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Original Articles

The Chemiluminescence Response of Normal Human Leukocytes to Chlamydia trachomatis

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The aim of the present study was to investigate the phagocytic response of normal human polymorphonuclear leukocytes (PMN) and monocytes (MN) to eight serotypes of C trachomatis (B,C,D,E,F,I,J, and L2) using a chemiluminescence (CL) assay, with luminol and lucigenin as amplifiers.

The magnitude of the phagocytic cell CL response was proportional to the phagocyte-to-chlamydiae ratio, with a poor CL response detected at a ratio of 1:125 and progressively larger CL responses up to ratios of 1:50,000. The durations of the CL responses to all chlamydiae serotypes tested were considerably longer than that for zymosan. The PMN demonstrated a relatively greater CL response to all chlamydiae serotypes tested when compared with MN. The PMN and MN CL responses to "genital serotypes" (D, E, F, I, and J) (as well as lymphogranuloma venereum serotype L2) were greater than that for "ocular" serotypes (B and C). Inactivation of serum complement and specific chlamydial antibody absorption reduced the CL responses of both PMN and MN.

This is the first study to characterize the CL responses of normal human PMN and MN cells to C trachomatis, and it indicates the important role of oxygen dependent antimicrobial systems in the phagocytosis of this common human pathogen.

Key words: chemiluminescence, human leukocyte, Chlamydia trachomatis

INTRODUCTION

Chlamydiae are a group of obligate intracellular micro-organisms which are increasingly recognised as human pathogens. They have been implicated in a variety of infections including trachoma, nongonococcal urethritis (NGU), pneumonitis [1], perihepatitis, meningitis, endocarditis [2], and Reiter's syndrome [3] as well as being recently implicated in the pathogenesis of anterior uveitis [4].

Chlamydiae have the capacity to provoke both acute and chronic inflammatory responses. Polymorphonuclear leukocytes (PMN) are the first cells to be detected histologically in response to primary ocular and genital tract infections in man [5]; however, the role of these phagocytic cells in the initial phase of chlamydial infection in the normal host has not been extensively investigated. Similarly, Taverne and Blyth [6] postulated that the degenerative changes seen in cells during chronic ocular trachoma may be due in part to damage by macrophages following ingestion of chlamydiae. Such epithelial cell damage may be due to oxygen radicals released by phagocytic cells following phagocytosis of elementary bodies, or the direct effects of the chlamydiae on infected cells. However, the role of oxygen radicals in the phagocytosis of C trachomatis remains uncertain. A previous report examining the PMN phagocytic response to C trachomatis [7] indicated that oxygen-dependent antimicrobial systems were not essential for the chlamydicidal activity of PMN. The present study was undertaken to investigate further the role of the oxygen-dependent systems in the phagocytosis of chlamydiae by PMN and MN cells using a chemiluminescence assay. This assay is a sensitive measure of the light emitted during the metabolic events occurring in phagocytosis and is dependent on the inter-

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action of luminol and other amplifiers with oxygen species generated during the respiratory burst.

The results of the present study indicate a significant generation of CL from both PMN and MN during the phagocytosis of C trachomatis and lead us to postulate that oxygen-dependent mechanisms are involved in this interaction.

MATERIALS AND METHODS

Phagocytic Cells

Peripheral blood was obtained from healthy adults who had no previous history of chlamydial infection and no detectable serum antibodies to C trachomatis. The PMN and MN cells were separated from 30 ml of heparinised blood utilising Ficoll-Paque centrifugation [8] followed by dextran sedimentation. Briefly, 30 ml blood was diluted with 60 ml phosphate-buffered saline (Ca^{2+} Mg^{2+} free) (PBS) [9] and 20 ml layered onto 10 ml Ficoll-Paque (Pharmacia Fine Chemicals, Upsala, Sweden) in 50 ml centrifuge tubes (Corning Glass Works, Corning, NY) and centrifuged at 600 × g for 30 min. The interface containing the MN was removed as well as excess Ficoll-Paque. These cells were washed three times in PBS and suspended in Hank's balanced salt solution (HBSS) at a concentration of 1×10^6 ml.

To the bottom layer of the Ficoll-Paque separation, containing PMN and red blood cells, was added 10 ml PBS and 3 ml of 3% dextran T500 in saline (Pharmacia Fine Chemicals, Upsala, Sweden). These cells were mixed and placed in a 37°C water bath for 40 min. The buffy coat was removed and centrifuged at 200 × g for 10 min, after which the remaining red cells were lysed with 10% PBS in distilled water for 30 seconds. The PMN were washed three times in PBS containing 15% foetal calf serum (FCS). After the final wash, PMN were counted using a trypan blue stain and suspended in HBSS at a concentration of 1×10^6 PMN/ml. Preparations of PMN demonstrated 95–97% purity and viability.

Opsonising Sera

Sera collected from ten healthy adults, who had no antibodies to C trachomatis, were pooled and stored in 5-ml volumes at −85°C until required. Inactivation of various complement components was achieved by one of three methods: heating at 56°C for 30 min (total complement inactivation) and heating at 50°C for 30 min (inactivation of factor B, alternative pathway of complement). Classical complement pathway inactivation was achieved by incubating serum at 37°C in the presence of 10 mM Mg-EGTA.

In a separate set of experiments, specific chlamydial antibody was absorbed from high-titre patient serum (micro-IF >640) by incubating the sera at 37°C for 60 min with the corresponding C trachomatis elementary bodies. After centrifugation at 30,000 × g for 60 min to precipitate the elementary bodies, the absorbed sera were filtered through 0.2-μm filters (Millipore Corp., Bedford, MA) before use.

Chlamydia trachomatis

C trachomatis serotypes A/MRC-17(G-17)/OT, B/HAR-2(SA-2)/OT, C/TW-3/OT, D/MRC-1(G-1)/OT, E/LB-4/ON, F/Cal-9(IC-Cal-3/ON, I/UW-12/Ur, and J/UW-36/Cx were grown in yolk sacs of developing chick embryos. L2/SA-f was grown in L cells. Since only one suspension of each serotype was used throughout the study, each is referred to by its laboratory designation or serotype.

Purification of C trachomatis Suspensions

Suspensions of yolk sacs from uninfected live 17-day-old chick embryos, uninfected L and HeLa 229 cells, and heavily infected suspensions were semi-purified by treatment with 2 M KCl [10], and suspended in PBS [11]. Pellets of C trachomatis were resuspended in small volumes of sucrose potassium glutamate saline (SPG) [12] and stored at −60°C. These were further purified by substituting Angiografin (Schering Pty. Ltd.) for renografin in the method of Howard et al [13]. Preparations were layered on 17.5% angiografin and centrifuged at 24,000 × g for 2 hours in a 42.1 rotor of a Beckman ultracentrifuge (Model L5-65). Pellets were resuspended in PBS, sonicated, layered on 28.5% angiografin, and centrifuged again. Pellets were resuspended in 30 ml PBS and centrifuged 10,000 × g, 30 min at 4°C in a Lourdes B-fuge model A-2. Final pellets were resuspended in small volumes of PBS or 15% egg albumin-glycerol 1:1 (EAG) [14], sonicated for 60 seconds at 4°C and stored in small aliquots at −20°C. Following this preparation, elementary bodies of C trachomatis were noninfective as measured by infectivity assays in HeLa cells.

Elementary bodies were counted by Giemsa stain darkfield microscopy [15]. Bacterial contamination was not detected. For use in CL assays, suspensions were thawed at 37°C and diluted with HBSS to obtain the required ratio of C trachomatis to leukocytes.

Titration and Serotyping of Antibodies to C trachomatis

Antibodies in human sera against C trachomatis were identified with a micro-immunofluorescence assay (micro-IF) test [16] using purified elementary body suspensions grown in yolk sac or HeLa 229 cells and suspended in EAG [14]. Fluorescein isothiocyanate conjugated sheep-anti-human immunoglobulins prepared against all immunoglobulins were obtained from Wellcome, Australia; those prepared against the major immunoglobulin classes in human sera were obtained from Dakopatts.

CL Assay Procedure

All CL measurements were performed on a Packard 2660 liquid scintillation counter (LSC) (Packard Instrument Co., Downers Grove, IL) using our previously described methods [17]. Minor modifications to this method included a reduction of the reaction vial volume to 800 μL. To each vial was added 400 μL PBS, 200 μL PMN or MN, 50 μL luminol (10^{-4} M) (Sigma Chemical Co., St. Louis, MO) or 50 μL lucigenin (10,10'-dimethyl-biacridinium dinitrate, 10^{-4} M; Sigma), 100 μL pooled normal serum, and 50 μL of the activating agent. Cells, buffer, serum, and luminol were preincubated at 30°C for 10 min prior to the addition of the activator. Each measurement was performed on duplicate vials.

The CL measurements were made automatically for 6-second intervals on each of ten vials up to a total of 200-min duration, with a constant amount of agitation between measurements. The temperature was maintained at a constant 30 ± 0.2°C by a thermostatically controlled device installed inside the LSC (AST Electronics, Sydney, Australia). Assay vials (standard 20-mm scintillation vials) were preheated to 30°C. Vials were not dark-adapted nor were assays carried out in a darkened room (except for the removal of fluorescent lights) because these factors were found not to influence luminol-assisted CL (unpublished observations).

Data Analysis

The CL responses as measured in counts per minute (cpm) were recorded on line to a Hewlett-Packard 85 computer (Hewlett-Packard), which provided a visual curve and six-parameter analysis based on peak height, time to peak, initial slope, maximum slope, integral to peak height, and total integral counts. Statistical analysis was performed on data using the Student's t-test.

RESULTS

Incubation of chlamydiae with both PMN and MN resulted in a significant increase in luminol-enhanced CL. Decreasing the ratio of phagocytes to chlamydiae from 1:125 to 1:50,000 caused a corresponding increase in the peak height and integral counts. With reducing numbers of chlamydiae, the time to reach peak height increased considerably (Fig. 1). Chemiluminescence responses could not be detected with less than 125 chlamydiae per phagocyte. In order to investigate the maximum luminol-enhanced CL responses of phagocytic cells to C trachomatis, all serotypes were tested at ratios of phagocytes to chlamydiae up to 1:50,000. For reasons of economy of chlamydial suspensions, and since the CL responses were consistent and reproducible, all subsequent measurements were made at a ratio of 1:1,250. Each chlamydial serotype was measured at this ratio, and Figure 2 shows

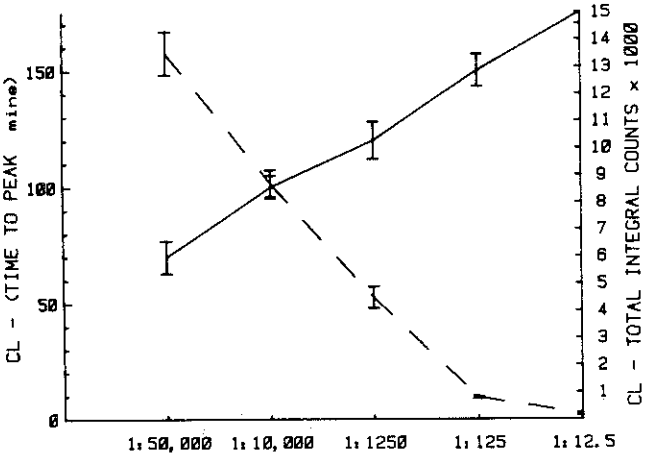

Fig. 1. Effect of reducing phagocyte-to-chlamydiae ratio on total integral CL (---) and time to peak height (—). Each point is the mean of four individual experiments with PMN cells using a single strain of C trachomatis (serotype F). Bars indicate mean ± SE of the mean.

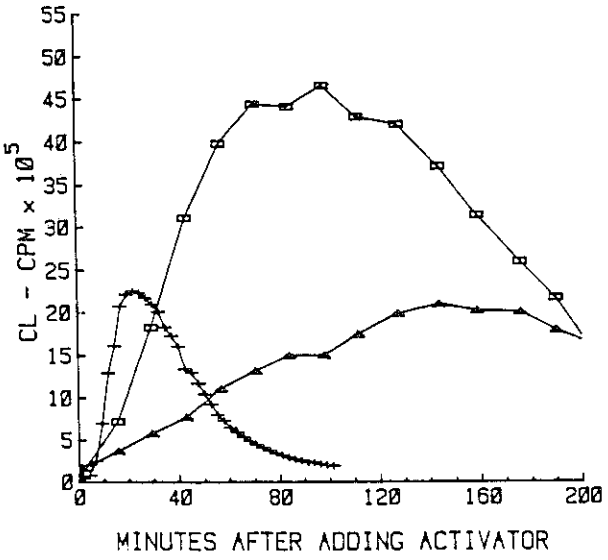

Fig. 2. Comparison of normal PMN CL response to zymosan (ratio PMN:zymosan, 1:500) (++), and two serotypes of C trachomatis at a ratio of PMN to chlamydiae of 1:50,000. The respective ratios were used since they gave the maximum CL responses for zymosan and chlamydiae. Shown are two serotypes which produced a wide variation in CL response. □—□, F serotype (genital isolation); ▲—▲, C serotype (ocular isolation).

representative curves for two serotypes (C and F), and for comparison the response to zymosan (500 particles per phagocyte) is included. The two serotypes represented indicate the large variation of CL responses within which the eight serotypes tested fell. Normal yolk sac suspen-

sions and HeLa 229 cells used for culturing chlamydiae were included as a control; however, they failed to evoke a CL response.

The CL responses of MN to several serotypes of C trachomatis in the presence of either luminol or lucigenin are shown in Figure 3. All serotypes tested produced a greater CL response with luminol than those using lucigenin as an amplifier. All following experiments utilised luminol-amplified CL.

The peak heights of luminol-amplified PMN CL responses activated by seven different serotypes of chlamydiae are shown in Figure 4a and the total integral counts for the same serotypes are shown in Figure 4b. Figure 4c indicates the relative difference between PMN and MN responses to the same serotypes of C trachomatis and at the same phagocyte:chlamydiae ratio. The PMN CL responses were greater than those for MN to each serotype tested ($P < 0.01$). The times to achieve peak heights were not altered for the seven serotypes tested. The CL responses produced in response to genital serotypes (E, F, I, and J) (as well as lymphogranuloma venereum serotype L2) were greater for both MN and PMN cells when compared with the CL responses evoked by ocular serotypes of chlamydiae (serotypes B and C) (Fig. 4a). Time to peak height for the CL response consistently occurred earlier with genital serotypes when compared with ocular serotypes. The integral counts (Fig. 4b) showed similar results.

The effect of various serum treatments on both PMN and MN CL responses to a single serotype of C trachomatis is shown in Figure 5. Compared with the CL response in normal pooled serum, heat inactivation (56°C) reduced the response an average of 52% for PMN and 35% for MN. Similar decreases were produced by Mg-EGTA (PMN 38%, MN 42% of controls) and heating serum at 50°C for 30 min (PMN 52%; MN 35% of controls). Absorption of chlamydial serum antibodies reduced the luminol-enhanced CL responses of PMN and MN to C trachomatis by 72% and 61%, respectively, compared with unabsorbed high titre serum.

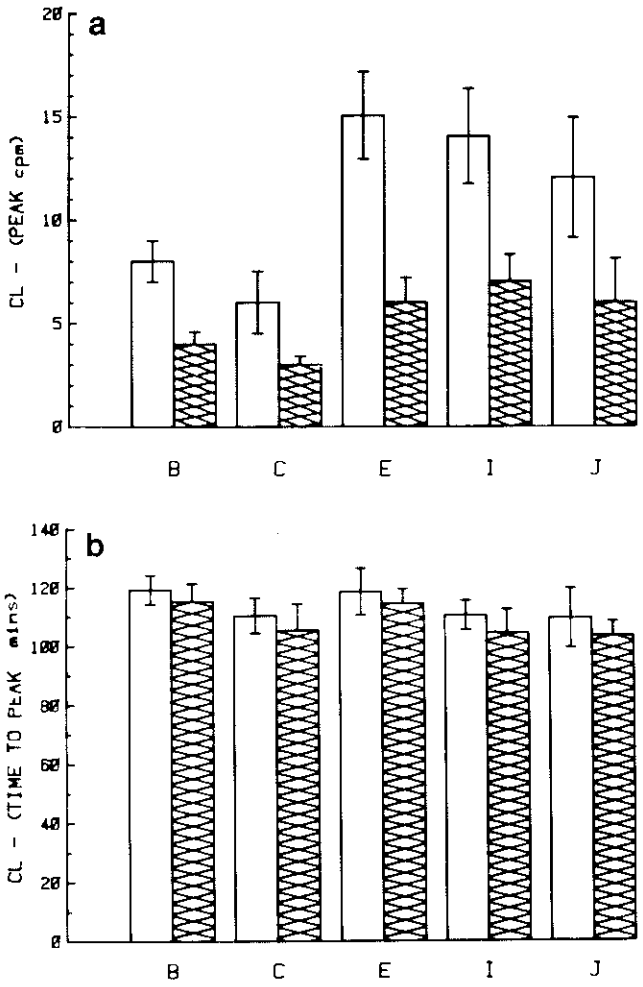

Fig. 3. Open histograms show luminol CL and hatched histograms lucigenin-enhanced CL responses of MN to five serotypes of C trachomatis (ocular serotypes B and C, and genital serotypes E, I, and J). a. Peak CL for each serotype. b. Times for peak heights. Bars indicate means ± 1 SE of the mean. The differences in peak responses were significant for each serotype tested when comparing luminol- and lucigenin-amplified CL ($P < 0.05$). Differences in time to peak were not significantly different between either luminol- or lucigenin-enhanced MN CL, indicating similar kinetics.

DISCUSSION

Despite evolving awareness of the prevalence of chlamydial infections, little is known of the cellular mechanisms involved in the phagocytosis and intracellular killing of this microorganism by phagocytic cells. Polymorphonuclear leukocytes are involved in the local tissue response to C trachomatis and may therefore be important in the initial phagocytosis and elimination of this microorganism at mucosal surfaces [18]. The role of oxygen radicals in the intracellular destruction of these micro-organisms is not well established.

A previous study by Yong et al [7] concluded that the oxidative microbicidal mechanisms of PMN were not essential in the destruction of C trachomatis. However, under the conditions of the present study, both PMN and MN cells were found to exhibit a significant CL response, indicating a possible role for oxygen radicals in the phagocytosis of C trachomatis. The present study differed in several ways from that of Yong et al. Firstly, we used both PMN and MN cells and measured a direct phagocyte response rather than an indirect assay. Secondly, several serotypes of C trachomatis representative of both ocular and genital strains were compared. Thirdly, the phagocytic cells were not exposed to extreme conditions of centrifugation (up to 60 min) used in the Yong et

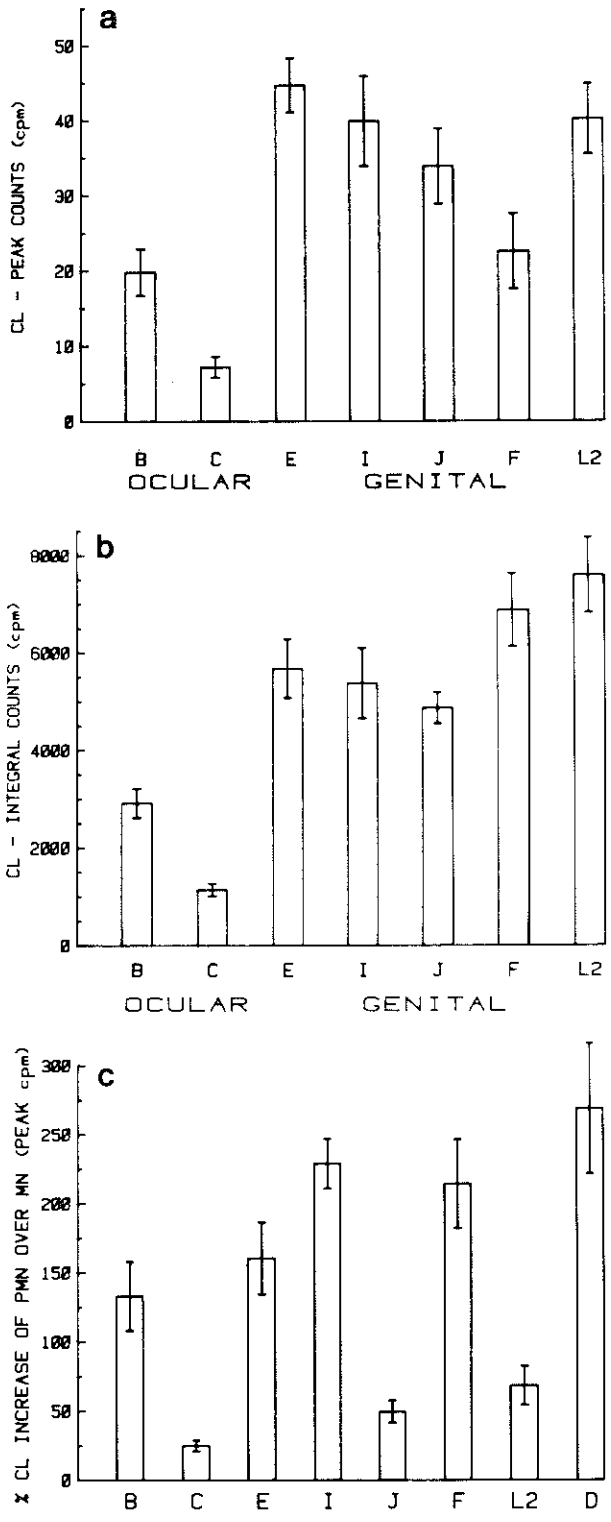

Fig. 4. PMN CL responses to several different serotypes of C trachomatis. a. Peak heights. b. Total integral counts. c. A comparison of PMN and MN response showing percentage increases of the PMN CL response compared with the MN CL response to the same serotype and ratio of chlamydiae. Shown also is the difference between isolates of C trachomatis from either ocular (serotypes B, C) or genital infections (serotypes E, I, J, F, and L2). Differences between PMN and MN responses were significant for each serotype ($P < 0.05$): however there was a large range in the responses.

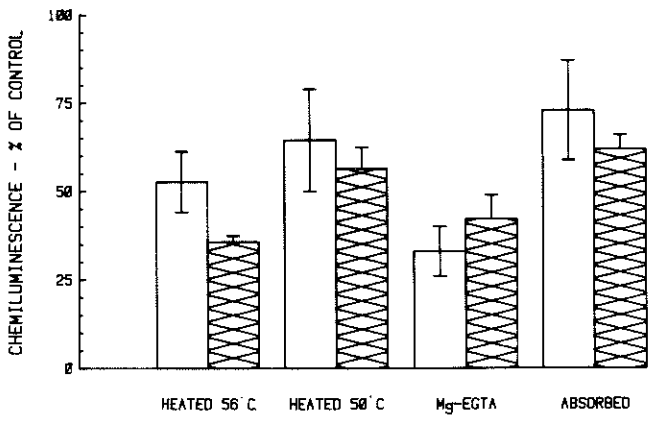

Fig. 5. The peak CL responses of PMN (open histograms) and MN (hatched histograms) after treatment of serum as described. Each treatment is compared with the response of C trachomatis in the presence of pooled normal serum. Each determination was made using the one serotype of C trachomatis (F serotype) and phagocytes isolated from five individual subjects. In the case of absorption experiments, serotype F was used to absorb specific chlamydial antibodies and CL measured to that same serotype. Bars indicate means ± 1 SE.

al study, which may have been destructive to the functional integrity of phagocytes.

The results of the present study outline several unusual features of the CL response of phagocytic cells to a variety of serotypes of C trachomatis. A major difference between the CL response to chlamydiae and that of other micro-organisms appears to be the lack of a measurable response at ratios of phagocytes to chlamydiae of less than 1:60. We have previously demonstrated that a good CL response can be obtained at this ratio using numerous other micro-organisms [16]. It is possible that as a result of the small size of chlamydiae (250–300 nm), collisions with individual phagocyte membranes are less frequent and thus any oxidative response is undetectable using the LSC. Alternatively, a larger number of chlamydiae (compared with bacteria) may be required to bind to the phagocyte membrane in order to activate the membrane oxidase sufficiently to induce the CL response. The CL response to C trachomatis was characterized by an initial lag period followed by a prolonged response over a period of several hours. This pattern of response has not been observed with other intracellular micro-organisms and may represent a unique interaction between the phagocyte membrane receptors and the elementary body of C trachomatis [19].

The larger CL response of PMN to C trachomatis when compared with the MN CL response is similar to that observed in the CL response to a number of other pathogenic micro-organisms [20] and reflects the ability of PMN to mount a more rapid and effective phagocytosis and killing reaction compared with the MN.

The kinetics of the interaction between chlamydiae and phagocytic cells may depend upon particular cell wall biochemical characteristics of the invading micro-organism as, for example, the major outer membrane proteins of C trachomatis [21]. It is likely that there is a unique process by which chlamydiae are bound and internalised by phagocytic cells and that this explains the large number of chlamydiae per cell required to induce a measurable CL response, and the prolonged CL response of PMN and MN to C trachomatis when compared with that induced by bacteria, viruses, and zymosan. We have reported both peak height and total integral counts as an indication of the total responsiveness of phagocytes to C trachomatis. Because of the prolonged activation time, the integral data may more accurately reflect the total oxidative potential of phagocytes activated by C trachomatis.

Genital serotypes of C trachomatis produced phagocytic cell CL responses several times the magnitude of that produced by ocular serotypes. This may reflect differences between these micro-organisms in a component(s) of the cell wall of the elementary bodies which promote interactions at the level of the initial phagocytic event that induces CL. One particular ocular serotype, C, was a very poor activator of CL. This may be of considerable interest, since serologically this micro-organism is closely related to J serotype which induced a strong CL response. The comparative inability of phagocytic cells to mount an effective CL response to the ocular serotypes of C trachomatis may be an important factor in the pathogenesis of the primary infection and continual reinfections seen with diseases such as trachoma.

Luminol and lucigenin are reported to utilise different oxygen species and therefore measure different reactions in the metabolic pathway of respiratory burst. Luminol is oxidised by a mechanism involving the MPO-H_2O_2-Cl system in phagocytes whereby MPO mediates the formation of intracellular HOCl [22]. Lucigenin appears to operate by a different mechanism. McCapra and Hann [23] proposed that reduced lucigenin reacted with singlet molecular oxygen yielding a dioxetane product whose subsequent decomposition yielded light from excited carbonyl groups. Allen [24] suggested that the superoxide anion serves as the univalent reducing agent for lucigenin. The data presented here for chlamydiae indicate that despite similar PMN and MN CL kinetic patterns, the luminol-enhanced CL responses were of a greater magnitude than those of lucigenin. This may be related to the nature of the phagocyte membrane interaction produced during chlamydial phagocytosis. The differences observed between luminol and lucigenin in CL responses induced by a number of serotypes may result from the type of recognition signals evoked by the chlamydiae, or be due to the inaccessibility of the amplifier to the site of production of reactive oxygen species produced. The increased luminol-enhanced CL may result from greater accessability of this amplifier to appropriate oxygen species or may reflect the fact that the MPO-H_2O_2-Cl system is preferentially activated during chlamydial phagocytosis.

The role of opsonins in the phagocytosis of C trachomatis has not been extensively studied. Our preliminary results implicate both the classical and alternative pathways of complement activation in the phagocytosis of C trachomatis. Heating serum to inactivate complement significantly reduced the CL response of both PMN and MN cells. The absence of factor B (alternative pathway) and Ca^{2+} (classical pathway) significantly reduced the CL responses of both PMN and MN cell types to a greater extent than removal of specific chlamydial antibody. These data suggest that the opsonic requirements for efficient phagocytosis and killing of C trachomatis appear to involve both complement components as well as specific chlamydial antibody.

The present study has defined the characteristics of the CL response of normal human PMN and MN to eight serotypes of C trachomatis and provides the methodology for the further investigation of the phagocytic response to these important micro-organisms.

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