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Nonoxidative Antimicrobial Effects of Human Polymorphonuclear Leukocyte Granule Proteins on *Chlamydia* spp. In Vitro

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Proteins from isolated granules of human polymorphonuclear leukocytes were assessed for their nonoxidative microbicidal effect on chlamydiae by two different methods: a radioisotope assay for elementary body integrity and a biological assay for inclusion development. Crude granule extract, which consisted of a mixture of all granule proteins, caused a 20 to 30% decrease in infectivity and a 52% decrease in infectivity when incubated with *Chlamydia psittaci* CAL-10 and *Chlamydia trachomatis* serovar E, respectively. To define more specifically the components that were damaging to chlamydiae, crude granule extract was subjected to Sephadex G-75 column chromatography and isolated granule fractions were obtained. Only fractions containing lysozyme as the major component consistently caused reductions in infectivity of *C. trachomatis* elementary bodies. In contrast, fractions collected after the lysozyme fraction, containing proteins with molecular masses of 13,000 daltons or less, had detrimental effects on *C. psittaci* infectivity. Additional experiments using highly purified human polymorphonuclear leukocyte lysozyme confirmed its infectivity-reducing action upon *C. trachomatis* but not upon *C. psittaci*.

Chlamydia psittaci and *Chlamydia trachomatis* are intracellular pathogens responsible for a variety of diseases in humans. Infection of humans with either organism elicits an inflammatory response in which infiltration of polymorphonuclear leukocytes (PMN) is a prominent feature (7, 32, 38). Studies performed by other investigators (23) and by us (36) have indicated that complement opsonization of chlamydiae leads to the recruitment of these phagocytes and the ingestion of chlamydiae, both in vitro (36, 58) and in vivo (9). Furthermore, at least in vitro, the majority of internalized *Chlamydia* spp. are rapidly killed (36, 58).

The ability of PMN to kill and degrade microorganisms is derived from an intracellular arsenal of toxic substances; some of these substances depend upon oxygen metabolism, and others are oxygen independent (14, 46). Yong et al. (60) assessed the importance of one oxygen-dependent system in the killing of *C. trachomatis* by human PMN. They found that a cell-free myeloperoxidase-H_2O_2-halide system was toxic for two biovars of the organism. However, both myeloperoxidase-deficient PMN and PMN from patients with chronic granulomatous disease, which are incapable of generating a respiratory burst, were still strongly chlamydiacidal. Therefore, myeloperoxidase systems and other oxygen-dependent systems are not solely responsible for the killing of chlamydiae by human PMN. The aim of the present study was to examine in vitro the nonoxidative antimicrobial potential of isolated human neutrophil granule proteins against *C. psittaci* and *C. trachomatis*.

MATERIALS AND METHODS

Growth and purification of *Chlamydia* spp. The CAL-10 meningopneumonitis strain of *C. psittaci* was grown in L-929 suspension cells by the method of Tamura and Higashi (50). Elementary bodies (EB) were harvested at 48 h postinfection. Intracellular organisms were released by sonication of infected cells and were pooled with the cell-free supernatants. EB were purified by differential centrifugation and by centrifugation through 30% sucrose cushions and discontinuous gradients of 38, 44, and 59% Renografin-76 (E. R. Squibb & Sons, Princeton, N.J.), as described previously (57).

C. trachomatis biovar E/UW-5/Cx was grown in McCoy cell monolayers, harvested, and purified essentially by the method of Newhall et al. (28). Radioactive chlamydiae, labeled internally, were obtained by the method of Eissenberg et al. (8) by using [^{3}H]uridine (1 mCi/liter of culture).

The infectivity titers of *C. psittaci* and *C. trachomatis* were determined by using the inclusion-forming unit (IFU) technique (18). Dilutions of *C. psittaci* EB added to L cell monolayers and dilutions of *C. trachomatis* EB added to McCoy cell monolayers on 12-mm^2 cover slips in 1-dram vials were centrifuged at 282 × *g* for 30 min. Inclusions stained with Giemsa (Gurr; BDH Chemicals Ltd., Poole, England) were counted after 24 h of incubation at 37°C (for *C. psittaci*) or after 48 h of incubation at 35°C (for *C. trachomatis*). The percent infectivity was calculated as follows: (number of infectious particles/number of total particles [determined spectrophotometrically]) × 100 (56).

Preparation of PMN granule proteins. Human PMN were collected from patients with chronic granulocytic or myelocytic leukemia at Emory University Medical Center, Atlanta, Ga. Crude granule extracts were prepared in the laboratory of J. K. Spitznagel by extraction of the granules with 0.2 M sodium acetate, pH 4.0, overnight at 4°C (25). The granule proteins were concentrated by ultrafiltration through an Amicon YM-5 filter. A portion of each batch of crude extract was fractionated by Sephadex G-75 column chromatography (35), and the fractions were pooled and concentrated by filtration through an Amicon Y5-05 filter. Each fraction was analyzed as follows. Protein concentra-

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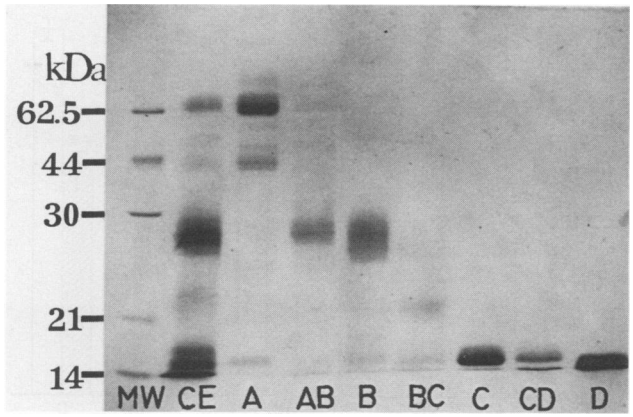

FIG. 1. SDS-PAGE (10% polyacrylamide) of human PMN granule proteins, stained with Coomassie blue. Lane 1 contains protein standards of known molecular mass, expressed here in kilodaltons. Lane 2 contains crude granule extract (CE). Lanes A to D contain fractionated granule proteins. The initial fraction (A) contains predominately myeloperoxidase: the 59.5-kDa heavy chain and the 45- and 15-kDa light chains. The major proteins in successive fractions (lanes) are as follows: cationic proteins (AB); elastase, cathepsin G, and other neutral proteases (B and BC); lysozyme (C); and low-molecular-weight peptides (CD and D).

tion was determined by the method of Bradford (3). Lysozyme content was determined by the lysoplate method of Osserman and Lawlor (33). The 57- and 37-kilodalton (kDa) cationic antimicrobial proteins were detected by enzyme-linked immunosorbent assay; myeloperoxidase was also detected immunologically by using rabbit antibody to human myeloperoxidase (42). Elastase and cathepsin G enzyme activities were measured by the method of Starkey and Barrett (47, 48). Crude extract (50 μg) and each fraction thereof (25 μg) were further analyzed on sodium dodecyl sulfate (SDS)-polyacrylamide gels (10% polyacrylamide) (19), under reducing conditions, stained with 0.25% Coomassie brilliant blue R-250 (Fig. 1). Duplicate gels were stained with silver to visualize more readily the 57- and 37-kDa proteins (44). Portions of the samples, on ice, were air expressed to Chapel Hill, N.C., and stored at 4°C. Prior to use, crude and fractionated granule extracts were dialyzed against phosphate-buffered saline (PBS), pH 7.4.

Purified human PMN lysozyme. Several fraction C portions (Fig. 1) from the same patient were further subjected to Sephadex G-75 column chromatography to obtain purified PMN lysozyme. This lysozyme was analyzed by the lysoplate method of Osserman and Lawlor (33), by the spectrophotometric method of Shugar (45), and by SDS- polyacrylamide gel electrophoresis (PAGE) (10% polyacrylamide) and cationic gels stained with Coomassie blue and silver. In experiments requiring inactivated lysozyme, the method of Laible and Germaine (20) was used. Briefly, 100 μg of the purified lysozyme per ml was incubated in 0.5 mM potassium phosphate, pH 7.0, containing 10 mM dithiothreitol (DTT) for 20 h at 37°C. Mock-inactivated lysozyme was generated by incubation under the same conditions but without DTT. Lysozyme was then dialyzed (MWCO 2000; Spectrum Medical Industries, Inc., Los Angeles, Calif.) against 0.5 mM potassium phosphate, pH 7.0, for 24 h at 4°C, with numerous changes of buffer to remove DTT. Enzymatic activity was assessed before and after inactivation by using the spectrophotometric assay (45). A solution of dried *Micrococcus lysodeikticus* (Sigma Chemical Co., St. Louis, Mo.) in 0.066 M potassium phosphate, pH 6.24, was prepared such that the A_{450} was between 0.6 and 0.7. The decrease in absorbance after addition of lysozyme was recorded for 2 to 4 min. The initial linear rate of the decrease was used to calculate units of activity per milligram of protein by the following equation: $(\Delta A_{450}/\text{minute})/[(0.001 \times \text{milligrams of protein})/\text{milliliters of reaction mixture}]$. In addition, the PMN lysozyme, as well as DTT-treated and mock-treated preparations of it, was analyzed by 6 to 18% gradient SDS-PAGE stained with Coomassie brilliant blue R-250.

Bactericidal assays. Damage to infectious chlamydial EB was assessed by two different methods: (i) a radioisotope assay and (ii) a biological assay. For the radioisotope assay, chlamydial nucleic acid was labeled with [^{3}H]uridine. Aliquots of the internally labeled EB were exposed to crude or fractionated granule extract (0.2 μg of protein per 2×10^3 EB infectious units, equivalent to 250 μg of protein) for 60 min at 37°C. The samples were then placed on ice, and an equal volume of cold 10% trichloroacetic acid (TCA) was added. Precipitates were collected on glass fiber filters (Whatman 934-AH filters) and prepared for scintillation counting as previously described (56). Damage to chlamydiae was defined as the appearance of radioisotope in an acid-soluble form; conversely, radioisotope in an acid-insoluble (TCA-precipitable) form was assumed to remain associated with intact EB (36).

For the biological assay, PMN crude granule extract, fractions thereof (100 μg/ml), or purified PMN lysozyme was incubated with *C. psittaci* or *C. trachomatis* EB of known particle count or IFU titer, at pH 7.4 or 5.5, for 60 min at 37°C with intermittent agitation. The total reaction volume was 200 μl per sample. The organisms were subsequently pelleted by centrifugation in a Microfuge (Beckman Instruments, Inc., Fullerton, Calif.) at 13,000 × *g* for 20 min at 4°C. After the supernatants were removed, the pellets were suspended in 0.2 ml of Dulbecco minimum essential medium and briefly sonicated in a water bath to obtain an even suspension of the chlamydiae, and the EB were titrated on L cell or McCoy cell monolayers.

pH gradient. For some of the later bactericidal experiments, it was essential to establish a pH gradient rather than to use a fixed pH of 7.4 or 5.5. A 100-μl drop of PBS (pH 7.4) was deposited on the surface of a microdialysis membrane filter (type VS, ca. 25-mm diameter; Millipore Corp., Bedford, Mass.), and the filter was floated on 0.01 M sodium phosphate buffer, pH 8.0, in a Pyrex dish (Corning Glass Works, Corning, N.Y.). After 15 min, the pH of the 100-μl drop rose to 7.8. The filter was then transferred and floated on 0.01 M phosphate buffer, pH 6.0, which was less damaging to McCoy cells in the biological assay than was pH 5.5. After a 45-min incubation, the pH of the 100-μl drop decreased to 6.0. In control experiments, EB suspended in PBS were incubated for 1 h in the pH gradient. The suspension droplet was lifted from the filter, and the filter was washed three times with Dulbecco minimal essential medium. The wash fluids were pooled with the original sample, centrifuged, and resuspended in Dulbecco minimum essential medium, and the EB were titrated for IFU as described above. In test experiments, granule extract or purified human PMN lysozyme in PBS (pH 7.4) was mixed with *C. psittaci* or *C. trachomatis* EB, and 100 μl of each suspension was deposited onto the surface of the microdialysis membrane filter. The filters were incubated via the pH gradient and processed as the controls.

Statistics. The one-tailed Student *t* test was used to ana-

TABLE 1. Effect of human PMN crude granule extract on *C. psittaci* and *C. trachomatis* infectivity

Species	Crude granule extract protein concn (μg/ml)	% Inclusions formed (*P*)[a] at:	
		pH 7.4	pH 5.5
C. psittaci	30	75 (0.03)	92 (NS)
	100	70 (0.01)	80 (0.01)
	150	70 (0.05)	82 (0.0001)
C. trachomatis	150	48 (0.001)	ND

[a] EB incubated under identical conditions but in the absence of granule proteins served as controls. Results are calculated on the basis of the number of inclusions in test samples compared with the number of inclusions in controls, expressed as a percentage. Each inclusion percentage is derived from the average of three to six determinations. Significance values (in parentheses) compared the percent inclusions in test samples to the percent inclusions in controls (*t* test for independent means). *P* values less than 0.05 were considered significant. NS, Not significant; ND, not done.

lyze and compare differences in the effects of crude granule extract or of individual fractions thereof on EB infectivity, as measured by (i) percentages of inclusions in L cells or McCoy cells or (ii) percentages of TCA-precipitable ^3H counts. Results for which *P* was <0.05 were defined as significant.

RESULTS

PMN granule proteins. The susceptibility of *Chlamydia* spp. to damage by nonoxidative microbicidal mechanisms was tested first by exposing purified EB to various concentrations of crude extract, pH 7.4, for 60 min at 37°C and then by determining the EB titer for retention of biological activity, i.e., the ability to form inclusions in tissue culture cells. At concentrations of crude granule extract ranging from 30 to 150 μg/ml, there was a significant decrease in *C. psittaci* EB infectivity, resulting in a 25 to 30% decrease in inclusion formation ($P < 0.01$ to $P < 0.05$; Table 1). Since the pH of the phagolysosome is known to decrease considerably over time and since a number of PMN granule proteins have a low pH optimum, the experiments were repeated at pH 5.5. Crude granule extract ≥100 μg/ml also had a significant effect on infectious *C. psittaci* EB at this pH. The acidic conditions were somewhat detrimental to the tissue culture cells, causing them to round up and stain more intensely and thereby making the chlamydial inclusions more difficult to identify. As a consequence, most experiments were carried out at pH 7.4. Crude granule extract was more damaging to *C. trachomatis* than to *C. psittaci* at pH 7.4, causing a 52% reduction ($P < 0.001$) in inclusions in McCoy cells (Table 1).

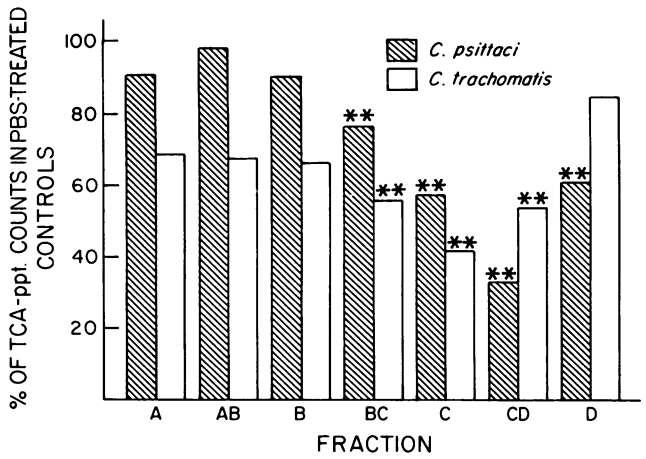

FIG. 2. Effect of human PMN fractionated granule extract on TCA-precipitable counts associated with ^3H-labeled chlamydiae. Radiolabeled organisms were incubated with each fraction (0.2 μg of protein per 2×10^3 EB infectious units) for 1 h at 37°C. After samples were placed on ice, an equal volume of 10% TCA was added to each. EB incubated under identical conditions but in the absence of granule proteins served as controls. Results are calculated on the basis of the number of TCA-precipitable counts in test samples compared with the number of TCA-precipitable counts in controls, expressed as a percentage. Data were analyzed by the *t* test for independent means. **, $P < 0.01$.

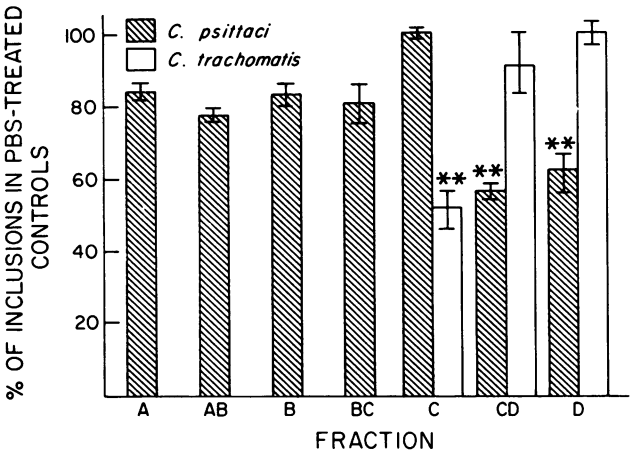

FIG. 3. Effect of human PMN fractionated granule extract on chlamydial infectivity. *C. psittaci* or *C. trachomatis* EB were treated with 100 μg of each fraction per ml at pH 7.4 for 1 h at 37°C and then assessed for inclusion-forming ability in tissue culture cells. EB incubated in PBS under identical conditions but in the absence of granule proteins served as controls. Results are calculated on the basis of the number of inclusions in test samples compared with the number of inclusions in PBS controls, expressed as a percentage. Each bar represents the average of four to eight determinations. Data were analyzed by the *t* test for independent means. **, $P < 0.01$.

Fractionated granule extract. In an attempt to define more specifically which components of the crude granule protein mixture were damaging to these gram-negative bacteria, the chlamydiae were exposed to separate fractions of the crude granule extract at pH 7.4. Damage to infectious EB was assessed by two different methods: (i) a radioisotope assay and (ii) a biological assay. Fractions C, CD, and D consistently caused significant losses in the number of TCA-precipitable counts associated with radiolabeled *C. psittaci*, with the greatest loss occurring in fraction CD ($P < 0.0002$). The results of a single representative experiment are illustrated in Fig. 2; however, each experiment was performed an average of two to six times. When ^3H-labeled *C. trachomatis* was used, the greatest decrease (58%) in TCA-precipitable counts occurred after incubation with fraction C ($P < 0.003$). Fraction C contained predominantly lysozyme, whereas fractions CD and D contained lower-molecular-mass peptides, of approximately 13,000 Da or less.

In the biological assay, the ability of *C. psittaci* and *C.*

TABLE 2. Comparison of the effect of human PMN lysozyme and HEWL on the infectivity of *C. trachomatis*

Lysozyme source	Protein concn (μg/ml)	Unit concn (U/ml)	% Inclusions formed (*P*)[a]
Human PMN	49	17,229	65.7 (0.01)
Hen egg white	49	2,729	54.1 (0.009)
Hen egg white	309	17,229	90.1 (NS)

[a] EB incubated under identical conditions but in the absence of lysozyme served as controls. Results are calculated on the basis of the number of inclusions in test samples compared with the number of inclusions in controls, expressed as a percentage. The results represent the average of at least six determinations. For an explanation of significance values, see Table 1, footnote *a*.

trachomatis to establish productive infection in tissue culture cells was assayed after incubation of EB with fractionated granule extract for 1 h at 37°C (Fig. 3). With *C. psittaci*, fractions CD ($P < 0.01$) and D ($P < 0.01$) had the greatest effect on inclusion-forming ability (38 to 42% reduction). Only fraction C ($P < 0.002$) consistently affected the ability of *C. trachomatis* to form inclusions. For reasons which are not entirely clear, the cultured McCoy cells were more susceptible than were L cells to the proteases in fractions A, AB, B, and BC; thus, reproducible inclusion counts were difficult to obtain. However, the pattern of results obtained in the biological assay was very similar to that obtained in the radioisotope assay.

Purified human PMN lysozyme. Freeze-fracture studies reveal a thin wall layer between the outer membrane and the cytoplasmic membrane of chlamydiae, but chemical analyses detect little (11, 34) to no (1, 13) muramic acid, although D-alanine is present. Binary fission of reticulate bodies within inclusions in infected cells is interrupted in the presence of penicillin (16, 22). Thus, the presence and function of a classical peptidoglycan matrix in chlamydiae are areas of controversy. The finding that *C. trachomatis* infectivity was reproducibly and significantly altered by human PMN lysozyme was somewhat surprising. Thus, we chose to investigate this phenomenon further.

Several fraction C aliquots from the same patient were subjected to Sephadex G-75 column chromatography. The resulting more purified human PMN lysozyme (100 μg/ml) was preincubated with *C. trachomatis* at pH 7.4 for 1 h at 37°C. The exposed EB were then tested for infectivity in McCoy cells. Only 24% ($P < 0.001$) of the organisms were still able to develop inclusions relative to EB incubated for 1 h in PBS. Although all muramidases hydrolyze the β1-4 linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine, muramidases from different sources display differences in specificity for peptidoglycan substrates (37, 49). For comparison, hen egg white lysozyme (HEWL) (Sigma) and mutanolysin (Miles Laboratories, Inc., Elkhart, Ind.) were also tested for their effects on *C. trachomatis* EB. HEWL (pH 7.4) at concentrations of 25, 50, and 100 μg/ml reduced EB inclusion-forming ability to 58% ($P < 0.001$), 51% ($P < 0.002$), and 51% ($P < 0.1$), respectively. Mutanolysin, at 50 μg/ml, reduced EB inclusion-forming ability by 70% ($P < 0.0005$).

Two published studies examining the effect of HEWL on chlamydial infectivity report conflicting results. Kondo et al. (15) found that 2 mg of HEWL per ml consistently produced a significant reduction in infectivity, whereas Welch et al. (55) observed no reduction in infectivity with EB exposed to 6 to 11 mg of HEWL per ml. In the latter study, the concentration of lysozyme in human tears was essentially equivalent to 1.7 mg of HEWL per ml. Since the enzymatic activity of human lysozymes has been reported to be five to eight times that of HEWL (51, 61), and since we also found that to be true (human PMN lysozyme = 194,293 U/mg; HEWL = 41,600 U/mg), we performed one set of experiments in which the effect of a PMN lysozyme standard was compared with that of HEWL at equal protein concentration or equal enzyme unit concentration. When these solutions were incubated with *C. trachomatis* for 60 min at 37°C prior to titration on McCoy cells, only the mixture of HEWL equal in protein concentration to the PMN lysozyme standard, i. e., eight times less enzymatically active, caused an equivalent and significant decrease in infectivity (Table 2).

Since lysozyme is such a basic protein (pI = 10.5 to 11.0), it is possible that its effect on chlamydiae is not a result of muralytic activity but of altered charge phenomena. Thus, we attempted to reverse the effect of lysozyme by stripping it from the bacterial cell surface (6) before testing the infectivity of the treated EB. In one experiment, EB exposed to HEWL (50 μg/ml) were subsequently incubated for an additional 30 min at 37°C with 250 μg of trypsin per ml prior to inoculation onto McCoy cells. This concentration of trypsin does not alter EB infectivity. Infectivity of the lysozyme-treated EB was less than 10% of the control untreated EB; infectivity of the trypsin-treated lysozyme-exposed EB was 5.4% ($P < 0.0005$). Rather than restoring EB infectivity, the trypsin treatment enhanced the effect of lysozyme.

To confirm that the small amounts of lysozyme which contaminated fractions CD and D were not responsible for reductions in *C. psittaci* EB infectivity, the experiments using purified human PMN lysozyme were repeated with *C. psittaci*. Incubation of *C. psittaci* EB with concentrations of lysozyme ranging from 0.01 to 40 μg/ml failed to alter the infectivity of this species; 100% inclusion-forming capability was retained. Similar results were obtained with HEWL (data not shown).

Segal and colleagues (39) and Cech and Lehrer (5) reported that there is an initial transient rise in PMN

TABLE 3. Summary of the effect of the interaction of human PMN crude granule extract and purified PMN lysozyme in a pH gradient on *C. trachomatis* and *C. psittaci* infectivity

Fraction and pH	% Inclusions formed (*P*)[a] by:	
	C. trachomatis	*C. psittaci*
PBS controls		
pH 7.4	100	100
pH gradient	79	68
Crude granule extract (150 μg/ml)		
pH 7.4	52 (0.003)	70 (0.005)
pH gradient	27 (0.0001)	29 (0.00006)
Purified PMN lysozyme (100 μg/ml)		
pH 7.4	24 (0.001)	100 (NS)
pH gradient	12 (0.0005)	100 (NS)

[a] The number of inclusions obtained in tissue culture cells after incubation of chlamydiae in PBS at a fixed pH of 7.4 for 1 h served as the standard 100% control and the control for fixed pH 7.4 experiments with crude granule extract and lysozyme. The number of inclusions obtained after incubation of chlamydiae in PBS in the pH gradient (79 and 68%), readjusted to 100%, served as the control for the effect of the crude granule extract or purified lysozyme on chlamydiae in the pH gradient. For an explanation of significance values, see Table 1, footnote *a*.

phagolysosomal pH before the environment becomes acidic. Narita and colleagues (26, 27) reported that when *C. psittaci* was incubated in buffers at a high pH, a protein-carbohydrate-lipid complex was liberated from the cell surface. Although the release was maximal at pHs 10 to 12, it was also observed at pH 8. Could this envelope-perturbing manipulation unmask an apparently lysozyme-resistant phenotype? Therefore, we devised a micro-pH gradient to mimic conditions more likely to exist in vivo. *C. trachomatis* or *C. psittaci* EB were mixed with PBS, crude granule extract (150 μg/ml), or purified human PMN lysozyme (100 μg/ml), and 100-μl aliquots were subjected to pH gradient incubation. The exposed EB were then assayed for inclusion formation in their eucaryotic host cells. The data are compiled and summarized in Table 3. In control experiments *C. trachomatis* EB, suspended in PBS and subjected to the pH gradient incubation, lost about 21% of their infectivity compared with EB subjected to a 60-min incubation at a fixed pH of 7.4. Inclusion-forming potential dropped from 52% ($P < 0.003$) to 27% ($P < 0.0001$) in crude granule extract-exposed *C. trachomatis* EB during pH gradient incubation compared with incubation at a fixed pH (7.4), and it dropped from 24% ($P < 0.001$) to 12% ($P < 0.0005$) in purified PMN lysozyme-exposed EB. Inclusion-forming potential dropped from 70% ($P < 0.005$) to 29% ($P < 0.00006$) in crude granule extract-exposed *C. psittaci* EB during pH gradient incubation. Yet again, when *C. psittaci* EB were exposed to purified human PMN lysozyme, even under pH gradient conditions, there was no significant alteration of infectivity. These data tend to confirm that one or more granule proteins other than lysozyme, with molecular masses of approximately 13,000 Da or less, were responsible for damage to *C. psittaci*.

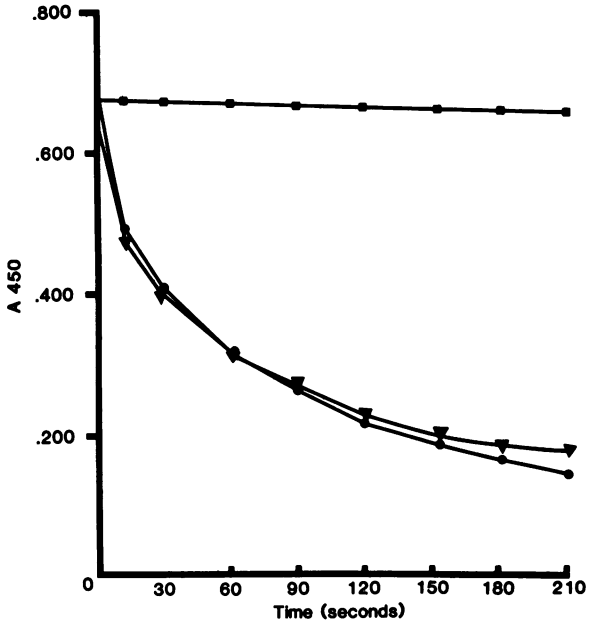

FIG. 4. Enzymatic inactivation of human PMN lysozyme. Purified human PMN lysozyme was incubated in phosphate buffer for 20 h at 37°C in the presence or absence of 10 mM DTT. After dialysis to remove DTT, 4 μg of lysozyme treated with the reducing agent (■) or incubated similarly in its absence (▼) was tested for the ability to decrease the A_{450} of a suspension of *M. lysodeikticus*. For comparison, 4 μg of untreated human PMN lysozyme was also used (●).

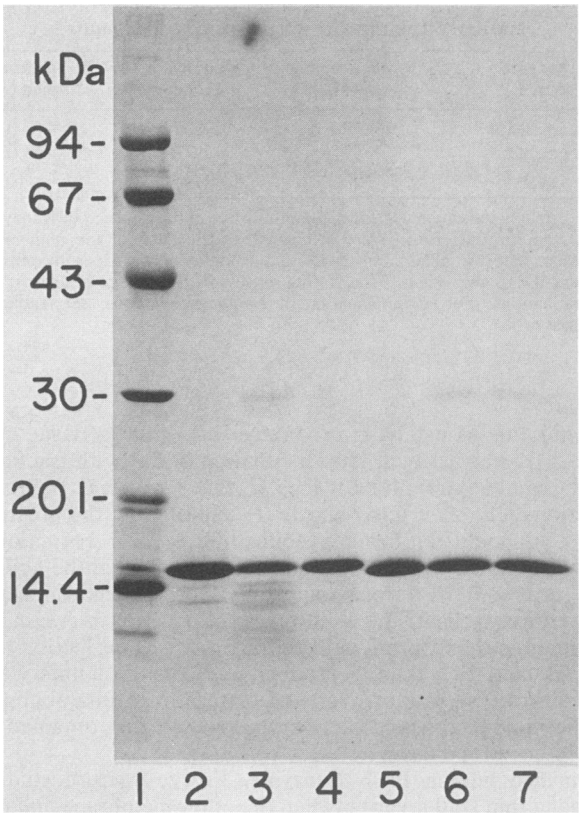

FIG. 5. SDS-PAGE 6 to 18% gradient gel of human PMN lysozyme and HEWL stained with Coomassie blue. Lanes: 1, molecular mass standards (expressed in kilodaltons); 2, mock-inactivated PMN lysozyme; 3, DTT-inactivated PMN lysozyme; 4, untreated, Sephadex G-75 column chromatography-purified PMN lysozyme; 5, mock-inactivated HEWL; 6, DTT-inactivated HEWL; 7, untreated HEWL.

Finally, PMN lysozyme was rendered enzymatically inert by treatment with the disulfide bond-disrupting agent, DTT, by the method of Laible and Germaine (20). When PMN lysozyme was incubated with DTT at 37°C for 20 h, virtually complete loss of muramidase activity resulted (Fig. 4). Mock-inactivated PMN lysozyme, incubated under the same conditions but in the absence of DTT, remained as active as untreated lysozyme. HEWL, treated identically, served as a control, and the results were similar to those obtained with PMN lysozyme (data not shown). Analysis of the lysozymes by 6 to 18% gradient SDS-PAGE revealed a predominant band with a molecular mass of approximately 14,000 Da (Fig. 5). Since lysozyme consists of a single 14,000-Da polypeptide chain, disruption of the four disulfide bridges by DTT does not alter its electrophoretic mobility. The few faint bands below the 14,000-Da human PMN lysozyme bands indicated that the chromatography-purified fraction C lysozyme contained some impurities, which were more visible after dialysis of the samples (Fig. 5, lanes 2 and 3). These minor contaminating proteins were not likely to alter chlamydial infectivity since the data obtained were the same as those obtained with the commercially purified HEWL, in which there were no apparent contaminating proteins.

When the mock-inactivated PMN lysozyme (50 to 75

μg/ml) was incubated with *C. trachomatis* EB, there was a 45% reduction ($P < 0.001$) in EB infectivity in the biological assay. In contrast, 98.2% of the EB exposed to DTT-inactivated lysozyme were still capable of inclusion formation.

DISCUSSION

Interaction of human PMN with *C. psittaci* and *C. trachomatis* for 10 h results in killing of 80 to 95% of the chlamydiae (36). Yong et al. (59) reported that EB of both *C. trachomatis* biovars were located in PMN phagolysosomes within 15 to 30 min postinoculation. This was true when the EB were incubated either in the absence of serum or in the presence of normal or immune sera which had not been heat inactivated. Although fusion of chlamydiae-containing phagosomes with PMN lysosomes has not been demonstrated directly in our studies, we presume that that is what occurred, even though the EB were nonopsonized. In vitro analysis of the effect of fractionated PMN granule components on purified EB allowed preliminary identification of proteins detrimental to chlamydiae: lysozyme (for *C. trachomatis*) and lower-molecular-mass proteins (for *C. psittaci*).

C. psittaci is just one of a host of microorganisms found to be susceptible to low-molecular-mass human PMN granule proteins. Modrzakowski and Paranavitana (24) reported the lethal effects of a 9,000-Da protein on an *Acinetobacter* sp. Subsequently, other investigators documented the sensitivity of *Proteus mirabilis* to a granule protein fraction containing peptides of <14,000 Da (S. Engle, W. Shafer, and J. K. Spitznagel, Abstr., Annu. Meet. Am. Soc. Microbiol. 1985, D117, p. 74). Most recently, Ganz et al. (10) described three cationic peptides with molecular weights of 3,500 or less which were toxic to gram-positive and gram-negative bacteria, *Cryptococcus neoformans*, and herpes simplex virus. Amino acid sequence analysis revealed that these peptides are homologous to a previously characterized family of cationic peptides derived from rabbit granulocytes (40). Although cationicity may be important, it alone does not seem to account for the microbicidal effects of the peptides since there was not a direct correlation between activity and net charge (41). Which, if any, of the aforementioned proteins is (are) responsible for damage to *C. psittaci* remains unclear pending additional fractionation and purification of these components.

A number of other PMN granule proteins display antimicrobial activity. Weiss et al. (54) described a 59-kDa noncatalytic protein that is lethal to *Escherichia coli* and *Salmonella typhimurium* and that appears to alter the permeability of these organisms. Modrzakowski and Spitznagel (25) reported a 37-kDa cationic protein which displayed antimicrobial activity against gram-negative organisms. Shafer and colleagues (43, 44) subsequently isolated this protein, as well as a 57-kDa protein with similar activity. Numerous laboratories have reported that *S. typhimurium* and *E. coli* which have been genetically or environmentally manipulated to decrease expression of their O antigens and core polysaccharides exhibit increasing sensitivity to cationic granule proteins (46, 53). Additional data suggested that, at least for binding of the 37- and 57-kDa proteins, accessibility of anionic pyrophosphate groups of lipid A was responsible for the various degrees of sensitivity in these organisms (43, 44). Since chlamydial lipopolysaccharide (LPS) appears to be most similar to an Re *Salmonella* chemotype which lacks all core polysaccharide except 2-keto-3-deoxyoctulosonic acid (4, 30), one might expect chlamydiae to display similar sensitivity to these cationic granule proteins. The apparent inactivity observed in this study may be explained by a number of possibilities; chlamydial LPS simply may lack the specific sites required for binding of these proteins, or the binding sites may be inaccessible, even though the LPS is truncated. Outer membrane proteins of chlamydiae are extensively cross-linked via disulfide bonds (12, 29). This cross-linking could result in a shielding of potential binding sites on LPS. At least one group of investigators has reported great difficulty in isolating chlamydial LPS unless EB were first reduced and alkylated (4).

The results obtained in our laboratory with *C. trachomatis* and lysozyme are in agreement with some earlier reports but disagree with others. Certain reports in the literature document a damaging effect of lysozyme-containing body fluids on this organism (2, 21), although Mardh et al. (21) found that purified human lysozyme from urine did not inhibit inclusion formation. Differences in experimental conditions, in composition of external milieu, and especially in concentrations of muramidases per EB infectious unit pose difficulties in making valid comparisons of results among different laboratories.

The susceptibility of *C. trachomatis* to human lysozyme may account for its different growth patterns in professional phagocytes versus fibroblasts. The organism does not thrive within lysozyme-rich macrophages and PMN. However, lysozyme-deficient fibroblasts are permissive for the growth of *C. trachomatis*. *C. psittaci* is known to avoid phagolysosome fusion in macrophages (56, 57) and fibroblasts (8) and proceeds to develop mature inclusions. At the present time, there is no evidence that *C. trachomatis* also possesses the ability to escape phagolysosome fusion; the limited growth of *C. trachomatis* in mouse macrophages (17) is consistent with this idea. Upon infection of fibroblasts, *C. trachomatis* develops into one or two large inclusions, even at a high multiplicity of infection, whereas *C. psittaci* is capable of forming numerous inclusions within a single cell. It has been proposed that during early intracellular development *C. trachomatis*-containing endosomes fuse with one another (52, 58). If *C. trachomatis* does promote endosome-endosome fusion, perhaps endosome-lysosome fusion also occurs (59).

It is not clear at present whether any of the low-molecular-weight chlamydiacidal cationic proteins of PMN granules are also found in fibroblasts and human macrophages. However, the absence of phagolysosome fusion in *C. psittaci*-infected macrophages and fibroblasts would protect this organism from their destructive effects. The reasons for the apparent inability of *C. psittaci* to prevent phagolysosome fusion in human PMN are not known. In any case, the PMN appears to be of primary importance in attempting to prevent the establishment and spread of chlamydial infection.

The sequential fusion of specific and then azurophil granules with the phagosomes, combined with changes in the phagolysosomal milieu resulting from increased oxygen consumption, results in a dynamic, rapidly changing intracellular environment. Maximum bactericidal activity is likely to occur only when granule proteins are allowed to interact with one another and with oxygen-dependent bactericidal mechanisms. One excellent example is that of elastase, which has been found to enhance greatly both oxygen-dependent and other oxygen-independent systems (31). The in vitro system used here with fractionated granule extract, for example, may not favor the optimal activity of certain granule proteins that are, nevertheless, toxic in vivo. Progress in our understanding of how the chlamydiae interact

with their eucaryotic host cells will depend a great deal upon our ability to manipulate experimental systems to mimic more closely in vivo conditions.

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