellular survival is decreased in the presence of neutrophils.

---

**Table 1. Properties of S. typhimurium LT-2 strains used**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Type of LPS</th>
<th>Structure of LPS</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SH 2201 S (O-4,5,12) (oligosaccharide, repeating unit: ( n = 2 ) through 30)</td>
<td>Gal</td>
<td>Hep</td>
<td>KDO</td>
</tr>
<tr>
<td>SL 901 SR (O-4,5,12) (oligosaccharide, repeating unit: ( n = 1 ))</td>
<td>GlcNAc</td>
<td>Gal</td>
<td>Hep</td>
</tr>
<tr>
<td>his 515 Ra</td>
<td>GlcNAc</td>
<td>Gal</td>
<td>Hep</td>
</tr>
<tr>
<td>SH 5014 Rb2</td>
<td>Gal</td>
<td>Glc</td>
<td>Hep</td>
</tr>
<tr>
<td>SL 1032 Rd1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SL 1102 Re</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Oligosaccharide consists of d-galactose, l-rhamnose, d-mannose, and abequose. Gal, d-galactose; Glc, d-glucose; GlcNAc, N-acetylgalactosamine; Hep, heptose.*
TABLE 2. Factorial designs for experiments

<table>
<thead>
<tr>
<th>Complement</th>
<th>Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>NHS</td>
</tr>
<tr>
<td>+</td>
<td>ABS</td>
</tr>
<tr>
<td>-</td>
<td>HIS</td>
</tr>
<tr>
<td>-</td>
<td>HABS</td>
</tr>
</tbody>
</table>

* +, Present; −, absent. Complement activity in the presence of antibody, NHS − HABS. Complement activity in the absence of antibody, ABS − HABS. Antibody alone, HIS − HABS. Effect of antibody on complement activity = Complement activity when antibody present/Complement activity when antibody absent.

**Designs and analysis.** Since only two *Salmonella* strains could be tested together in the same experiment, and since the source of neutrophils necessarily had to be different for each experiment, a balanced incomplete block design was used, in which each strain was compared with every other strain in the same experiment the same number of times, namely once (3). In addition, factorial designs (Table 2) were used and analysis on log values was carried out by analysis of variance. A factor is an agent which may influence the value of an index, and an effect is the ratio of the geometric mean value of the index in the presence of the factor to the geometric mean value of the index in its absence.

By the method used here, antibody-dependent complement activity cannot be measured directly, whereas direct measurement of complement activity in the presence of antibody (i.e., total heat-labile activity) and complement activity in the absence of antibody (i.e., antibody-independent complement activity) is possible. Moreover, the effect of antibody on complement activity can be determined by the difference between complement activity in the presence and in the absence of antibody.

**RESULTS**

**Preliminary investigation.** The basic experiments on phagocytosis of six strains of *S. typhimurium* were per-

![FIG. 1. Serum antibodies (immunoglobulin G) interacting with mutant strains of *Salmonella typhimurium* determined by indirect immunofluorescence assay. HBSS was used as a control; the fluorescence reading was similar to that for ABS or HABS (not shown on the graph). Because of the susceptibility of strain SL 1102 (Re) to lysis by higher concentrations of serum, HIS, and HABS were compared. Symbols represent the means of four values.](http://iai.asm.org/)
formed in the presence of different serum concentrations (1, 4, 7, and 10%) and at three reaction time intervals (10, 20, and 30 min). It was found that the difference in ingestion of bacteria by neutrophils was greatest in the presence of 1% NHS and at a reaction time of 20 min.

Fluorescence measurement of serum antibodies. To directly demonstrate the presence of serum antibodies capable of interacting with the surface of the Salmonella strains, a quantitative fluorescence immunoassay was set up. As the concentration of NHS increased, so did that of antibody, and almost linearly; concentrations of ABS, however, had little effect on the fluorescence reading (Fig. 1). Thus, NHS contains immunoglobulin G antibodies which bind to these strains of Salmonella. Absorption with the homologous organisms completely depletes serum of the same immunoglobulin G.

Effect of normal serum on five indices. In the presence of 1% NHS, five bactericidal indices were determined (Table 3). For VIS, VIC, VIO, and VEC, the higher the value of the index, the less is the killing. For PI, the higher the value of the index, the greater is the ingestion.

Serum alone had no effect on strain SH 2201 (S), but the VIS of all R mutants was reduced, and this was especially marked for strain SL 1102 (Re). Otherwise, there was no difference between the R strains.

The phagocytic index (PI) varied from 25% (the S strain) to 91.5% (the Re strain). Although there was a significant difference between strains his 515 (Ra) and SH 5014 (Rb) 2 (P < 0.01), no difference was noted between either SL 901 (SR) and his 515 (Ra) or SL 901 (SR) and SH 5014 (Rb). In addition, no difference was found between SL 1032 (Rd) and SL 1102 (Re).

VIC was highest for strain SH 2201 (S), but even so, only a small percentage of bacteria survived after ingestion. Moreover, a difference among the four strains (S, SR, Ra, and Rb) was not detectable. VIC decreased to 0.06% for SL 1032 (Rd) and 0.004% for SL 1102 (Re).

VEO steadily decreased from 63.8% for SH 2201 (S) to 0.4% for SL 1102 (Re).

Similarly, the extracellular killing effect of neutrophils increased, VEC being 92% for SH 2201 (S) and 21.9% for SL 1102 (Re); this means that the extracellular survival of SL 1102 decreased fivefold in the presence of neutrophils.

Effect of serum factors on the five indices. (i) VIS. The viability of each rough mutant in 1% NHS was reduced significantly by complement activity in the presence of antibody (Table 4). Complement activity in the absence of antibody was important for the killing of most R mutants, but antibody alone contributed only to deep R mutant (Re).

(ii) PI. Complement activity in the presence of antibody was important for the opsonization of all strains, although this was so to a lesser extent for strains his 515 (Ra) and SL 1102 (Re) (Table 5). Complement activity, particularly in the absence of antibody, influenced the ingestion of SL 1032 (Rd). Antibody enhanced the complement activity on ingestion, especially for SH 2201 (S), SL 901 (SR), and SH 5014 (Rb) (see Table 9). Antibody alone had no effect.

(iii) VIC. Complement activity in the presence of antibody reduced the VIC for all strains (Table 6). Moreover, in the absence of antibody, complement activity reduced the VIC of all R mutants. Only SL 901 (SR) and SH 5014 (Rb) were affected by antibody alone.

(iv) VIO. No serum factors contributed significantly to a reduction of the overall viability of strain SH 2201 (S) (Table 7).

---

TABLE 3. Five indices in 1% NHS for six strains of S. typhimurium

<table>
<thead>
<tr>
<th>Strain</th>
<th>VIS</th>
<th>PI</th>
<th>VIC</th>
<th>VIO</th>
<th>VEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>SH 2201 (S)</td>
<td>97.6 ± 1.7</td>
<td>25.4 ± 5.5</td>
<td>3.8 ± 2.7</td>
<td>63.8 ± 5.4</td>
<td>91.9 ± 8.7</td>
</tr>
<tr>
<td>SL 901 (SR)</td>
<td>73.3 ± 7.4</td>
<td>56.8 ± 5.7</td>
<td>2.0 ± 1.9</td>
<td>17.3 ± 3.6</td>
<td>51.3 ± 8.3</td>
</tr>
<tr>
<td>his 515 (Ra)</td>
<td>73.0 ± 5.5</td>
<td>51.6 ± 2.4</td>
<td>2.1 ± 0.9</td>
<td>17.1 ± 1.6</td>
<td>45.8 ± 6.5</td>
</tr>
<tr>
<td>SH 5014 (Rb 2)</td>
<td>71.6 ± 6.5</td>
<td>65.3 ± 7.2</td>
<td>1.8 ± 1.6</td>
<td>10.0 ± 3.4</td>
<td>40.6 ± 8.9</td>
</tr>
<tr>
<td>SL 1032 (Rd)</td>
<td>62.4 ± 7.8</td>
<td>86.5 ± 3.8</td>
<td>0.06 ± 0.02</td>
<td>2.5 ± 1.3</td>
<td>31.4 ± 8.8</td>
</tr>
<tr>
<td>SL 1102 (Re)</td>
<td>24.3 ± 5.4</td>
<td>91.5 ± 4.0</td>
<td>0.004 ± 0.002</td>
<td>0.4 ± 0.1</td>
<td>21.9 ± 6.4</td>
</tr>
</tbody>
</table>

* Mean ± standard deviation of five experiments.

---

TABLE 4. Geometric mean effect of serum factors on VIS for six strains of S. typhimurium

<table>
<thead>
<tr>
<th>Strain</th>
<th>Complement activity in presence of antibody</th>
<th>Complement activity in absence of antibody</th>
<th>Antibody alone</th>
</tr>
</thead>
<tbody>
<tr>
<td>SH 2201 (S)</td>
<td>0.97</td>
<td>0.95</td>
<td>0.96</td>
</tr>
<tr>
<td>SL 901 (SR)</td>
<td>0.76&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.92</td>
<td>0.95</td>
</tr>
<tr>
<td>his 515 (Ra)</td>
<td>0.76&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.88&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.91</td>
</tr>
<tr>
<td>SH 5014 (Rb 2)</td>
<td>0.74&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.83&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.95</td>
</tr>
<tr>
<td>SL 1032 (Rd)</td>
<td>0.64&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.76&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.92</td>
</tr>
<tr>
<td>SL 1102 (Re)</td>
<td>0.29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.63&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.82&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> An effect is the ratio of the geometric mean value in the presence of the factor to the geometric mean value in its absence.

<sup>b</sup> P < 0.01.

<sup>ab</sup> P < 0.001.

<sup>c</sup> P < 0.05.

---

TABLE 5. Geometric mean effect of serum factors on PI for six strains of S. typhimurium

<table>
<thead>
<tr>
<th>Strain</th>
<th>Complement activity in presence of antibody</th>
<th>Complement activity in absence of antibody</th>
<th>Antibody alone</th>
</tr>
</thead>
<tbody>
<tr>
<td>SH 2201 (S)</td>
<td>25.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.58</td>
<td>1.00</td>
</tr>
<tr>
<td>SL 901 (SR)</td>
<td>20.84&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.92&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.64</td>
</tr>
<tr>
<td>his 515 (Ra)</td>
<td>2.00&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.25</td>
<td>1.21</td>
</tr>
<tr>
<td>SH 5014 (Rb 2)</td>
<td>34.57&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.63</td>
<td>1.36</td>
</tr>
<tr>
<td>SL 1032 (Rd)</td>
<td>12.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.42&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.21</td>
</tr>
<tr>
<td>SL 1102 (Re)</td>
<td>2.25&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.36</td>
<td>1.07</td>
</tr>
</tbody>
</table>

<sup>a</sup> Those organisms outside but in contact with the neutrophils were not counted. An effect is the ratio of the geometric mean value in the presence of the factor to the geometric mean value in its absence.

<sup>b</sup> P < 0.001.

<sup>c</sup> P < 0.01.

<sup>d</sup> P < 0.05.
TABLE 6. Geometric mean effect of serum factors on VIC for six strains of *S. typhimurium*<sup>a</sup>

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serum factors</th>
<th>Antibody alone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Complement activity in presence of antibody</td>
<td>Complement activity in absence of antibody</td>
</tr>
<tr>
<td>SH 2201 (S)</td>
<td>0.45&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.92</td>
</tr>
<tr>
<td>SL 901 (SR)</td>
<td>0.37&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.61&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>his 515 (Ra)</td>
<td>0.29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.81</td>
</tr>
<tr>
<td>SH 5014 (Rb&lt;sub&gt;2&lt;/sub&gt;)</td>
<td>0.38&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.69&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>SL 1032 (Rd&lt;sub&gt;1&lt;/sub&gt;)</td>
<td>0.36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.73</td>
</tr>
<tr>
<td>SL 1102 (Re)</td>
<td>0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.90</td>
</tr>
</tbody>
</table>

<sup>a</sup> An effect is the ratio of the geometric mean value in the presence of the factor to the geometric mean value in its absence.

<sup>b</sup> <i>P</i> < 0.001.

<sup>c</sup> <i>P</i> < 0.01.

7). However, complement activity in the presence of antibody reduced the overall viability of all R mutants; and this was due to antibody-independent complement activity or to the enhancement of antibody on complement activity (see Table 9). Antibody alone had no effect.

(v) VEC. The reduced extracellular survival of bacteria in the presence of neutrophils was enhanced by complement activity in the presence of antibody for two strains only, SL 901 (SR) and his 515 (Ra) (Table 8). Other serum factors had little effect on VEC in this series of experiments.

The effects of antibody on complement activity on all indices for all strains are shown in Table 9.

**DISCUSSION**

Serum-mediated killing of the six strains of *S. typhimurium* was observed in the presence of different concentrations of NHS, but the opsonizing effect became more difficult to evaluate as the concentration of NHS increased. This difficulty was due to the large or even complete lysis of strain SL 1102 (Re) in the presence of higher concentrations of NHS. Also, for higher serum concentrations a difference in the PI among the six strains of *S. typhimurium* was hardly discernible (data not shown). On the other hand, in the presence of 1% NHS ingestion was distinguishable among the mutant strains. Therefore, 1% serum was used in this series of experiments.

TABLE 7. Geometric mean effect of serum factors on VIO for six strains of *S. typhimurium*<sup>a</sup>

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serum factors</th>
<th>Antibody alone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Complement activity in presence of antibody</td>
<td>Complement activity in absence of antibody</td>
</tr>
<tr>
<td>SH 2201 (S)</td>
<td>0.69</td>
<td>0.94</td>
</tr>
<tr>
<td>SL 901 (SR)</td>
<td>0.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.90</td>
</tr>
<tr>
<td>his 515 (Ra)</td>
<td>0.27&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.82</td>
</tr>
<tr>
<td>SH 5014 (Rb&lt;sub&gt;2&lt;/sub&gt;)</td>
<td>0.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.79</td>
</tr>
<tr>
<td>SL 1032 (Rd&lt;sub&gt;1&lt;/sub&gt;)</td>
<td>0.13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.79</td>
</tr>
<tr>
<td>SL 1102 (Re)</td>
<td>0.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.96</td>
</tr>
</tbody>
</table>

<sup>a</sup> An effect is the ratio of the geometric mean value in the presence of the factor to the geometric mean value in its absence.

<sup>b</sup> <i>P</i> < 0.001.

<sup>c</sup> <i>P</i> < 0.01.

In the presence of 1% NHS, there was no difference in VIS between strains SL 901 (SR) and his 515 (Ra). This pattern did not change as the serum concentration was increased to 10% (data not shown). This suggests that the presence of O-antigen side chains in the SR strain does not contribute further resistance to serum killing. The outer membrane of the Ra strain consists only of 2-keto-3-deoxyoctonate (KDO) and lipid A, which is so defective and vulnerable that this organism can be killed rapidly and efficiently after exposure to human serum. In the presence of 1 or 10% NHS, the VIS values for SL 1102 strain were 24 and 0.05% (data not shown), respectively.

Although it is sometimes debated whether ingested bacteria (as seen in the cytocentrifuge preparations) are in fact ingested and not overlying the cells, the PI was used in this paper for two main reasons. First, extracellular bacteria, even if attached to the neutrophils, were not counted. Second, in the absence of serum no bacteria overlying the cells could be seen.

It was evident that complement activity in the presence of antibody was the main factor contributing to the bactericidal and opsonizing effects on R mutants. Antibody alone, however, had virtually no effect; this might be due to the small amount of absorbable substances in 1% serum, although complement activity in the presence of antibody was usually greater than complement activity in the absence of antibody. The presence of antibodies (immunoglobulin G) interacting with surface structures of all six strains of *S. typhimurium* was demonstrated by a quantitative immunofluorescence assay. It is unlikely that rough mutants are ever encountered

**TABLE 8. Geometric mean effect of serum factors on VEC for six strains of *S. typhimurium*<sup>a</sup>**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serum factors</th>
<th>Antibody alone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Complement activity in presence of antibody</td>
<td>Complement activity in absence of antibody</td>
</tr>
<tr>
<td>SH 2201 (S)</td>
<td>1.00</td>
<td>0.99</td>
</tr>
<tr>
<td>SL 901 (SR)</td>
<td>0.56&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.99</td>
</tr>
<tr>
<td>his 515 (Ra)</td>
<td>0.52&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.94</td>
</tr>
<tr>
<td>SH 5014 (Rb&lt;sub&gt;2&lt;/sub&gt;)</td>
<td>0.64</td>
<td>0.84</td>
</tr>
<tr>
<td>SL 1032 (Rd&lt;sub&gt;1&lt;/sub&gt;)</td>
<td>1.37</td>
<td>0.86</td>
</tr>
<tr>
<td>SL 1102 (Re)</td>
<td>0.96</td>
<td>1.20</td>
</tr>
</tbody>
</table>

*The actual values are given in Table 2. This table gives the effects of serum factors on those values. Thus, for the SR strain, complement activity in the presence of antibody reduced VEC by a factor of 0.56.

<sup>b</sup> <i>P</i> < 0.01.

**TABLE 9. Effect of antibody on complement activity on five indices for six strains of *S. typhimurium***

<table>
<thead>
<tr>
<th>Strain</th>
<th>Effect&lt;sup&gt;c&lt;/sup&gt; on indices:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VIS</td>
</tr>
<tr>
<td>SH 2201 (S)</td>
<td>1.60</td>
</tr>
<tr>
<td>SL 901 (SR)</td>
<td>0.83&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>his 515 (Ra)</td>
<td>0.87</td>
</tr>
<tr>
<td>SH 5014 (Rb&lt;sub&gt;2&lt;/sub&gt;)</td>
<td>0.89</td>
</tr>
<tr>
<td>SL 1032 (Rd&lt;sub&gt;1&lt;/sub&gt;)</td>
<td>0.85&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>SL 1102 (Re)</td>
<td>1.46&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>c</sup> Effect = Complement activity when antibody present/Complement activity when antibody absent.

<sup>d</sup> <i>P</i> < 0.01.
naturally, so that the antibodies involved may, for example, have been generated against some other organisms but may cross-react with similar epitopes on these salmonellae (1, 11).

Complement activity in the presence of antibody facilitated the ingestion of strains his 515 (Ra) and SL 1102 (Re). In the absence of serum factors, ingestion of Ra and Re strains were still 20 and 40%, respectively (data not shown). This might be due to the more hydrophobic nature of LPS on the bacterial surface (17, 23, 24) or to unidentified carbohydrate receptors on the surface of neutrophils (15).

SL 1032 (Rd), having additional heptoses to the heptoseless LPS of Re strain, was ingested readily by neutrophils, largely by complement activation in the presence or absence of antibody. However, the presence of antibody did not enhance its ingestion. Instead, SH 5014 (Rb), having galactose as the terminal sugar in the structure of LPS, was ingested by neutrophils only through complement activation in the presence of antibody. Similarly for SH 2201 (S), which has multiple repeating units of O-antigen side chains, ingestion was not as efficient as that of R mutants. SL 901 (SR), possessing one unit of O-antigen side chain, was ingested by neutrophils also largely by complement activation in the presence of antibody and to some extent by complement activation in the absence of antibody. These findings suggest that different strains of S. typhimurium are ingested by neutrophils through different mechanisms of complement opsonization or even independently of opsonization (Ra and Re strains). These different mechanisms of complement opsonization are largely dependent on the specific LPS composition rather than on the length of LPS.

Modrzakowski and Spitznagel showed that susceptibility of the R mutants to granule cationic proteins of human neutrophils increased as sugar residues decreased in the LPS (12). However, whether the oxygen-dependent bactericidal mechanisms also kill R mutants more than S strains is still unsettled (14, 18). Once bacteria are ingested by neutrophils, the intracellular killing mechanisms are highly effective (2). In this study, it was shown that intracellular killing of the S strain was less than that of some of the R mutants (Rd, Re strains), but the killing among the S, SR, Ra, and RB strains was not different. This suggests that the presence of O-antigen side chains in the S and SR strains does not contribute further resistance to intracellular killing.

When both neutrophils and serum are present, the overall viability of bacteria is the sum of those surviving extracellularly and those surviving intracellularly (9). It has been observed repeatedly in this laboratory that the VIC is always low; thus the overall survival depends almost entirely on the degree of ingestion and the degree of extracellular killing. It is clear that the susceptibility to overall killing of these six strains increased as LPS became shorter.

The relationship among the five indices has been established, and the effect of neutrophils on extracellular killing is expressed as follows: VEC = [VIO - (PI × VIC)]/[1.0 - PI × VIS].

Previous work has shown that neutrophils enhance the extracellular killing of Salmonella strains; this might be due partly to complement activation by soluble neutrophil products (N. P. Luo and H. Rowland, Trans. R. Soc. Trop. Med. Hyg., in press). In the current study, significant reduction of VEC by complement activation in the presence of antibody was found only for the SR and Ra strains. The reduction of VEC in the other rough mutants may be due directly to some soluble neutrophil products.

The LPS structure of S. typhimurium is the most intensively studied portion of the outer membrane related to the immunological response. This study is therefore concerned with changes in LPS structure only. The results of the present study are in keeping with the view that detailed structure rather than LPS length alone determines the host defense mechanisms against S. typhimurium. The role of the two pathways of complement activation in the opsonization of mutant strains of S. typhimurium is being investigated.

LITERATURE CITED