# Reduction in Chlamydial Infectivity by Lysozyme[1] (36974)

LeRoy Kondo,[2] Lavelle Hanna, and Hermine Keshishyan
(Introduced by Ernest Jawetz)
*Department of Microbiology, University of California, San Francisco, San Francisco, California 94122*

The chlamydiae of trachoma-inclusion conjunctivitis (TRIC agents, *Chlamydia trachomatis*) produce infections in man which are prominent in the conjunctiva, a site bathed in fluids rich in lysozyme. It was therefore of interest to determine whether lysozyme might possess an antichlamydial action, similar to its antibacterial action which is believed to have some protective value for the host. Lysozyme acts principally on gram-positive bacteria by cleaving a beta-1,4 glycosidic linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine in the peptidoglycan which is typical of bacterial cell walls. Lysozyme affects gram-negative bacteria to a lesser extent because their lysozyme-sensitive glycopeptide is made inaccessible by being covered with a lipoprotein–lipopolysaccharide layer (1).

Although once considered viruses, chlamydiae are now considered to be gram-negative bacteria which are obligate parasites of eukaryotic cells. Their obligate parasitism is presumably due to a dependence on the host cell for metabolic energy (2). Chlamydiae appear to possess typical bacterial (1) cell walls containing *N*-acetylmuramic acid which has been detected in chlamydial cell walls by some investigators (3, 4) but not by others (5). If chlamydiae were lysed by lysozyme, this would further support the presence of typical bacterial peptidoglycan in their cell wall. Since the unequivocal demonstration of lysis in suspensions of chlamydial particles may be difficult, this study attempted to establish the reduction of chlamydial infectivity in cell culture as a preliminary step in such an approach.

*Materials and Methods. Chlamydial inoculum.* The LB-1 strain (TRIC//GB/MRC-1/G) (6) of inclusion conjunctivitis agent, derived from a genital isolate in London, was employed in the sixtieth passage (7). It was propagated in 7-day-old embryonated eggs, the yolk sacs of which were harvested when 50% of eggs had died on Day 12. A 50% yolk sac suspension, suitably diluted in Eagle's minimum essential medium (MEM) with 10% added fetal calf serum and 2% dextrose (maintenance medium), served as the inoculum for cell culture.

*Cell culture.* Strain L 929 cells were grown in prescription bottles in MEM with 10% fetal calf serum without added antibiotics. Cells were scraped from the glass walls and suspended in the same medium in a density of 25,000 cells/ml. Leighton tubes containing coverslips were seeded with 1 ml of suspension and incubated at 35° for 48 hr. At that time, the coverslips were covered by a monolayer of healthy appearing L cells.

*Lysozyme preparation.* Hen egg white lysozyme (20,000 units/mg; 3× crystallized, dialyzed, and lyophilized) was obtained from Sigma Chemical Co. It was dissolved in Tris buffer in a concentration of 4000 μg/ml, then filter-sterilized through an 0.45 μm membrane. The plain tris(hydroxymethyl)aminomethane (Tris) buffer (Sigma Chemical Co.) (pH 8.0) served as control.

*Experimental procedure.* Monolayers of L cells were infected with varying dilutions of LB-1 suspensions mixed either with Tris buffer, or with lysozyme in Tris buffer (final lysozyme concentration: 2000 μg/ml). The mixtures were held at 37° for 1 hr, then 0.4

[1] Supported in part by grants from the NIH (EY 00186 and AI 00299) and from the Burroughs Wellcome Fund.

[2] Submitted in partial fulfillment of the requirement for the MA degree in Microbiology, University of California, San Francisco.

ml of the mixture was inoculated into each Leighton tube and permitted to adsorb for 3 hr at 20°. The inocula were then removed, the monolayers were washed twice with Hanks' basic salt solution, 1 ml of maintenance medium was added, and the tubes were incubated at 35° for 48 hr. At the end of this incubation period, the coverslips were removed from the tubes, washed twice in 0.01 *M* phosphate buffered saline (pH 7.1), and fixed in alcohol–formalin (1:9 v/v plus 0.05% calcium acetate) for 15 min. The coverslips were then removed and covered with absolute methanol for 30 min. For staining, the methanol was removed and replaced with 5% iodine in absolute ethanol for 20 min. The coverslips were then placed, cell sheet down, on a glass microscope slide with 1 drop of glycerine–iodine mounting fluid [equal parts of glycerine and of a solution of 10 g KI, 10 g $I_2$, 50 ml 95% ethanol, and 50 ml distilled water (personal communication, A. Quan)].

The wet-mount iodine-stained coverslips were examined with 200× high dry objective for the presence of darkly stained inclusions within the cytoplasm of L cells. Ten fields were counted on each coverslip and 5 coverslips were examined for each of the 3 dilutions of LB-1 which had been exposed to Tris buffer, or to lysozyme.

*Results and Conclusions.* The mean values of the number of inclusions per field were calculated for each treatment group and submitted to computer analysis using a hierarchical design (8). This method ("nested design") involves the comparison of the two fixed treatment group means, taking into consideration the random variation of mean slide counts within treatment groups and the random variation of counts on slides within each treatment group. An *F* ratio was computed which represents the variation due to treatment, divided by the random count variation. The greater the *F* ratio, the more significant the difference between treatment group means. An example of the results of experiments analyzed in this fashion is given in Table I.

The only known variable between control and experimental groups is the presence of hen egg white lysozyme. While the lysozyme failed to inactivate a majority of the infective chlamydial particles, it consistently produced a significant reduction in chlamydial infectivity. This supports other chemical evidence that the cell walls of infective chlamydial particles do contain glycopeptide (3, 4, 9) and therefore are, in fact, structurally very closely related to the cell walls of bacteria. The limited degree of inactivation, however, suggests that lysozyme alone will not suffice to prevent ocular chlamydial infection, and if lysozyme is implicated at all in a "protective defense mechanism" against chlamydiae, it must operate in conjunction with other tear constituents (10).

TABLE I. Effect of Lysozyme on Chlamydial Infectivity in Cell Culture.

| LB-1 inoculum dilution | Mean no. of inclusions/field | | *F* ratio | *p* value |
|---|---|---|---|---|
| | Tris control | Lysozyme | | |
| 1:500 | 47 | 34 | 13.96 | <0.01 |
| 1:1000 | 30 | 22 | 7.37 | <0.05 |
| 1:1500 | 14 | 9 | 19.16 | <0.01 |

The authors are grateful to Dr. David C. Heilbron for performing the statistical analysis of the experimental data.

1. Salton, R. J., "The Bacterial Cell Wall," 293 pp. Elsevier, New York (1964).

2. Moulder, J., "The Psittacosis Group as Bacteria," 95 pp. Wiley, New York (1964).

3. Jenkin, H. M., J. Bacteriol. **80**, 639 (1960).

4. Perkins, H. R., and Allison, A. C., J. Gen. Microbiol. **30**, 469 (1963).

5. Manire, G. P., and Tamura, A., J. Bacteriol. **94**, 1178 (1967).

6. Hanna, L., Merigan, T. C., and Jawetz, E., Proc. Soc. Exp. Biol. Med. **122**, 417 (1966).

7. Jawetz, E., and Hanna, L., Proc. Soc. Exp. Biol. Med. **105**, 207 (1960).

8. Winer, B. J., *in* "Statistical Principles in Experimental Design" (N. Garmezy, R. L. Solomon, L. V. Jones and H. V. Stevenson, eds.), 2nd ed., p. 184. McGraw-Hill, San Francisco (1971).

9. Tribby, I. I. E., J. Bacteriol. **104**, 1176 (1970).

10. Mull, J., and Peters, J. H., *in* "Trachoma and Related Disorders" (R. L. Nichols, ed.), p. 211. Excerpta Medica, Amsterdam (1971).

Received Sept. 1, 1972. P.S.E.B.M., 1973, Vol. 142.