INTERACTION OF THE LEGIONNAIRES' DISEASE BACTERIUM (*LEGIONELLA PNEUMOPHILA*) WITH HUMAN PHAGOCYTES

I. *L. pneumophila* Resists Killing by Polymorphonuclear Leukocytes, Antibody, and Complement*

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We have previously reported that virulent egg yolk-grown *Legionella pneumophila*, Philadelphia 1 strain, multiplies intracellularly in human blood monocytes and only intracellularly under tissue culture conditions (1). Because this bacterium can multiply extracellularly on complex media, *L. pneumophila* is a facultative intracellular parasite.

Patients with Legionnaires' disease respond to the infection by producing antibody against *L. pneumophila*. In fact, the diagnosis of Legionnaires' disease is most often made serologically by demonstrating a rise in antibody titer to *L. pneumophila* during the course of the disease. However, the role of antibody in prevention or control of the disease has not been defined.

In this study, we have examined the interaction between virulent egg yolk-grown *L. pneumophila* and human polymorphonuclear leukocytes (PMN),[1] antibody, and complement in vitro under antibiotic-free conditions. We shall demonstrate that *L. pneumophila* bacteria are resistant to the bactericidal effects of serum and that these bacteria, even when coated with antibody and complement, are only partially susceptible to killing by PMN. These findings suggest that PMN, even in conjunction with the humoral immune system, do not play a decisive role in defense against the Legionnaires' disease bacterium.

Materials and Methods

Media. Egg yolk buffer (EYB), EYB that contained 1% bovine serum albumin (EYB-BSA), Hanks' balanced salt solution (HBSS), Dulbecco's phosphate-buffered saline with Ca^{++} and Mg^{++} ions (PBS), and RPMI 1640 medium were obtained or prepared as described previously (1, 2). No antibiotics were added to any medium in any of the experiments.

Agar. Modified charcoal yeast extract agar (CYE agar) was prepared in 100- × 15-mm bacteriologic Petri dishes as described (1).

Antisera. Goat anti-human complement C3, IgG fraction, rhodamine-conjugated (N. L. Cappel Laboratories, Inc., Cochranville, Pa.); rabbit anti-*L. pneumophila*, Knoxville 1 strain,

* Supported by grant AI 08697 from the National Institutes of Health.

‡ This work was performed while Dr. Horwitz was a U. S. Public Health Service postdoctoral fellow (fellowship AI 05629).

[1] *Abbreviations used in this paper:* CFU, colony-forming units; CYE agar, modified charcoal yeast extract agar; EYB, egg yolk buffer; EYB-BSA, egg yolk buffer that contained 1% bovine serum albumin; HBSS, Hanks' balanced salt solution; IFA, indirect fluorescent antibody assay; PBS, phosphate-buffered saline with Ca^{++} and Mg^{++} ions; PMN, human polymorphonuclear leukocytes.

 J. Exp. Med. © The Rockefeller University Press · 0022-1007/81/02/0386/12 $1.00
Volume 153 February 1981 386–397

(Group 1) antiserum, globulin fraction, and fluorescein-conjugated globulins from the same lot, were obtained from the Center for Disease Control, U. S. Public Health Service, Atlanta, Ga. The nonconjugated antiserum was diluted 1:9 in PBS and dialyzed three times against 100 volumes of PBS; 0.6 μl of this antiserum preparation agglutinated 1 ml of a suspension of *L. pneumophila* (prepared by culturing egg yolk-grown organisms for 72 h on CYE agar and suspending the bacteria in EYB at a concentration of 0.30 OD U at $A_{540\ nm}$ on a Coleman 44 spectrophotometer [Perkin-Elmer Corp., Instruments Div., Norwalk, Conn.]). Consequently, 0.6 μl of this antiserum preparation is referred to in this paper as 1 agglutinating U. In our studies, we used 8.5 agglutinating U/ml; at the concentrations of *L. pneumophila* ($\leq 10^6$ colony-forming units [CFU]/ml) used in our studies, this amount of antibody did not agglutinate the bacteria.

Serum. Venous blood was obtained, clotted, and serum separated and stored at −70°C until used as described (2). Normal human serum with an indirect fluorescent antibody (IFA) anti-*L. pneumophila* titer of <1:64 (3) was obtained from an adult donor not known to have ever had Legionnaires' disease. Immune human serum with an IFA anti-*L. pneumophila* titer of 1:4,096 was obtained from an adult donor who had recently recovered from Legionnaires' disease; Dr. John Marr of the New York City Department of Health kindly assisted in locating this person. Ms. Helen Kravitz of the New York City Department of Health and Dr. Karim Hechemy of the New York State Health Department, Albany, N. Y. determined serum IFA anti-*L. pneumophila* titers.

Complement was inactivated by heating the serum at 56°C for 30 min just before use.

Bacteria. L. pneumophila Philadelphia 1 strain were grown in embryonated hens' eggs, harvested, tested for viability and for the presence of contaminating bacteria, stored at −70°C, and partially purified by differential centrifugation just before use, as described (1). A single batch was used in all experiments.

Escherichia coli, serotype 09:K29:H^-, strain Bi 161-42, an encapsulated, serum-resistant strain (2), was cultured and prepared for experiments as described (2).

Human PMN. Blood from a single healthy donor (type AB^+) was collected in a heparinized syringe and mixed 1:1 with 0.9% sodium chloride (normal saline); PMN were separated by centrifugation over a discontinuous gradient of Ficoll and sodium diatrizoate by the method of English and Anderson (4). Residual erythrocytes were hypotonically lysed and isotonicity restored as described (2). PMN were resuspended in HBSS, counted, and adjusted to the required concentration; their viability was >99% as measured by trypan blue exclusion (2). The cells (>99% PMN) were used within 30 min of purification.

Serum Bactericidal Activity for L. pneumophila. L. pneumophila in concentrations ranging from 10^3 to 10^6 CFU/ml were incubated in 0, 10, 25, or 50% fresh normal human serum in plastic test tubes (0.4 ml final vol) for 1 h at 37°C on a gyratory shaker at 250 rpm. In experiments in which serum bactericidal activity was measured in the presence of antibody, 8.5 agglutinating U/ml (see above) of dialyzed rabbit anti-*L. pneumophila* antiserum or 20% heat-inactivated immune human serum was added to test tubes that contained the bacteria and serum. At the end of the incubation, the contents of each tube were serially diluted in EYB-BSA and CFU of *L. pneumophila* were determined on CYE agar as described (1).

Complement Fixation to the L. pneumophila Surface. 3×10^6 CFU of *L. pneumophila* was incubated for 15 min at 37°C in 600 μl of medium that contained 25% fresh normal human serum, 25% fresh immune human serum, 25% heat-inactivated immune human serum, or 25% heat-inactivated immune human serum plus 25% fresh normal human serum. In some experiments, 8.5 agglutinating U/ml of dialyzed, nonconjugated rabbit antiserum were added instead of heat-inactivated immune human serum. The bacteria were then washed twice with PBS (4°C) and incubated with rhodamine-conjugated goat anti-human C3 IgG as described (2). The bacterial suspension was then air-dried on glass slides, incubated with 10 μl of fluorescein-conjugated rabbit anti-*L. pneumophila* antiserum for 20 min at 37°C in a humidified chamber, washed, incubated for 10 min at 22°C in phosphate-buffered saline (pH 7.6) (5), air dried, and examined with a Zeiss Fluorescent Photomicroscope III (Carl Zeiss, Inc., New York). *L. pneumophila* bacteria were first identified by fluorescein fluorescence and then these bacteria were examined for the presence of C3 on their surfaces by rhodamine fluorescence.

Anti-*L. pneumophila* antibody fixes to the surface of *L. pneumophila* when the bacterium is

incubated with antibody at 37°C for 15 min in the presence or absence of fresh normal human serum. This was determined by fluorescence microscopy, using fluorescein-conjugated rabbit anti-*L. pneumophila* antiserum.

Capacity of PMN to Bind or to Ingest L. pneumophila. 5×10^6 PMN and 5×10^5 CFU of *L. pneumophila* were added to plastic tubes that contained 10% fresh normal human serum, 10% fresh immune human serum, or 10% heat-inactivated immune human serum. The tubes were brought to a final vol of 900 μl with HBSS, gassed with 5% CO_2-95% air so that the pH was 7.4, as judged by the color of the phenol red indicator dye, capped, sealed with Parafilm (American Can Co., Greenwich, Conn.), and incubated for 30 min at 37°C on a gyratory shaker at 250 rpm. At the end of the incubation, the tubes were placed in an ice-water bath to stop the reaction, and the cells were centrifuged onto glass slides using a sealed cytocentrifuge specimen container especially designed for use with biohazardous material (Cytospin and Accessories, Shandron Southern Instruments, Inc., Sewickley, Pa.). The cells were fixed with Diff-Quick Fixative (Harleco, American Hospital Supply Corp., Gibbstown, N. J.), stained with fluorescein-conjugated rabbit anti-*L. pneumophila* antiserum as described (1), and examined by fluorescence microscopy. The percentage of 200 consecutive PMN with associated bacteria, and the average number of bacteria/PMN were determined.

For electron microscopy, PMN and bacteria were incubated together at the same ratio and under the same conditions as described above. The cells were fixed and processed for electron microscopy essentially as described (1) except that they were centrifuged into 2% agarose before dehydration and embedding. The sections were stained with lead citrate and uranyl acetate (1) and examined with a Philips 300 electron microscope (Philips Electronic Instruments, Inc., Mahwah, N. J.).

PMN-killing Assay. In all killing assays, reactants (total vol 900 μl) were added sequentially to plastic tubes. The tubes were gassed with 5% CO_2-95% air (final pH of contents of each tube = 7.4), sealed, and incubated for 1, 2, or 3 h as described above under capacity of PMN to bind or ingest *L. pneumophila*. *L. pneumophila* (2.5×10^5 or 5×10^5 CFU) and/or *E. coli* (5×10^5 CFU) in 100 μl HBSS, the serum preparation (90 μl fresh normal human serum, 90 μl fresh immune human serum, 90 μl heat-inactivated immune human serum plus 90 μl fresh normal human serum, or 4.5 μl of dialyzed rabbit anti-*L. pneumophila* antiserum [8.5 agglutinating U/ml] plus 90 μl fresh normal human serum) and enough PBS to bring the vol to 400 μl were added first and incubated 10 min at 22°C. Then 2.5 or 5×10^6 PMN in 500 μl HBSS (or the sonicate from an equivalent number of PMN or HBSS alone as controls) were added to each tube, after which the tubes were gassed, sealed, and incubated. A separate tube was used for each measurement; thus tubes were not opened until the end of the incubation. At that time, the tubes were placed in an ice-water bath to stop the reaction, and the contents were sonicated under sterile conditions for 15 sec continuously with a micro-tip attached to a sonicator (Heat-Systems Ultrasonics, Inc., Plainview, N. Y.) set at the 3 position; this amount of energy was sufficient to lyse the PMN completely but did not reduce bacterial CFU (1, 2). The CFU of *L. pneumophila* on CYE agar and of *E. coli* on tryptic soy broth agar or CYE agar were determined as described (1, 2); *E. coli* grew equally well on both nutrient agars, but *L. pneumophila* grew only on CYE agar.

Separation of PMN-associated and Nonassociated L. pneumophila by Differential Centrifugation. PMN and *L. pneumophila* were incubated with normal human serum or immune human serum as described above under PMN-killing assay. At the end of the incubation, 9.1 ml of PBS (4°C) was added to each tube, and the contents transferred to a conical centrifuge tube and centrifuged at 45 *g* for 10 min at 4°C. When PMN were mixed with *L. pneumophila* at 4°C in medium that contained normal human serum and centrifuged under these conditions, >99% of PMN were pelleted but >99% of *L. pneumophila* remained in the supernate. The supernate was removed and the pellet was resuspended in 2 ml PBS. The supernate and resuspended pellet were then sonicated as described above under PMN-killing assay and the total CFU in the pellet and supernatant fractions determined. Three replicate tubes were used for each measurement with immune serum and two replicate tubes for each measurement with normal serum. Additional replicate tubes, the contents of which were not fractionated after the incubation, were used to measure killing of *L. pneumophila* as in the PMN-killing assay described above; two tubes were used for each measurement.

Results

L. pneumophila Is Resistant to the Bactericidal Effects of Serum Even in the Presence of High Concentrations of Antibody. We incubated 10^3–10^6 CFU/ml *L. pneumophila* in medium that contained various concentrations of fresh normal human serum (lacking antibody to *L. pneumophila* by the IFA assay) for 1 h at 37°C on a gyratory shaker. There was no reduction in CFU of *L. pneumophila* (Table I A). Similarly, when we incubated *L. pneumophila* in medium that contained 0–50% fresh normal human serum and either 8.5 agglutinating U/ml of rabbit anti-*L. pneumophila* antiserum (Table I B) or 20% heat-inactivated immune human serum (Table I C), the CFU of *L. pneumophila* were not reduced. The fresh normal human serum used in these experiments reduced CFU of a serum-sensitive strain of *Neisseria gonorrhea* by >4 logs.

Thus, virulent egg yolk-grown *L. pneumophila* are resistant to the bactericidal effects of human serum even in the presence of high concentrations of rabbit or human anti-*L. pneumophila* antibody.

Antibody Is Required to Fix Complement to the Surface of L. pneumophila. To determine whether anti-*L. pneumophila* antibody is required to fix complement to the surface of *L. pneumophila*, we incubated these bacteria with fresh normal human serum (a source of complement) in the presence and absence of heat-inactivated human or rabbit immune serum (sources of anti-*L. pneumophila* antibody) or with fresh immune human serum. We assayed complement fixation to the bacterial surface by fluorescence microscopy, using rhodamine-conjugated goat anti-human C3 IgG to visualize this complement component. To facilitate identification of all the bacteria, we then incubated the bacteria with fluorescein-conjugated rabbit anti-*L. pneumophila* antiserum.

Over 90% of *L. pneumophila* bacteria fixed C3 to their surfaces, as evidenced by rhodamine fluorescence, after incubation with a source of both antibody and complement. None of the *L. pneumophila* exhibited rhodamine fluorescence after incubation with antibody or complement alone. However, the intensity of fluorescence exhibited by *L. pneumophila* after incubation with high-titer specific antibody (human or rabbit) and complement was weak in comparison to that exhibited by a serum-resistant encapsulated strain of *E. coli* (*E. coli* 09:K29:H^-) (2). This suggests that the anti-*L. pneumophila* antibodies found in human immune serum or in rabbit antiserum are not capable of fixing as much C3 to the surface of *L. pneumophila* as human anti-*E. coli* antibodies are capable of fixing to the surface of the *E. coli* strain.

PMN Phagocytose L. pneumophila Only in the Presence of Both Antibody and Complement. We incubated PMN with *L. pneumophila* in the presence of serum that contained anti-*L. pneumophila* antibody and/or complement. We then centrifuged the PMN onto glass slides, stained the PMN-associated bacteria with fluorescein-conjugated rabbit anti-*L. pneumophila* antiserum, and examined them by fluorescence microscopy (Table II).

With complement alone or antibody alone, few PMN (21 and 14%, respectively) bound or ingested *L. pneumophila*, and those that did had an average of less than two bacteria/PMN (Table II). With both antibody and complement, 93.5% of PMN bound or ingested *L. pneumophila*; these had an average of 5.1 *L. pneumophila*/PMN (Table II and Fig. 1 A).

In these experiments, the ratio of bacterial cells:PMN was ~5:1. (The CFU:PMN ratio was 1:10, but there are ~50 nonviable bacterial cells for each viable one [CFU]

TABLE I

Viability of L. pneumophila in the Presence of Human Serum and Rabbit or Human Anti-L. pneumophila Antibody

(A) Viability in the presence of fresh normal human serum

Initial number of *L. pneumophila*	Number of *L. pneumophila* after a 1-h incubation in medium that contained 0–50% serum			
	0%	10%	25%	50%
CFU/ml	*CFU/ml*			
4.0×10^6	3.4×10^6	3.8×10^6	4.9×10^6	5.6×10^6
3.2×10^5	3.6×10^5	6.3×10^5	6.1×10^5	3.8×10^5
4.7×10^4	1.6×10^4	5.6×10^4	5.8×10^4	6.3×10^4
1.3×10^3	1.7×10^3	2.5×10^3	3.1×10^3	3.1×10^3

(B) Viability in the presence of fresh normal human serum and rabbit anti-*L. pneumophila* antiserum

Initial number of *L. pneumophila*	Number of *L. pneumophila* after a 1-h incubation in medium that contained 0–50% serum and 8.5 agglutinating doses/ml anti-*L. pneumophila* antiserum			
	0%	10%	25%	50%
CFU/ml	*CFU/ml*			
1.0×10^5	1.2×10^5	0.9×10^5	2.6×10^5	5.4×10^5
1.1×10^4	0.6×10^4	3.6×10^4	2.8×10^4	4.2×10^4
1.8×10^3	0.9×10^3	1.2×10^3	2.6×10^3	2.1×10^3
4.6×10^2	1.6×10^2	3.4×10^2	5.6×10^2	4.0×10^2

(C) Viability in the presence of fresh normal human serum and heat-inactivated immune human serum

Initial number of *L. pneumophila*	Number of *L. pneumophila* after a 1-h incubation in medium that contained 0–50% serum and 20% heat-inactivated immune human serum			
	0%	10%	25%	50%
CFU/ml	*CFU/ml*			
2.7×10^6	2.7×10^6	2.4×10^6	3.0×10^6	2.1×10^6
3.2×10^5	3.1×10^5	2.8×10^5	3.5×10^5	2.9×10^5
2.7×10^4	3.6×10^4	3.1×10^4	3.4×10^4	3.1×10^4
2.8×10^3	3.1×10^3	2.8×10^3	3.0×10^3	2.8×10^3

L. pneumophila at the indicated concentrations of CFU/ml were incubated for 1 h at 37°C on a gyratory shaker in medium that contained 0, 10, 25, or 50% fresh normal human serum and (A) No antibody, (B) 8.5 agglutinating doses/ml of dialyzed rabbit anti-*L. pneumophila* antiserum, or (C) 20% heat-inactivated immune human serum with an IFA anti-*L. pneumophila* titer of 1:4,096. CFU were determined initially and at the end of the incubation.

in egg yolk-grown cultures [6]). That PMN bound or ingested an average of 4.75 bacterial cells in the presence of antibody and complement (Table II) indicates that nearly all the bacterial cells in the culture were bound to or ingested by PMN under these conditions. That this conclusion is valid for viable bacteria is supported by our finding (Fig. 4 B) that nearly all the viable *L. pneumophila* in the culture are bound to or ingested by PMN in the presence of antibody and complement.

TABLE II

Capacity of PMN to Bind or to Ingest L. pneumophila in the Presence of Antibody and/or Complement

Serum preparation	Percentage of PMN binding or ingesting *L. pneumophila*	Average number of *L. pneumophila*/PMN with ≥1 associated bacteria	Index of PMN-*L. pneumophila* association*
Normal serum	21	1.2	25
Heat-inactivated immune serum	14	1.8	25
Fresh immune serum	93.5	5.1	475

5×10^6 PMN and 5×10^5 CFU of *L. pneumophila* (~2.5×10^7 bacterial particles) were incubated in the presence of 10% of the indicated serum preparation for 30 min at 37°C on a gyratory shaker. At the end of the incubation, the cells were cytocentrifuged on to glass slides, fixed, stained with fluorescein-conjugated rabbit anti-*L. pneumophila* antiserum, examined by fluorescence microscopy, and 20 consecutive PMN counted.

* The index of PMN-*L. pneumophila* association is the percentage of PMN with bound or ingested *L. pneumophila* multiplied by the average number of *L. pneumophila*/ PMN with ≥1 associated bacteria.

To determine whether PMN phagocytosed *L. pneumophila* when incubated with them in the presence of antibody and complement, we examined the cells by electron microscopy. We found 25 PMN-associated *L. pneumophila* upon examination of ~200 leukocyte profiles. Of these, 23 were intracellular (Fig. 1 B and C) and 2 were adhered to the surface of PMN (and appeared fixed in the process of being ingested). This indicates that in preparations of PMN incubated with *L. pneumophila* in the presence of both antibody and complement (Table II), most of the PMN-associated bacteria were intracellular.

These results and our finding that antibody was required to fix complement to *L. pneumophila*, indicate that PMN effectively phagocytose *L. pneumophila* only if complement is fixed to the bacterial surface.

PMN Require Both Antibody and Complement to Kill Any L. pneumophila. We incubated PMN with *L. pneumophila* in the presence of fresh normal human serum, heat-inactivated immune human serum (data not shown), or fresh immune human serum (Fig. 2). PMN killed *L. pneumophila* only when both a source of antibody and of complement were present (Fig. 2). Even then, PMN reduced CFU of *L. pneumophila* by only 0.5 log (70%) (five experiments). Increasing the ratio of PMN:*L. pneumophila* from 10:1 to 20:1 or to 40:1 or the percentage of immune human serum from 10 to 20% or 40% did not improve PMN killing of *L. pneumophila.* Most of the killing occurred in the 1st h of incubation (Fig. 4 B). Some additional killing occurred when the incubation was extended from 1 to 4 h (Fig. 4 B), but no additional killing occurred when the incubation was further extended from 4 to 6 h (data not shown).

L. pneumophila Resists Killing by PMN under Conditions in Which PMN Effectively Kill a Serum-resistant Encapsulated Strain of E. coli. To compare PMN killing of *L. pneumophila* with PMN killing of a serum-resistant encapsulated strain of *E. coli*, we incubated PMN with *E. coli* alone, *L. pneumophila* alone, or both types of bacteria in the presence of fresh normal human serum with or without a source of rabbit or human anti-*L. pneumophila* antibody (Fig. 3). In the experiment described in Fig. 3, we used rabbit

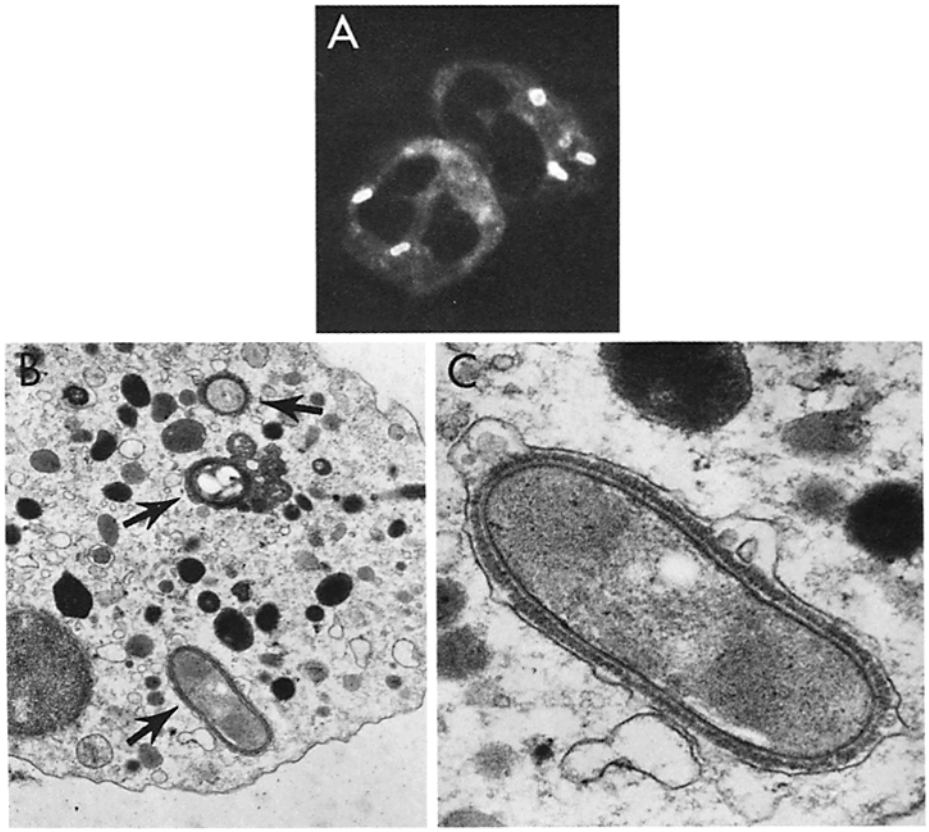

FIG. 1. PMN bind and ingest *L. pneumophila* in the presence of antibody and complement. (A) PMN bind opsonized *L. pneumophila*. PMN were incubated with *L. pneumophila* in the presence of fresh immune human serum for 30 min at 37°C on a gyratory shaker and centrifuged onto glass slides. The PMN-associated bacteria were stained with fluorescein-conjugated rabbit anti-*L. pneumophila* antiserum and the cells were examined by fluorescence microscopy. × 1,400. (B) and (C) PMN ingest opsonized *L. pneumophila*. PMN and *L. pneumophila* were incubated as in A, fixed in glutaraldehyde and osmium, and processed for electron microscopy. (B) PMN with three intracellular *L. pneumophila* bacteria (arrows). (× 11,580). C. The bacterium at the lower portion of the PMN shown in B is seen at higher magnification to be enclosed within a membrane-bound vacuole × 41,690.

antiserum, but we obtained similar results when we used immune human serum. In the presence of fresh normal serum and antibody, PMN failed to kill *L. pneumophila* effectively. Under these same conditions, PMN reduced CFU of *E. coli* by 2.5 logs. (PMN require both antibody and complement to kill *E. coli* [2]; both are present in fresh normal human serum, which contains low levels of natural antibody against this strain of *E. coli* [2].) When the *E. coli* and *L. pneumophila* were mixed together in the same test tube, PMN killed the *E. coli* as effectively as when the *E. coli* were present alone, indicating that the killing capacity of PMN was not reduced in cultures containing *L. pneumophila*.

L. pneumophila Resists PMN Killing Even after the Bacteria are PMN-associated. When PMN are incubated with *L. pneumophila* in the presence of antibody and complement, they bind or ingest nearly all the bacterial cells (Table II); however, most of the

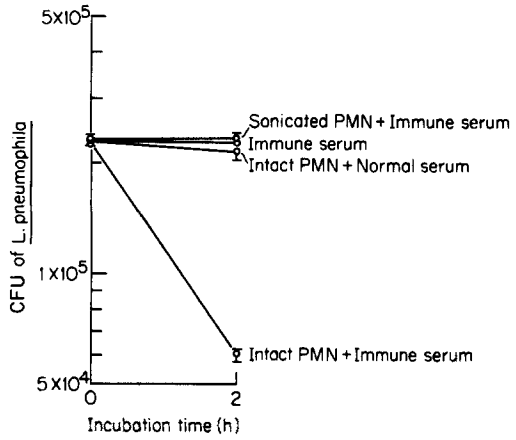

FIG. 2. PMN require both antibody and complement to kill any *L. pneumophila*. PMN (5×10^6) and 2.5×10^5 CFU of *L. pneumophila* were incubated at 37°C in 5% CO_2-95% air on a gyratory shaker for 2 h in 0.9 ml medium (final volume) containing 10% fresh normal human serum (with an IFA anti-*L. pneumophila* titer of <1:64) or 10% fresh immune human serum (with an IFA anti-*L. pneumophila* titer of 1:4,096). In some tubes, the sonicates from 5×10^6 PMN or buffer were added as controls for intact PMN. CFU in each tube were determined initially and at the end of the incubation. Each point represents the average for four replicate tubes ± SE.

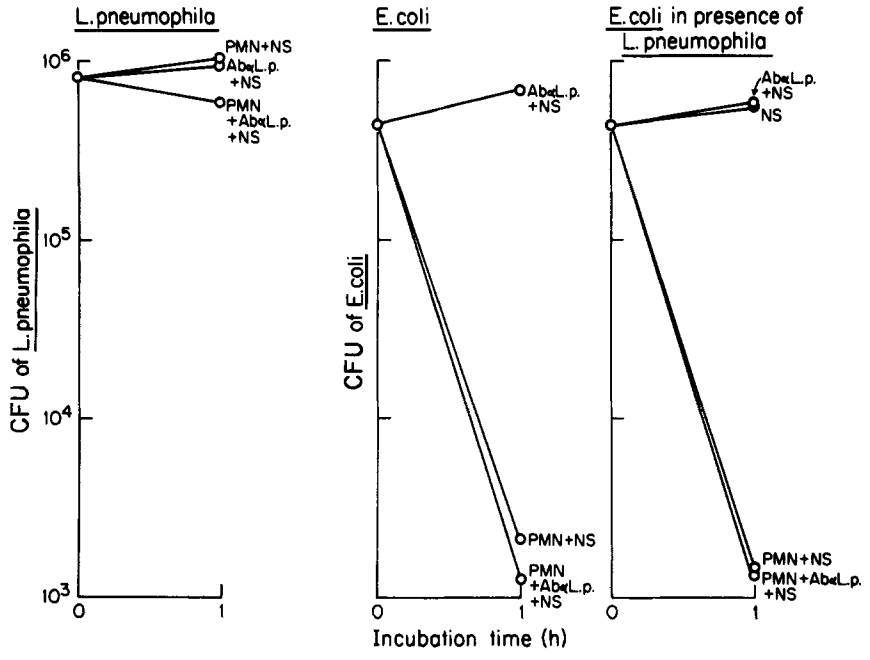

FIG. 3. *L. pneumophila* resists killing by PMN under conditions in which PMN effectively kill a serum-resistant encapsulated strain of *E. coli*. PMN (2.5×10^6) and 5×10^5 CFU of *L. pneumophila* and/or *E. coli* were incubated at 37°C for 1 h on a gyratory shaker in 0.9 ml medium (final volume) containing 10% fresh normal human serum (NS) alone or 10% NS plus 8.5 agglutinating units/ml of dialyzed rabbit anti-*L. pneumophila* antiserum (Abα-L.p.). CFU of *E. coli* and *L. pneumophila* were determined initially and at the end of the incubation.

bacterial cells from egg yolk-grown cultures are nonviable (6). Thus, from the studies described in Table II, we did not know whether PMN, in the presence of antibody and complement, bind or ingest that small minority of bacterial cells that are viable. We undertook this study to clarify this issue, and to determine whether the resistance of opsonized *L. pneumophila* to PMN killing is a result of a failure of PMN to bind the viable bacteria or of a failure of PMN to kill these bacteria after binding them. We incubated PMN with *L. pneumophila* for varying periods of time, separated PMN-associated and nonassociated *L. pneumophila* by differential centrifugation, and determined the number of CFU in each fraction.

When we incubated PMN and *L. pneumophila* together in the presence of both antibody and complement (fresh immune human serum) (Fig. 4B), PMN killed a

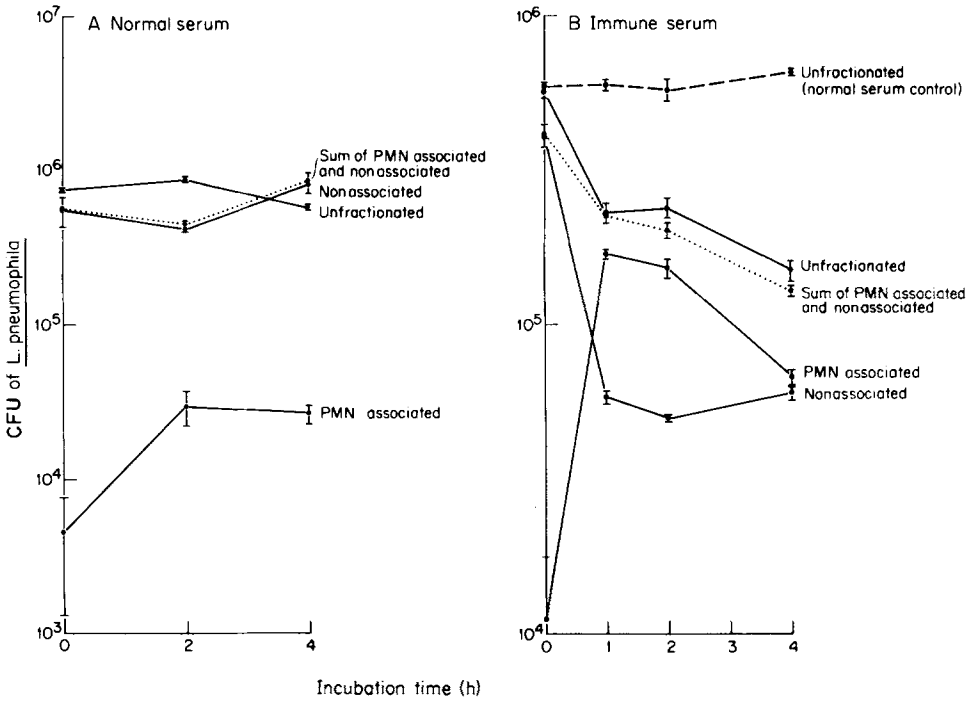

FIG. 4. Failure of PMN to kill *L. pneumophila* effectively: (A) In the absence of antibody, PMN fail to bind *L. pneumophila* and, subsequently, to kill the bacteria. (B) In the presence of antibody, PMN rapidly bind *L. pneumophila* but nevertheless fail to kill a large proportion of the bacteria. PMN (5×10^6) were incubated in plastic tubes with 5×10^5 CFU of *L. pneumophila* in 0.9 ml media (final vol) that contained 10% fresh normal human serum (A) or 10% fresh immune human serum (B) at 37°C in 5% CO_2-95% air on a gyratory shaker for 0, 1, 2, or 4 h. (In the experiment depicted in (B) one set of control tubes [dashed line] contained normal serum instead of immune serum.) At each time point, the contents of some replicate tubes were immediately assayed for the total number of CFU in each tube after lysing the PMN by sonication (Unfractionated samples). The contents of other replicate tubes were first centrifuged to separate PMN-associated and nonassociated *L. pneumophila*, as described in Materials and Methods; each of these fractions was then sonicated and assayed for the total number of PMN-associated CFU and nonassociated CFU of *L. pneumophila*. The points on the lines labeled Sum of PMN-associated and nonassociated are the mathematical sums of the experimentally determined values for PMN-associated and nonassociated CFU of *L. pneumophila* at each time point. Each point on the lines labeled Unfractionated represents the average for two replicate tubes ± SE. Each point on the lines labeled PMN-associated and nonassociated represents the average for three replicate tubes ± SE.

limited proportion of the *L. pneumophila* and most (>75%) of the surviving bacteria were PMN-associated after 1 h of incubation. The additional reduction in CFU of *L. pneumophila* that occurred between 1 and 4 h after the start of the incubation appeared to result primarily from the death of PMN-associated bacteria. Thus, the resistance of opsonized *L. pneumophila* to PMN killing was a result of the failure of PMN to kill bacteria that were associated with them.

In contrast, when we incubated PMN and *L. pneumophila* together in the presence of only complement (fresh normal human serum) (Fig. 4A), nearly all (93%) of the CFU of *L. pneumophila* failed to associate with PMN and, as expected from previous results (Fig. 2), PMN did not kill *L. pneumophila* under these conditions. Thus, the complete resistance of *L. pneumophila* to PMN killing in the absence of antibody was a result of a failure of PMN to bind these unopsonized bacteria.

PMN Do Not Support the Growth of L. pneumophila. To determine whether PMN, like monocytes, support the growth of *L. pneumophila* (1, 7), we incubated *L. pneumophila* with PMN in the presence of normal or immune serum on a gyratory shaker for 1 h, and without shaking thereafter, and determined CFU daily. *L. pneumophila* failed to multiply under either of these conditions.

Because PMN are relatively short-lived in comparison to monocytes, we repeated the experiment just described except we added fresh PMN and either normal or immune human serum to the cultures daily. After adding fresh cells and serum, we incubated the cultures on a gyratory shaker for 1 h and then returned them to stationary incubation for the remainder of the day. *L. pneumophila* did not multiply under these conditions either.

Discussion

Our experiments show that virulent egg yolk-grown *L. pneumophila* are completely resistant to the bactericidal effects of human serum even in the presence of high-titer rabbit or human anti-*L. pneumophila* antibody. In contrast, agar-adapted *L. pneumophila* have been reported to be sensitive to serum (8). Egg yolk-grown and agar-adapted bacteria differ in that agar-adapted bacteria are avirulent for guinea pigs and eggs, whereas egg yolk-grown bacteria cause lethal infections in both. In their virulence, egg yolk-grown *L. pneumophila* more closely resemble a human pathogen than do agar-adapted bacteria. Consequently, we suggest that *L. pneumophila* that are pathogenic for man are also serum resistant.

Antibody is required to fix complement to *L. pneumophila* and only complement-coated bacteria are efficiently bound to or ingested by PMN. In this respect, *L. pneumophila* resembles encapsulated *E. coli*, an extracellular pathogen (2). The similarity ends here, however, because complement-coated *E. coli* are efficiently killed by PMN but complemented-coated *L. pneumophila* are not. A significant proportion of *L. pneumophila* resist killing by PMN even after the bacteria are bound to the PMN surface by phagocytosis-promoting ligands.

The capacity of *L. pneumophila* to resist completely killing by human serum and PMN in the absence of antibody likely contributes to its pathogenicity for man. Because *L. pneumophila* are completely resistant to serum even in the presence of antibody, the degree to which antibody influences pathogenicity probably depends on how antibody affects the interaction of *L. pneumophila* with PMN and monocytes. In the case of PMN, the outcome of this interaction is inconclusive; some *L. pneumophila*

are killed but a significant proportion survive the encounter. Consequently, the critical questions regarding the influence of antibody on pathogenicity may be whether antibody influences the interaction of *L. pneumophila* with monocytes and, especially, whether antibody alters the capacity of *L. pneumophila* to multiply within monocytes. These questions are addressed in an accompanying paper (7).

Summary

We have previously reported that virulent egg yolk-grown *Legionella pneumophila*, Philadelphia 1 strain, multiplies intracellularly in human blood monocytes. We now report on the interaction between virulent *L. pneumophila* and human polymorphonuclear leukocytes (PMN), antibody, and complement, in vitro, under antibiotic-free conditions.

L. pneumophila in concentrations ranging from 10^3 to 10^6 colony forming units (CFU)/ml are completely resistant to the bactericidal effects of 0–50% fresh normal human serum, even in the presence of high concentrations of rabbit or human anti-*L. pneumophila* antibody.

L. pneumophila bacteria fix the third component of complement (C3) to their surfaces, as measured by fluorescence microscopy using rhodamine-conjugated goat anti-human C3 IgG, only when the bacteria are incubated with both specific anti-*L. pneumophila* antibody and complement. Similarly, *L. pneumophila* adhere to PMN, as measured by fluorescence microscopy, only in the presence of both specific antibody and complement. Electron microscopy revealed that these opsonized bacteria are phagocytosed by the PMN.

PMN require both antibody and complement to kill *L. pneumophila*; even then, PMN reduced CFU of *L. pneumophila* by only 0.5 log under conditions in which they reduce CFU of a serum-resistant encapsulated strain of *Escherichia coli* by 2.5 logs. Separation of PMN-associated and nonassociated CFU of *L. pneumophila* revealed that the major proportion of the surviving bacteria are PMN associated. Thus, the ineffective killing of opsonized *L. pneumophila* is a result of a failure of PMN to kill these bacteria after they become PMN-associated. With or without antibody, PMN do not support the growth of *L. pneumophila.*

These findings suggest that PMN, even in conjunction with the humoral immune system, do not play a decisive role in defense against the Legionnaires' disease bacterium.

We thank Ms. Darragh Kelvie for excellent technical assistance, and Ms. Judy Adams for preparing the electron microscopy specimens and printing the photographs in this paper.

Received for publication 16 October 1980.

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