INFECTION AND IMMUNITY, Apr. 1981, p. 153-159
0019-9567/81/040153-07$02.00/0

Vol. 32, No. 1

Shigella sonnei Phase I and Phase II: Susceptibility to Direct Serum Lysis and Opsonic Requirements Necessary for Stimulation of Leukocyte Redox Metabolism and Killing

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The synthesis of the lipopolysaccharide O-specific repeat polymer by *Shigella sonnei* phase I is a clearly defined bacterial virulence factor necessary for penetrating epithelial cells; *S. sonnei* phase II does not synthesize this antigen and is uniformly avirulent. The serum opsonic requirements, relative to differences in gross lipopolysaccharide structure, were investigated by quantification and comparison of polymorphonuclear leukocyte (PMNL) metabolism and PMNL-mediated microbicidal action to phase I and phase II organisms, using normal and immune serum. The stimulation of PMNL O_2-redox metabolism, as required for oxidative killing, was quantified by a chemiluminescent technique, using luminol as a chemilumigenic substrate. Susceptibility to direct serum or serum PMNL-mediated killing was evaluated by serum and serum-phagocytic killing assays. Stimulation of PMNL metabolism and phagocytic killing of *S. sonnei* phase I required opsonification by specific phase I antibody plus the classical pathway of complement. *S. sonnei* phase II was susceptible to direct complement-mediated serum killing. Likewise, opsonification of the phase II microbe, as measured by PMNL-associated chemiluminescence, was effected by complement in the absence of immune antibody. These data demonstrate the importance of the O-specific repeat polymer in protecting the microbe from the microbicidal action of PMNL and the bacteriolytic action of serum.

Shigellosis, or bacillary dysentery, is an acute gastrointestinal disease caused by bacteria of the genus *Shigella* (7). The ability of shigella to penetrate epithelial cells, considered to be the essential step in the pathogenesis of this disease (13), is under polygenic control, and is affected by a number of bacterial surface attributes, among which are the colonial morphology of the organism (9, 13, 15), the chemical composition of the surface lipopolysaccharide (LPS) O-repeat polymer (8), and expression of smooth LPS as exemplified by *Shigella sonnei* (7). Although such attributes are associated with the virulence of *Shigella* spp., the role of immune defense mechanisms in protection against shigellosis is less well understood.

S. sonnei is a model bacterium for the study of the gross LPS character and its role in providing resistance to host humoral defense mechanisms. Phase I and phase II colonies on agar medium are easily recognized when viewed under low-power stereomicroscopy; *S. sonnei* phase I colonies appear round and raised with a regular margin, whereas phase II colonies appear flat and rough with an irregular margin. Virulent *S. sonnei* phase I bacteria possess a complete (smooth) LPS structure with 2-amino-2-deoxy-L-altruronic acid as a constituent of the O-repeat polymer (11). Spontaneously occurring phase II bacteria do not synthesize the O-repeat polymer (18) and are uniformly avirulent.

The purpose of the present study was to determine differences in opsonic requirements necessary for phagocytosis and killing of virulent phase I and avirulent phase II bacteria by polymorphonuclear leukocytes (PMNL). The results presented herein indicate that specific phase I antibody and complement are necessary for phagocytic killing of *S. sonnei* phase I, whereas complement alone is required for killing of *S. sonnei* phase II.

MATERIALS AND METHODS

Bacteria. *S. sonnei* 53G was kindly provided by Peter Gemski, Department of Bacterial Diseases, Walter Reed Army Institute of Research, Washington, D.C. This strain, originally isolated in Japan (12), displays the typical biochemical and serological traits characteristic of *S. sonnei* and is virulent as shown by its ability to evoke keratoconjunctivitis in guinea pigs (positive Sereny test) (19). Separate phase I and phase II stock cultures were preserved in the lyophilized state and maintained on brain heart infusion agar (Difco) at 4°C. Isolated colonies were chosen for study with the assistance of low-power stereomicroscopy.

Serum. White New Zealand rabbits weighing 2 to 4 kg were bled by cardiac puncture before bacterial injections (normal rabbit serum) and after the completion of the injection protocol (immune rabbit serum). *S. sonnei* phase I and phase II cells used in the injections were grown separately overnight without aeration in brain heart infusion broth at 37°C. Overnight broth cultures were then centrifuged at 1,100 × *g* for 10 min, washed twice in equal volumes of cold saline (0.9%), and diluted to obtain a concentration of 1.0×10^6 to 5.0×10^6 viable bacteria per ml. This concentration of bacteria was used in the following daily injection protocol: day 1, 0.1 ml given subcutaneously; day 2, 0.2 ml given subcutaneously; day 3, 0.1 ml given intravenously; day 4, 0.2 ml given intravenously; day 5, 0.5 ml given intravenously; days 6 and 7, no injection; days 8 to 12, 0.5 ml given intravenously. On day 18, rabbits were bled by cardiac puncture. Blood was allowed to clot, and the serum was separated from the clot by centrifugation, pooled, sterilized by passage through a 0.45-μm (pore size) membrane filter (Millipore), and maintained in glass vaccine bottles at −20°C. Both normal and immune rabbit sera were heat inactivated at 56°C for 30 min.

Phase I- and phase II-specific antibodies were removed from anti-phase I immune serum by absorbing the serum twice with live phase I or phase II bacteria. The following protocol was used. Overnight broth suspensions of *S. sonnei* phase I and phase II were swabbed to the surface of brain heart infusion agar plates (40 plates per group) and incubated overnight at 37°C. The heavy, confluent bacterial growth of each group was then harvested in saline and packed by centrifugation with cells harvested from 20 plates in each new group. Then 2 ml of anti-phase I immune serum was added to one of the two tubes of packed phase I cells and one of the two tubes of packed phase II cells. The cells were suspended in serum and allowed to incubate at room temperature for 1 h. The suspensions were then centrifuged. The serum was decanted into the second tube of packed cells, suspended, and allowed to incubate again at room temperature for 1 h. These suspensions were then centrifuged, and the final twice-absorbed sera were sterilized by passage through a 0.45-μm membrane filter.

Preparation of human PMNL and CL. Human whole blood was obtained from volunteers by venipuncture. The leukocyte-rich plasma was isolated after dextran sedimentation of erythrocytes (20). After hypotonic lysis of the remaining erythrocytes (0.2% saline for 15 s) and two additional washes with Dulbecco phosphate-buffered saline (6) without Ca^{2+} or Mg^{2+}, pH 7.2, total and differential counts were done on the leukocyte suspension, and the volume was adjusted to yield a concentration of 1,000 PMNL/μl. The desired quantity of PMNL was added to each sterile, siliconized glass vial (24-ml capacity) containing 1.75 ml of barbital (Veronal)-buffered saline with Ca^{2+} Mg^{2+} (16), albumin (0.1% [wt/vol]), and glucose (0.1% [wt/vol]), pH 7.2.

The vials received 20 μl of diluted heat-treated normal or immune rabbit serum (as a source of antibody) or 10 μl of guinea pig serum (Microbiological Associates) (2.5 CH_{50} U of guinea pig complement) or both where indicated. One nanomole of luminol (3, 4, 21) was added to each vial just before measurement of background chemiluminescence (CL).

CL (2, 5) was quantified at room temperature (22°C), using the single-photon counting capability of a Beckman LS-150 scintillation counter equipped with EMI 9829A photomultiplier tubes (bialkali spectral response). The counter was operated in the out-of-coincidence mode with the tritium channel settings.

The CL intensity from each vial sample was measured for a 0.2-min period. The raw intensity values in counts per minute were converted to photons per minute by multiplying the counts per minute by a photon conversion factor, 14. This factor was established by calibrating the counter with an established blue photon emitter standard prepared by H. H. Seliger (14). The CL intensity from each preparation of unstimulated PMNL was measured for 21 min to establish the background CL activity. Stimulation was initiated at time zero by addition of either *S. sonnei* phase I or *S. sonnei* phase II as indicated in the results. The integral CL response was calculated from the CL intensity data by trapezoidal approximation.

Serum bactericidal and phagocytic killing assays. *S. sonnei* phase I and phase II susceptibilities to the bactericidal action of serum or the microbicidal action of PMNL were determined by a viable bacterial colony count method (10). The preparations in Falcon tissue culture tubes consisted of 0.4 ml of complete Veronal buffer, 5.0×10^5 PMNL suspended in 0.5 ml of phosphate-buffered saline, 0.1 ml of heat-treated normal or immune rabbit serum, and 20 μl of guinea pig serum (complement) where indicated. For controls, phosphate-buffered saline was substituted for PMNL or serum. To each tube were added 10^5 viable (late-log) *S. sonnei* phase I or phase II bacteria. After 0.1 ml was removed from each tube for zero time plate counts, all tubes were placed in a Dubnoff metabolic shaking incubator and incubated in water at 37°C with shaking. Samples (0.1 ml) were withdrawn at 30, 60, 90, and 120 min, diluted in appropriate volumes of cold normal saline, mixed on a Vortex mixer, and plated in triplicate on brain heart infusion agar. After overnight incubation at 37°C, bacterial colonies were counted either visually by using a hand held counter or electronically by using an automated colony counter (Biotran II; New Brunswick Scientific Co.). Triplicate counts from each specimen were averaged and normalized with respect to zero time, and the normalized values were then plotted against the interval of incubation in minutes.

RESULTS

Measurement of PMNL CL from leukocytes challenged with *S. sonnei* phase I. To determine the serum requirements necessary for opsonic recognition of *S. sonnei* phase I, the PMNL suspensions were challenged with *S. sonnei* phase I, and the rate and extent of activation of PMNL O_2-redox metabolism were quantified as the CL resulting from oxygenation of the chemilumigenic probe luminol (1, 3). The following serum sources were tested for opsonic capacity: (i) heat-treated normal rabbit serum, (ii)

heat-treated normal rabbit serum plus guinea pig serum (as a source of fresh complement), (iii) guinea pig serum (complement) alone, (iv) heat-treated anti-phase I immune rabbit serum, and (v) heat-treated anti-phase I immune rabbit serum plus complement.

No CL was detected from PMNL challenged with *S. sonnei* phase I in the absence of serum (Fig. 1A). Likewise, the addition of heat-treated normal rabbit serum to PMNL yielded no CL. A very low level of PMNL CL was detected when heat-treated anti-phase I immune rabbit serum (Fig. 1A), complement alone, or heat-treated normal rabbit serum plus complement was employed as an opsonic source (Fig. 1B). Opsonic activity was greatly increased when both heat-treated anti-phase I immune rabbit serum and complement were employed (compare Fig. 1B with A). These results indicate that specific immune serum was necessary for opsonification of *S. sonnei* phase I and that opsonification was greatly amplified in the presence of complement.

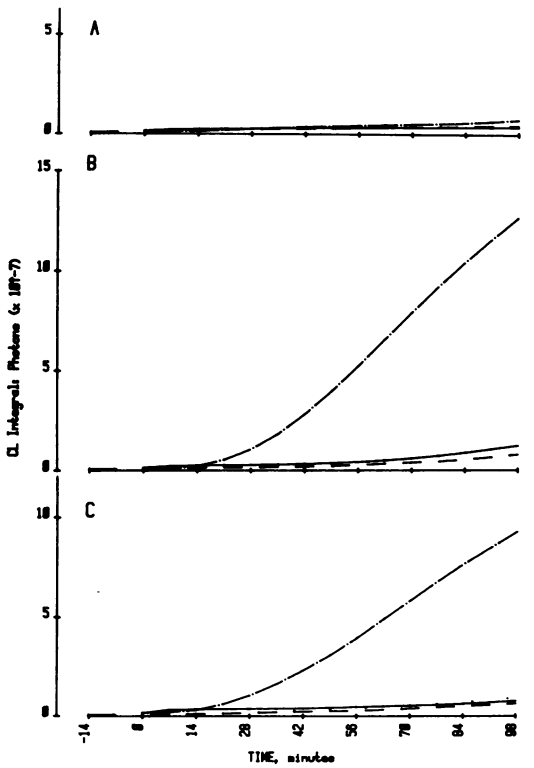

FIG. 1. *Integral CL response against time from PMNL challenged with S. sonnei phase I. Each vial contained 2.5 × 10⁴ PMNL, 2.5 × 10⁶ S. sonnei phase I, and 1 nmol of luminol in a final volume of 2.0 ml of complete Veronal buffer with the following additions. A. None (——); normal rabbit serum, 4 μl (·····); anti-phase I immune rabbit serum, 2 μl (-·-·). B. Complement, 10 μl (——); complement plus normal rabbit serum, 2 μl (·····); complement plus anti-phase I immune rabbit serum, 2 μl (-·-·). C. Complement plus anti-phase II immune rabbit serum, 2 μl (——); complement plus anti-phase I immune rabbit serum (absorbed with phase I bacteria), 2 μl (·····); complement plus anti-phase I immune rabbit serum (absorbed with phase II bacteria), 2 μl (-·-·); anti-phase I immune rabbit serum (absorbed with phase II bacteria), 2 μl (···).*

The relative requirements of *S. sonnei* phase I-specific antibody and complement for opsonification of *S. sonnei* phase I were further evaluated by determination of PMNL CL, employing either heat-treated anti-phase II immune rabbit serum or heat-treated anti-phase I immune rabbit serum previously absorbed with either *S. sonnei* phase I or phase II bacteria. As indicated in Fig. 1C, there was a very low level of CL from PMNL challenged with *S. sonnei* phase I in the presence of complement plus anti-phase II immune rabbit serum, complement plus anti-phase I immune rabbit serum absorbed with phase I bacteria, or anti-phase I immune rabbit serum absorbed with phase II bacteria (in the absence of complement). However, a large PMNL CL response was seen when complement and phase II-absorbed anti-phase I immune rabbit sera were employed. These results, using absorbed sera, support initial findings that *S. sonnei* phase I-specific antibody plus complement is required for optimum opsonification of *S. sonnei* phase I.

Susceptibility of *S. sonnei* phase I to killing by serum or PMNL. PMNL CL is a product of oxygenation reactions. Such oxygenations can affect microbicidal action. However, the opsonin-dependent stimulation of PMNL CL measures potential microbicidal action only; CL does not measure the loss of microbe viability. Therefore, a phagocytic killing assay was used to evaluate *S. sonnei* phase I viability after exposure to PMNL in the presence or absence of serum. Phagocytic killing of *S. sonnei* phase I was investigated by using heat-treated normal or immune rabbit serum with or without guinea pig complement. In control tubes (no PMNL), no serum killing of *S. sonnei* phase I was detected with complement alone or with complement plus heat-treated normal or immune rabbit serum (Fig. 2A and B). Similarly, in tubes containing PMNL, there was no detectable killing of *S. sonnei* phase I with complement, heat-treated normal rabbit serum (with or without added complement), or immune rabbit serum alone. However, killing of *S. sonnei* phase I by PMNL was effected with heat-treated immune rabbit serum supplemented with fresh guinea pig complement (compare the test and control data in Fig. 2B with those in A). Disrupting the PMNL

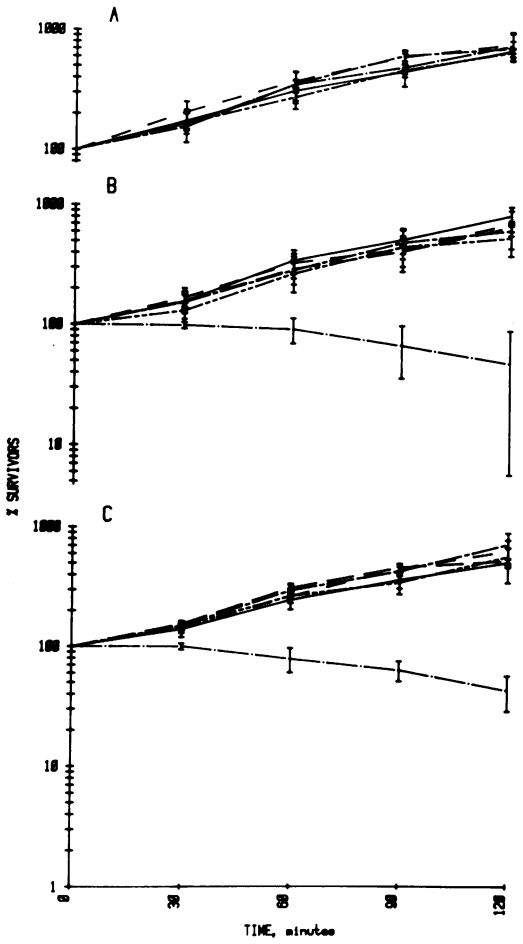

FIG. 2. *Susceptibility of S. sonnei phase I to killing by PMNL. Tubes contained 5.0 × 10⁵ PMNL and 1.0 × 10⁵ S. sonnei phase I in a final volume of 1.0 ml of complete Veronal buffer. Control tubes which lacked PMNL were substituted with an equal volume of phosphate-buffered saline. Points represent the mean values of three experiments performed on different days, and each vertical bar represents one standard deviation. Additions were made as follows. A. None (——); normal rabbit serum, 100 μl (·····); control (— — —); anti-phase I immune rabbit serum, 100 μl (-·-·-); control (—·—). B. Complement, 20 μl (——); control (—— ——); complement plus normal rabbit serum, 100 μl (— —); control (— — —); complement plus anti-phase I immune rabbit serum, 100 μl (—·—·-); control (—·—). C. Anti-phase II immune rabbit serum, 100 μl (——); control (—— ——); complement plus anti-phase I immune serum (absorbed with phase I bacteria), 100 μl (— —); control (— — —); complement plus anti-phase I immune rabbit serum (absorbed with phase II bacteria), 100 μl (—·—·-); control (—·—).*

by diluting samples in distilled water, rather than saline, did not alter these results.

The data indicate that *S. sonnei* phase I was killed by PMNL only with anti-phase I immune rabbit serum plus complement, that *S. sonnei* phase I was resistant to bactericidal action of complement, and that the results of PMNL CL and microbicidal studies were in agreement.

Additional killing studies, using heat-treated anti-phase II immune rabbit serum or anti-phase I immune serum absorbed with either *S. sonnei* phase I or phase II bacteria, were performed (Fig. 2C). No serum killing of *S. sonnei* phase I was observed in the absence of PMNL. In tubes containing PMNL, there was no killing of *S. sonnei* phase I with either complement plus anti-phase II immune serum or complement plus anti-phase I immune rabbit serum absorbed with phase I bacteria. However, a relatively large kill of *S. sonnei* phase I by PMNL was observed with complement in the presence of heat-treated anti-phase I immune serum absorbed with phase II bacteria (compare Fig. 2C with B).

Measurement of PMNL CL from PMNL challenged with *S. sonnei* phase II. Challenging PMNL with *S. sonnei* phase II resulted in a markedly different CL response relative to that observed with *S. sonnei* phase I. After prolonged incubation, a very low level of CL was detected from PMNL challenged with *S. sonnei* phase II in the absence of serum (Fig. 3A). Low levels of CL activity were observed when heat-treated normal rabbit serum or anti-phase II immune serum were employed. However, addition of complement (Fig. 3B) alone or in combination with heat-treated normal rabbit serum resulted in high levels of CL when PMNL were challenged with *S. sonnei* phase II (compare Fig. 3B with A). The highest level of CL was obtained from PMNL challenged with *S. sonnei* phase II in the presence of complement plus anti-phase II immune rabbit serum. These results indicate that efficient opsonification of *S. sonnei* phase II was effected by complement in the absence of immune serum; however, the rate and magnitude of the CL response were increased by the presence of immune serum.

Susceptibility of *S. sonnei* phase II to killing by serum. Results of controls in Fig. 4A show no bactericidal action of heat-treated normal rabbit serum or heat-treated anti-phase II immune rabbit serum against *S. sonnei* phase II. Additionally, no microbicidal activity against *S. sonnei* phase II was effected by PMNL with heat-treated sera. However, *S. sonnei* phase II, unlike phase I, was susceptible to the direct bactericidal action of complement (compare Fig. 4B with A). Bactericidal activity was greatest in tubes containing complement and anti-phase II immune serum. Therefore, the determination of PMNL-dependent microbicidal activity against *S. sonnei* phase II with complement could not be determined.

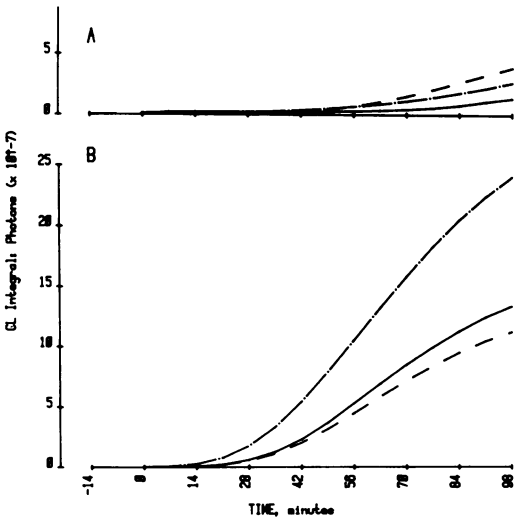

FIG. 3. *Integral CL response against time from PMNL challenged with S. sonnei phase II. Each vial contained 2.5 × 10⁴ PMNL, 2.5 × 10⁶ S. sonnei phase II, and 1 nmol of luminol in a final volume of 2.0 ml of complete Veronal buffer with the following additions. A. None (——); normal rabbit serum, 2 μl (·····); anti-phase II immune rabbit serum, 2 μl (—·—·). B. Complement, 10 μl (——); complement plus normal rabbit serum; 2 μl (·····); complement plus anti-phase II immune rabbit serum, 2 μl (—·—·).*

DISCUSSION

The present study was designed to investigate the role of specific and nonspecific humoral factors required for opsonification of *S. sonnei* phase I and phase II as measured by the CL resulting from activation of PMNL O_2-redox metabolism. The results of these investigations indicate that phase I and phase II organisms differ with respect to opsonic requirements necessary for activation of PMNL O_2-redox metabolism. Likewise, *S. sonnei* phase I and phase II are distinctly different with regard to susceptibility to direct serum killing.

A schematic depiction consistent with the presented data is shown in Fig. 5. For optimal opsonification of *S. sonnei* phase I, both heat-stable and heat-labile humoral factors are required; that is, humoral recognition, as assayed by the rate and extent of PMNL activation, is effected by phase I-specific immunoglobulins plus the classical pathway of complement. As previously reported by Reed (17), various *Shigella* strains that are resistant to direct serum killing are susceptible to PMNL microbicidal action in the presence of specific antibody alone. However, certain *Shigella* strains required both specific antibody plus complement for effective phagocytic killing.

Specific recognition was not required for opsonification of *S. sonnei* phase II. Stimulation of PMNL O_2-redox metabolism was effected by using *S. sonnei* phase II opsonified with complement in the absence of specific immune serum. However, complement plus specific phase II antibody effected an increased rate and magnitude of opsonification as assayed by PMNL CL.

Complement in the absence of immune serum was directly microbicidal for *S. sonnei* phase II. Therefore, the role of phagocytic killing could not be directly assessed. However, no detectable phagocytic killing was observed in the absence of complement.

The major chemically defined difference between *S. sonnei* phase I and phase II is the composition of their respective surface LPS. The LPS of *S. sonnei* phase I contains 2-amino-2-deoxy-L-altruronic acid (11). The synthesis and expression of phase I-specific antigen has been shown to be mediated by a large plasmid (12).

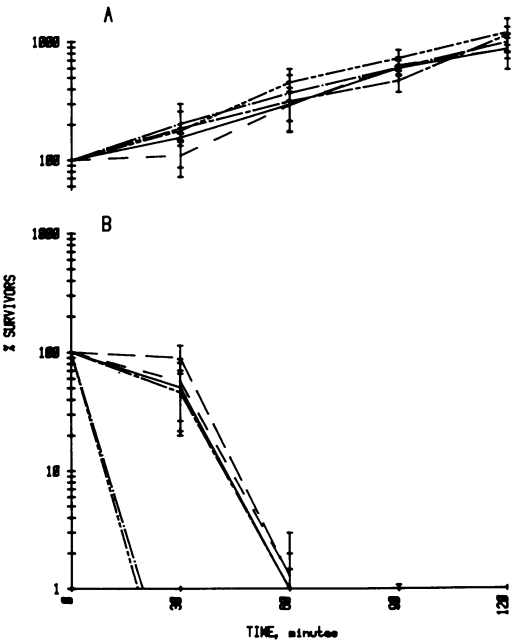

FIG. 4. *Susceptibility of S. sonnei phase II to killing by serum or PMNL. Tubes contained 5.0 × 10⁵ PMNL and 1.0 × 10⁵ S. sonnei phase II in a final volume of 1.0 ml of complete Veronal buffer. Control tubes contained no PMNL. Points represent the mean values of three experiments performed on different days, and each vertical bar represents one standard deviation. Additions were made as follows. A. None (——); normal rabbit serum, 100 μl (— —); control (— - —); anti-phase II immune rabbit serum, 100 μl (—·—·); control (—··—). B. Complement, 20 μl (——); control (—— ——); complement plus normal rabbit serum, 100 μl (— —); control (— - —); complement plus anti-phase II immune rabbit serum, 100 μl (—·—·); control (—··—).*

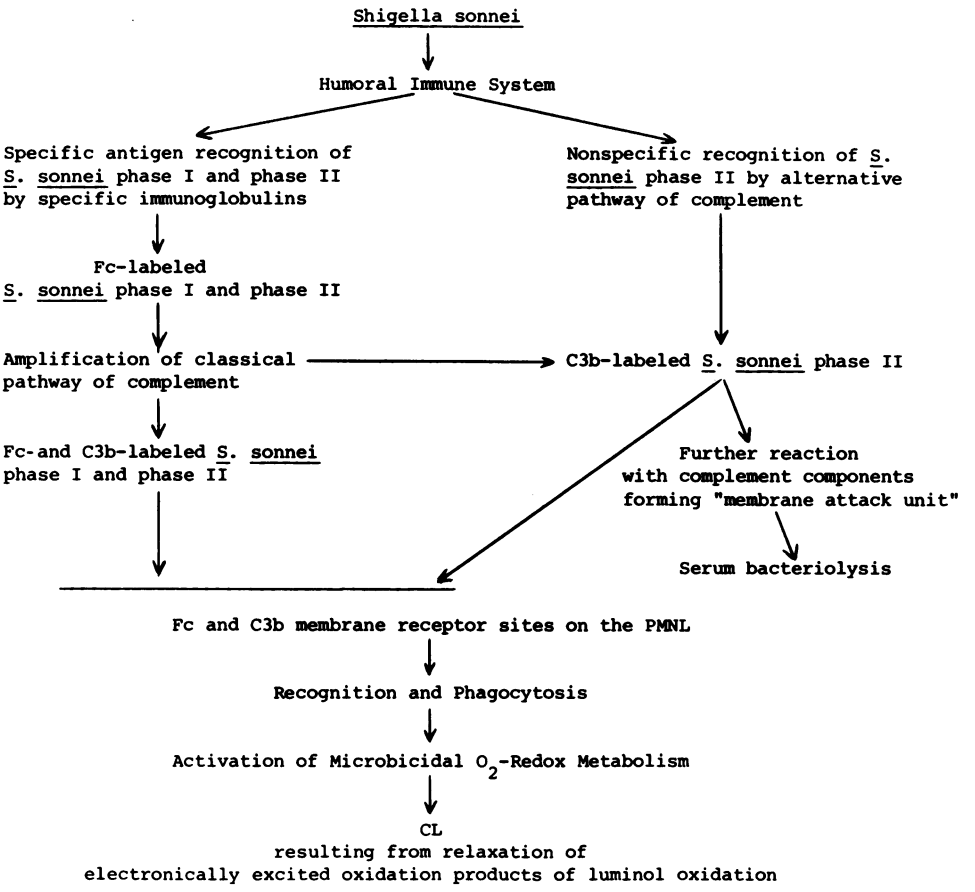

FIG. 5. *Schematic representation of data.*

Loss of this plasmid from *S. sonnei* phase I, which occurs at a relatively high frequency, results in loss of expression of the phase I-specific antigen.

The present data indicate that LPS expression determines the susceptibility to direct serum bacteriolysis and the opsonification requirements necessary for PMNL-mediated microbicidal action. Furthermore, the observed differences between *S. sonnei* phase I and phase II organisms, relative to serum lysis, serve as a basis for a useful technique, with regard to isolation of pure culture preparations of *S. sonnei* phase I.

ACKNOWLEDGMENTS

We thank Michael M. Lieberman for encouragement, constructive advice, and review of the manuscript; Dodie Bratten for expert manuscript preparation; Geralyn Strong for invaluable assistance in graphic representation of data; Jack Kelly, Karen M. Wolcott, and Gwendolyn L. Wright for cooperative technical assistance; Ricky Hickman, Department of Pathology and Area Laboratory Service, for necessary media preparation; and the Laboratory Animal Branch of the Institute of Surgical Research for animals, caretaker service, and facilities provided.

This research was supported by the Department of Clinical Investigation, Brooke Army Medical Center, under work unit C-26-79.

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