

## Differences among *Shigella* spp. in Susceptibility to the Bactericidal Activity of Human Serum

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(Received 5 January 1988)

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Clinical isolates of *Shigella* spp. were examined for their susceptibility to human serum. The susceptibility of the strains to immune and nonimmune human serum was dependent upon the size of the bacterial inoculum and the concentration of serum. There were differences among *Shigella* spp. in susceptibility to human serum: *S. sonnei* strains were the least susceptible, strains of *S. boydii* and *S. flexneri* serotype 6 were intermediate, and those of *S. flexneri* other than serotype 6 and *S. dysenteriae* were the most susceptible. Experiments in which heat-treated (56 °C for 30 min, or 50 °C for 20 min) serum was used, and analysis of activation of complement by lipopolysaccharides (LPS) from each *Shigella* sp., suggested that LPS composition, especially the O antigen polysaccharide chains, contributes to the differences among *Shigella* spp. in susceptibility to human serum.

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### INTRODUCTION

Diarrhoeal disease is a major cause of morbidity and mortality worldwide, especially in developing countries. *Shigella* is one of the most important causes of diarrhoeal disease (Blaser *et al.*, 1983; DuPont & Pickering, 1980; Stoll *et al.*, 1982). Shigellosis is caused by invasion of the epithelium and subsequent multiplication in the gut cells by virulent *Shigella* organisms, resulting in a massive inflammatory response in the gut mucosa (Keusch, 1979; LaBrec *et al.*, 1964; Ogawa *et al.*, 1967; Okamura *et al.*, 1983). Although *Shigella* rarely invades the blood stream and causes generalized infection (Keusch, 1979), several studies suggest that *Shigella* bacteraemia may occur more frequently, and may bear a high case fatality rate, in the immunocompromised host (Hallet & Scragg, 1978; Koshi *et al.*, 1979; Struelens *et al.*, 1985). Shigellosis due to *S. sonnei* is becoming more common (Blaser *et al.*, 1983; Parker, 1984) than shigellosis due to other *Shigella* spp., especially in the industrialized countries.

The mechanism of immunity against *Shigella* spp. remains unclear. There have been several reports on the role of host defence factors (polymorphonuclear leucocytes, macrophages, lymphocytes, antibodies and complement) in the pathogenesis of shigellosis (Lowell *et al.*, 1980;

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Abbreviation: GVB<sup>2+</sup>, gelatin/veronal buffer supplemented with Ca<sup>2+</sup> and Mg<sup>2+</sup>

Morgan *et al.*, 1984*a, b*; Reed, 1975, Reed & Albright, 1974). Human serum provides the host with antibodies and complement, both or either of which act as opsonins and facilitate killing by polymorphonuclear leucocytes. Complement also has bactericidal activity through the classical and alternative pathways, with or without the need for specific antibodies (Taylor, 1983). Fresh serum is capable of killing certain strains of *Shigella*. Wardlaw & Pillemer (1956) studied this bactericidal system using *S. dysenteriae* 1 strain and attributed it to the properdin system. Reed & Albright (1974) reported that there was a wide range of susceptibility among strains of *Shigella*, and that there was little difference in killing activity between individual sera and no relationship between antibody titres and killing capacity.

In the work described here, we determined the susceptibility of 174 *Shigella* strains to normal human serum. For five representative strains we studied the effect of serum concentration and size of bacterial inoculum on serum killing, and investigated the involvement of complement and bacterial LPS.

## METHODS

**Bacterial strains.** Strains of *S. dysenteriae* (13 strains, serotypes 1, 2, 3, 4 and 8), *S. flexneri* (94 strains, including all serotypes), *S. boydii* (9 strains, serotypes 1, 3, 4, 5 and 10), and *S. sonnei* (58 strains, including both form I and form II) were isolated from patients with dysentery at various hospitals in metropolitan Tokyo and the Yokohama and Osaka areas. The following were used as representative strains for more detailed studies of serum bactericidal activity; *S. dysenteriae* 2 M8, *S. flexneri* 2a T4, *S. flexneri* 6 M7, *S. boydii* 4 SH71, *S. sonnei* M23. Serotypes were always confirmed by agglutination test using specific antisera (Denka-Seiken Co.) prior to each experiment.

**Sera.** Pooled normal human serum was obtained from several laboratory volunteers who denied previous *Shigella* infection. To minimize potential differences in the bactericidal activity of normal human serum among donors, most of the experiments were done with a single pool of serum containing about 36–37 CH<sub>50</sub> units ml<sup>-1</sup> of haemolytic complement components. Normal individual sera and sera from patients with *Shigella* dysentery were also used and compared with each other to see if there were any differences in bactericidal activity among donors. Human serum was stored at -80 °C in 1 ml samples and was discarded after being thawed once.

**Treatment of sera.** Sera were treated with heat or chelator to examine the role of serum complement. Classical pathway activity of complement in pooled normal human serum was selectively inhibited by chelation with 0.2 M-EGTA (Sigma), pH 7.5, at a final concentration of 10 or 20 mM, with 2 or 5 mM-MgCl<sub>2</sub> (Fine *et al.*, 1972). Alternative pathway activity of complement was selectively inactivated by heat treatment (50 °C, 20 min). Both classical and alternative pathway activities were inhibited by heat at 56 °C for 30 min.

**Preparation of lipopolysaccharides (LPS).** LPS were isolated from acetone-dried cells by the phenol/water method of Westphal *et al.* (1952) and purified by repeated ultracentrifugation (105 000 *g*) and treatment with RNAase (20 µg ml<sup>-1</sup> in 25 mM-Tris/HCl buffer, pH 7.4) (Okamura *et al.*, 1983).

**Haemolytic complement assay.** The total haemolytic complement activity, expressed as the amount of complement required to lyse 50% of sensitized sheep erythrocytes (CH<sub>50</sub>), was measured according to the method of Mayer (1961) with a slight modification.

**Serum bactericidal assay.** A qualitative assay was first done to examine the susceptibility of all the *Shigella* strains to the bactericidal activity of pooled normal human serum. Exponential-phase cultures were inoculated into tryptone/glucose broth [0.5% tryptone (Difco), 0.5% glucose, 0.5% NaCl, pH 7.4] containing 10% (v/v) normal human serum as described by Moll *et al.* (1979), to give 10<sup>6</sup>–10<sup>7</sup> cells ml<sup>-1</sup>. Bromothymol blue was added to the cultures as a pH indicator. The cultures were then incubated at 37 °C for 6–7 h and overnight. If the bacteria are killed, the medium remains green, whereas if they survive and grow, the medium turns yellow.

Quantitative serum bactericidal assays were done as follows, using microtitre plates. Ten-microlitre portions of exponential-phase cultures of *Shigella* were inoculated into 0.2 ml of serum dilutions in tryptone/glucose broth, Hanks' solution, or gelatin/veronal buffer supplemented with Ca<sup>2+</sup> and Mg<sup>2+</sup> (GVB<sup>2+</sup>), and incubated at 37 °C. Viable counts of bacteria were done at appropriate intervals by the duplicate plate method using Trypticase soy agar (BBL).

**Measurement of anticomplementary activity of *Shigella* LPS.** The anticomplementary activity of LPS was measured as described by Shafer *et al.* (1984), with slight modifications. Serum was mixed with LPS (0.063–1 mg) suspended in GVB<sup>2+</sup> in a final volume of 0.2 ml and incubated with shaking at 37 °C for 2 h. Antibody-sensitized sheep erythrocytes in 0.2 ml GVB<sup>2+</sup> were added to a fourfold dilution of the treated serum and incubated for an additional 30 min in a 37 °C water bath. Ice-cold 0.85% saline (3 ml) was added to the mixture, the cells were pelleted by centrifugation (800 *g*, 5 min), and the absorbance of the supernatant was measured at 412 nm. The positive control was sensitized erythrocytes plus serum without added LPS, and the negative control was LPS plus erythrocytes without added serum.

*Inhibition of serum bactericidal activity by Shigella LPS.* *Shigella* LPS was suspended in distilled water to a final concentration of 2 mg ml<sup>-1</sup> and sonicated until the suspension cleared. LPS solution in the concentration range 0.05–1 mg ml<sup>-1</sup> was added to 0.2 ml serum in a well of a microtitre plate. The solution was incubated at 37 °C for 1 h, then 10 µl bacterial suspension in tryptone/glucose broth was added to the well (about  $2 \times 10^6$  organisms ml<sup>-1</sup>) and incubated at 37 °C for an additional 2 h before dilution plating.

*Reproducibility of results.* All the experiments reported were done at least twice. The results shown are from representative experiments.

## RESULTS

### *Serum bactericidal activity*

We first examined the 174 strains of *Shigella* for their susceptibility to 10% pooled normal human serum by the qualitative test. Almost all of the *S. sonnei* form I and six of the nine *S. boydii* strains grew in this reaction mixture. Six of the 11 *S. flexneri* 6 strains were not susceptible. *S. flexneri* strains other than serotype 6, and all the strains of *S. dysenteriae*, were susceptible. Strains of *S. sonnei* which were susceptible to normal human serum were all form II strains.

We then chose a representative strain from each species and from *S. flexneri* 6. These strains were examined for susceptibility to pooled normal human serum by doing viable counts at appropriate times. A serum concentration of 10% in tryptone/glucose broth and a bacterial inoculum of  $10^6$  organisms ml<sup>-1</sup> were first chosen. Fig. 1 shows the time course of the serum bactericidal assay. The *S. flexneri* 2a and *S. dysenteriae* 2 strains were killed rapidly, with a more than 1000-fold reduction in viable counts within 2 h. No decrease in viable counts was observed with the *S. sonnei* form I strain. The *S. flexneri* 6 and *S. boydii* 4 strains showed a slight decrease in viable counts at 2 h after inoculation, but kept growing thereafter (Fig. 1). These results were in agreement with those of the qualitative tests mentioned above. There were no differences in growth rate among the representative strains of *Shigella* (results not shown). We also examined the serum bactericidal activity with GBV<sup>2+</sup> or Hanks' solution as serum diluent, and obtained similar results to those with tryptone/glucose broth. However, better reproducibility and higher killing potency were achieved with tryptone/glucose broth as serum diluent, and this diluent was therefore used for most of the experiments unless stated otherwise. Exponential-phase bacteria were more susceptible to serum killing than were stationary-phase bacteria (data not shown).

The bactericidal capacity of pooled normal human serum at different concentrations was examined (Table 1). *S. flexneri* 6 and *S. boydii* strains were killed at a serum concentration of 20%, whereas none of the *S. sonnei* strains examined were susceptible at this concentration. As the concentration of serum was increased to 80%, the number of susceptible *S. sonnei* strains increased, but some strains remained resistant.

We then examined the killing effect of 10% human serum with various concentrations of bacterial inoculum. As the inoculum of bacteria increased, even the *S. dysenteriae* 2 and *S. flexneri* 2a strains became less susceptible to the serum killing (Fig. 2). On the other hand, even the *S. sonnei* M23 strain was killed efficiently when the inoculum was  $10^4$  organisms ml<sup>-1</sup> (not shown). Moreover, all of the 32 wild-type strains of *S. sonnei* form I tested were susceptible to 10% human serum with such an inoculum.

### *Bactericidal activity of individual sera and sera from dysentery patients*

The killing efficacy of individual human sera was examined to see if there were differences in serum bactericidal activity among donors. Four serum specimens from normal human volunteers and 24 serum specimens from dysentery patients (causative organisms: *S. sonnei*, 15 patients; *S. flexneri*, 7 patients; *S. boydii*, 2 patients) were examined. The total haemolytic complement activity of these sera ranged from 27 to 48 CH<sub>50</sub> units ml<sup>-1</sup>. The representative *Shigella* strains mentioned above were inoculated into 10% human serum in tryptone/glucose broth and viable bacteria were counted after 2 h incubation at 37 °C. Despite slight fluctuation with each serum, the representative *Shigella* strains showed the same ranking of susceptibility as had been found with the pooled sera (Fig. 3). Although some of the sera killed even *S. sonnei* M23 efficiently at a concentration of 10%, at lower concentrations they showed similar bactericidal activity to that of the pooled normal human serum.

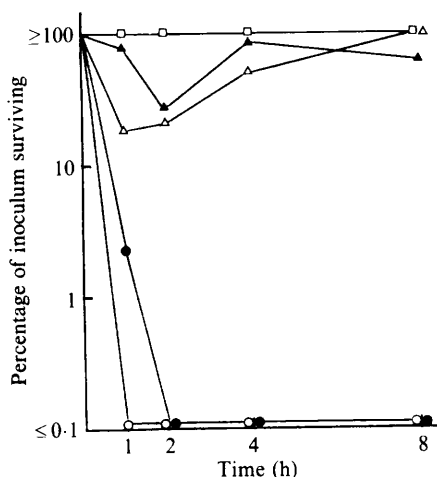


Fig. 1

Fig. 1. Kinetics of killing of *Shigella* spp. (initial cell density about  $10^6$  bacteria  $\text{ml}^{-1}$ ) by 10% pooled normal human serum in tryptone/glucose broth. ●, *S. dysenteriae* 2 M8; ○, *S. flexneri* 2a T4; ▲, *S. flexneri* 6 M7; △, *S. boydii* 4 SH71; □, *S. sonnei* M23.

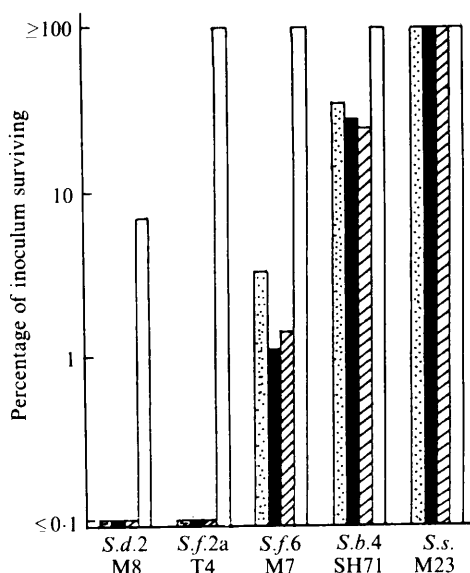


Fig. 2

Fig. 2. Kinetics of killing of *S. dysenteriae* 2 M8, *S. flexneri* 2a T4, *S. flexneri* 6 M7, *S. boydii* 4 SH71 and *S. sonnei* M23 by 10% human serum with various bacterial inocula (□,  $10^5$ ; ■,  $10^6$ ; ▨,  $10^7$ ; ▤,  $10^8$  organisms  $\text{ml}^{-1}$ ).

Table 1. Susceptibility of *Shigella* strains to various concentrations of normal human serum

The results are shown as number of 'resistant' strains/number of strains tested. Viability of more than  $10^{-1}$  of the inoculum at 4 h post-inoculation was considered resistant.

| Species*                         | Serum concn (%) |       |       |      |      |
|----------------------------------|-----------------|-------|-------|------|------|
|                                  | 5               | 10    | 20    | 40   | 80   |
| <i>S. dysenteriae</i>            | 1/7             | 0/11  | ND    | ND   | ND   |
| <i>S. flexneri</i> serotypes 1-5 | 1/16            | 0/42  | ND    | ND   | ND   |
| <i>S. flexneri</i> serotype 6    | 10/10           | 5/12  | 0/12  | ND   | ND   |
| <i>S. boydii</i>                 | 6/7             | 6/7   | 0/7   | ND   | ND   |
| <i>S. sonnei</i> (form I)        | ND              | 15/15 | 24/24 | 7/24 | 5/24 |

ND, Not done.

\* About  $5 \times 10^6$  organisms  $\text{ml}^{-1}$  in the reaction mixture.

#### Role of complement in bacterial killing

Heat inactivation at  $56^\circ\text{C}$  for 30 min completely destroyed the ability of human serum to kill *Shigella*. Serum heated at  $50^\circ\text{C}$  for 20 min, which depletes it of alternative complement pathway activity, was as potent as untreated control serum in killing *Shigella* strains (Fig. 4). On the other hand, serum treated with MgEGTA, which selectively inhibits classical complement pathway activity, showed considerably less potency than untreated control serum (Fig. 5). In both cases, however, the test strains showed the same ranking with regard to serum susceptibility as before (Figs 4 and 5).

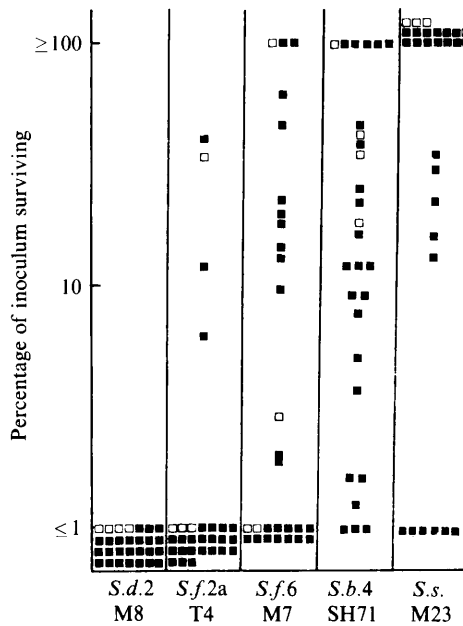


Fig. 3. Killing of *S. dysenteriae* 2 M8, *S. flexneri* 2a T4, *S. flexneri* 6 M7, *S. boydii* 4 SH71 and *S. sonnei* M23 by normal human sera (□) and sera from dysentery patients (■). The initial cell density was about  $10^6$  bacteria  $\text{ml}^{-1}$  in 10% serum.

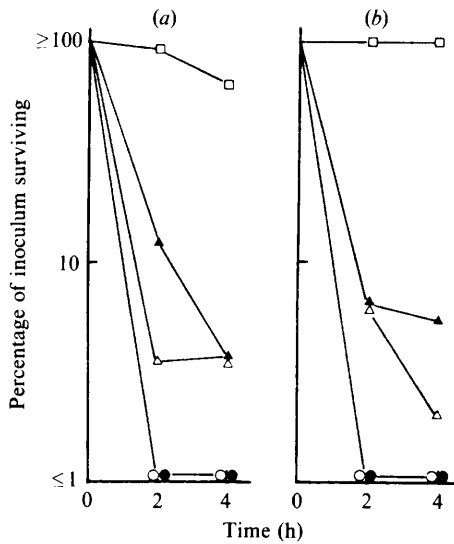


Fig. 4

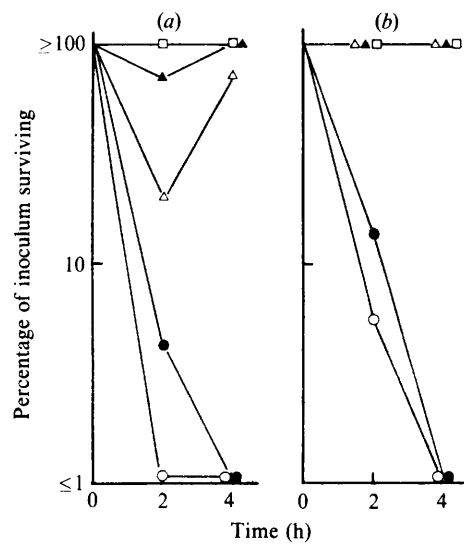


Fig. 5

Fig. 4. Role of the classical complement pathway in killing of *Shigella*. (a) 10% human serum in tryptone/glucose broth; (b) 10% heat-treated (50 °C, 20 min) human serum. ●, *S. dysenteriae* 2 M8; ○, *S. flexneri* 2a T4; ▲, *S. flexneri* 6 M7; △, *S. boydii* 4 SH71; □, *S. sonnei* M23. The initial cell density was about  $10^6$  bacteria  $\text{ml}^{-1}$ .

Fig. 5. Role of the alternative complement pathway in killing of *Shigella*. (a) 10% human serum in GVB<sup>2+</sup>; (b) 10% human serum in GBV with MgEGTA. ●, *S. dysenteriae* 2 M8; ○, *S. flexneri* 2a T4; ▲, *S. flexneri* 6 M7; △, *S. boydii* 4 SH71; □, *S. sonnei* M23. The initial cell density was about  $10^6$  bacteria  $\text{ml}^{-1}$ .

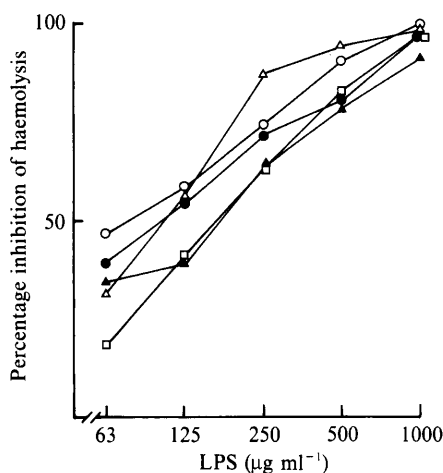


Fig. 6. Anticomplementary effect of LPS isolated from *S. dysenteriae* 2 M8 (●), *S. flexneri* 2a T4 (○), *S. flexneri* 6 M7 (▲), *S. boydii* 4 SH71 (△) and *S. sonnei* M23 (□).

Table 2. Inhibition of serum bactericidal activity against *Shigella* strains by homologous and heterologous LPS

The concentration of serum was 10% with strains M8 and T4, 20% with strain M7, and 30% with strain M23. The concentration of LPS was 200 µg ml<sup>-1</sup> except where indicated.

| LPS from                      | Percentage survival of strains: |      |     |       |
|-------------------------------|---------------------------------|------|-----|-------|
|                               | M8                              | T4   | M7  | M23   |
| <i>S. dysenteriae</i> 2 M8    | 210                             | 0.2  | 2.3 | 27.5* |
| <i>S. flexneri</i> 2a T4      | 0.2                             | 425  | 1.1 | <0.1* |
| <i>S. flexneri</i> 6 M7       | 0.6                             | 1.3  | 292 | 15.9* |
| <i>S. boydii</i> 4 SH71       | 0.5                             | 2.9  | 2.8 | 0.7*  |
| <i>S. sonnei</i> (form I) M23 | 0.6                             | 0.7  | 1.2 | 725*  |
| No LPS                        | 0.3                             | <0.1 | 0.5 | 1.2   |

\* LPS concentration 100 µg ml<sup>-1</sup>.

#### Anticomplementary activity of *Shigella* LPS

The anticomplementary activity of LPS of the representative *Shigella* strains was measured to determine whether their different susceptibility to human serum was due to differences in the depletion of complement-mediated haemolytic activity by LPS. The anticomplementary properties of *Shigella* LPS in sheep-erythrocyte haemolytic assays (Shafer *et al.*, 1984) was dose dependent, but there was no difference in depletion of complement-mediated haemolytic activity among the different *Shigella* LPS preparations (Fig. 6).

#### Inhibition of serum bactericidal activity by *Shigella* LPS

Table 2 shows the percentage survival of *Shigella* strains in pooled normal human serum treated with 100 or 200 µg LPS ml<sup>-1</sup> from the representative *Shigella* strains. Under conditions where untreated serum killed most of the *Shigella* organisms, the strains survived well in serum preincubated with LPS obtained from the homologous strains, suggesting the involvement of specific immunoglobulins in serum killing of *Shigella*.

## DISCUSSION

Kinetic studies with normal human serum showed that the lower the inoculum of bacteria and/or the higher the concentration of serum, the more susceptible were the *Shigella* strains to killing (Figs 1 and 2, Table 1). The *Shigella* spp. differed in susceptibility to human serum: *S. dysenteriae* and *S. flexneri* serotypes 1–5 were the most susceptible, *S. flexneri* serotype 6 and *S. boydii* were intermediate, and *S. sonnei* was the least susceptible. Electron microscopy showed that the *Shigella* strains were not only killed but also lysed by human serum (data not shown).

Susceptibility of a number of Gram-negative bacteria to the bactericidal activity of immune or nonimmune sera has been attributed to their LPS composition (Taylor, 1983; Shafer *et al.*, 1984; Schiller *et al.*, 1984). Our results also suggested that the variation in susceptibility could be due to O antigens of *Shigella* LPS. It is not surprising that *S. flexneri* 6 strains differed from other *S. flexneri* serotypes in susceptibility to human serum, since the LPS structures of the two groups are quite different (Lindberg, 1979). It is unlikely that other cell-surface components such as capsular antigens, pili or plasmid-encoded outer-membrane proteins are responsible for the differences in serum susceptibility, because the differences in susceptibility we observed correlated with serological differences in O antigens of LPS. We found no relationship between serum susceptibility and the presence of antibiotic-resistance plasmids or large plasmids responsible for invasiveness (unpublished observations).

The bactericidal effects of immune or nonimmune serum are mediated by activated components of the classical or alternative complement pathway (Taylor, 1983; Götze & Müller-Eberhard, 1971). Activation of either pathway can lead to membrane damage resulting in cell death (Taylor, 1983). Our study of the mechanism of complement activation by *Shigella* strains indicated that both classical and alternative pathways were involved in serum killing of *Shigella* organisms, but that the classical pathway was more important (Figs 4 and 5). In either case, however, the species showed the same ranking with respect to serum susceptibility. Other Gram-negative bacteria such as *Haemophilus influenzae* (Quinn *et al.*, 1977), *Salmonella* spp. (Joiner *et al.*, 1982), *Campylobacter* spp. (Blaser *et al.*, 1985), and *Escherichia coli* (Taylor & Parton, 1977) are known to activate both complement pathways, but *Neisseria gonorrhoeae* (Shafer *et al.*, 1984), *Pseudomonas aeruginosa* (Schiller *et al.*, 1984) and *Haemophilus ducreyi* (Odumeru *et al.*, 1985) activate mainly the classical pathway. •

We then asked whether the differences in susceptibility to normal human serum among *Shigella* spp. result from differential activation of human complement by different LPS, or whether differences in the LPS structure itself are responsible, by determining susceptibility to the membrane attack complex of human complement. The fact that we found no difference in depletion of complement-mediated haemolytic activity among different *Shigella* LPS preparations (Fig. 6) supports the latter possibility.

Although there were great variations among normal individual sera and sera from dysentery patients in antibody titre against *Shigella* LPS as assessed by enzyme-linked immunosorbent assay (unpublished observation), these sera showed similar bactericidal activity against *Shigella* spp. to that of the pooled normal human serum. On the other hand, treatment of human serum with *Shigella* LPS inhibited its bactericidal activity against homologous strains under conditions where untreated serum killed most of the *Shigella* strains. The results might suggest that antibodies in only minute amounts were sufficient for killing *Shigella* strains through the classical complement pathway, as also reported by Reed & Albright (1974). Natural antibodies directed against the surface antigens of a number of Gram-negative bacteria have also been demonstrated in nonimmune serum (Schoolnik *et al.*, 1976; Schwab & Reeves, 1966; Skarnes, 1978).

Reed & Albright (1974) reported that most *S. flexneri* 1 and *S. sonnei* strains are sensitive to serum, most *S. flexneri* 2 strains are intermediate, and all *S. flexneri* 3 strains tested are resistant, but there is also wide variation within some of the serogroups. They also reported that both classical and alternative complement pathways are responsible for the killing (Reed & Albright, 1974). Our findings on susceptibility of *Shigella* to human serum differ from theirs, possibly due to the different bactericidal assay systems: they used stationary-phase bacteria and gel Hanks'

solution, while we used exponential-phase bacteria, and, in most of the experiments, tryptone/glucose broth.

The possible role of serum killing of *Shigella* in curing or preventing shigellosis is still unclear. Shigellosis is only a minimally invasive disease, and these serum complement components may have little opportunity to come in contact with the disease-producing organisms, since complement is scarce in intestinal epithelial tissues. However, during the most extensive stage of the disease process acute massive inflammation takes place, resulting in ulceration of epithelial tissues and passage of blood and exudates in the stools, so serum complement components could well interact with invading organisms. Shigellosis is a localized enteric infection and, for unknown reasons, *Shigella* bacteraemia is very rare (Keusch, 1979). Nonetheless, episodes of *Shigella* bacteraemia have been reported in the literature (Hallet & Scragg, 1978; Koshi *et al.*, 1979; Struelens *et al.*, 1985; Martin *et al.*, 1983). There is controversy over which *Shigella* spp. are most liable to cause bacteraemia. Struelens *et al.* (1985) demonstrated a significantly higher frequency of *Shigella* bacteraemia with *S. dysenteriae* 1 as opposed to other serotypes in Bangladesh, whereas in other reports (Hallet & Scragg, 1978; Koshi *et al.*, 1979; Martin *et al.*, 1983) there is no evidence suggesting any preponderance of one species over the others as a causative agent of *Shigella* bacteraemia. Still epidemiologically unexplained also is the change in species prevalence in which *S. sonnei* has emerged as the most common isolate, especially in the industrialized countries (Blaser *et al.*, 1983; Parker, 1984). Our results may contribute to explaining such unsolved questions. They may also serve as a basis for the development of *Shigella* vaccines.

We thank T. Chida for his excellent technical assistance, and Y. Kudo and H. Watanabe for providing *S. boydii* and *S. dysenteriae* 1 strains.

This work was supported by a Grant-in-aid for Scientific Research (no. 61570223) from the Ministry of Education, Science, and Culture, and in part by the US-Japan Medical Science Program Cholera Panel.

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