Induction of DNA Strand Scissions in HeLa Cells by Human Polymorphonuclear Leucocytes Activated by Chlamydia trachomatis Elementary Bodies

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Incubation of human polymorphonuclear leucocytes (HPMN) with Chlamydia trachomatis elementary bodies (EB) or phorbol 12-myristate 13-acetate (PMA) resulted in the production of superoxide anions (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$). Exposure of HeLa cells to EB- or PMA-activated HPMN and to EB alone, for 2 h, resulted in the formation of DNA strand scissions (nicks) in the HeLa cells. The nicks were visualized by incorporation of biotin 11-dUTP with its detection by streptavidin-peroxidase, and quantified by using [3H]dCTP in the in situ nuclear nick-translation reaction. Catalase, and to a lesser extent superoxide dismutase, reduced the amount of nicks induced by the EB- or PMA-activated HPMN. The possible relationship between the activity of PMN in chlamydial infections and the development of chronic diseases is discussed.

INTRODUCTION

Members of the genus Chlamydia are obligate intracellular Gram-negative bacteria with a genome of 660 MDa (Sarov & Becker, 1969), which parasitize the host cell for nutrition and energy (Schachter & Caldwell, 1980). Different serovars of chlamydia have been associated with clinically distinct infections ranging from hyperendemic trachoma to sexually transmitted infections (Schachter, 1978). Chlamydial infection can be persistent, and recurrent infection commonly occurs (Hanna et al., 1968; Oriel & Ridgway, 1982). Associations have been described between chlamydial infection and chronic infections (Henry-Suchet et al., 1981), rheumatic diseases (Keat, 1986) and cervical neoplasia (Piura et al., 1985).

Histopathological studies have shown human polymorphonuclear leucocytes (HPMN) to be the predominant inflammatory cells which infiltrate the site of early infection with Chlamydia trachomatis (Monnickendam & Pearce, 1983). It was further shown that elementary bodies (EB) of the L2 serovars of C. trachomatis could bind to HPMN (Bard & Levitt, 1986), be phagocytosed and inactivated by these cells (Yong et al., 1982; Zvillich & Sarov, 1985), activate complement and stimulate chemotaxis of HPMN in vitro (Megran et al., 1985), and induce a chemiluminescent response in the HPMN (Soderlund et al., 1984; Zvillich & Sarov, 1985).

In the present study we examined the ability of C. trachomatis EB to activate the oxidative burst of HPMN to produce superoxide anion (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$), and the probable role of these radicals in inducing DNA strand scissions in the nuclei of co-cultivated HeLa cells.

Abbreviations: EB, elementary bodies; HBSS, Hanks' balanced salt solution; IFU, inclusion-forming units; HPMN, human polymorphonuclear leucocytes; PMA, phorbol 12-myristate 13-acetate; RU, relative units; SOD, superoxide dismutase.
METHODS

Growth conditions and purification of chlamydial EB. EB of C. trachomatis biotype lymphogranuloma venereum (L2/434/Bu), were grown on BGM cells (Flow Laboratories) and purified 48–72 h post-infection by a modification of the method of Caldwell et al. (1981) as previously described by Zvillich & Sarov (1985).

Immunoperoxidase assay for titration of C. trachomatis. C. trachomatis was titrated on BGM cells as described by Shemer & Sarov (1984). The cells were seeded at 5 × 10^6 per well in 96-microwell plates (Nunc). Twenty-four hours later, the cells were infected in triplicate with 10-fold dilutions of purified C. trachomatis. The progress of infection was followed by light microscopy: at 48 h post-infection, the cells were fixed with 100% ethanol, and an immunoperoxidase assay test was performed. The staining appeared in the inclusions as well as on the membrane of C. trachomatis-infected cells.

Isolation of HPMN. Heparinized whole blood was obtained from healthy human donors, diluted twofold with RPMI-1640 medium, layered onto Ficoll-Hypaque (Pharmacia; 1.077 g cm^-3 at 25 °C), and centrifuged at 400 g for 30 min, according to the method of Boyum (1968). The pellet was resuspended in an equal volume of 6% (v/v) dextran in RPMI-1640 medium. The erythrocytes were allowed to sediment at 37 °C for 30 min and the HPMN were removed and centrifuged for 5 min at 400 g. Contaminating erythrocytes were removed by osmotic shock. The HPMN (99% pure) were washed three times and resuspended in RPMI-1640 containing 10% (v/v) foetal calf serum. The viability was 99% as determined by 1% (w/v) trypan blue staining. After 24 h incubation, viability of the HPMN was 85%.

Assay for O2 production. O2 was measured by the reduction of ferricytochrome c (Goldstein et al., 1981; Pick & Keisari, 1981). HPMN (2 × 10^6) were suspended in 1 ml 80 μM-ferricytochrome c (type III, Sigma) in phenol-red-free HBSS and kept at 36.5 ± 0.5 °C. Stimulants, which included C. trachomatis EB (10^7 inclusion-forming units (IFU) ml^-1) or phorbol 12-myristate 13-acetate (PMA; Sigma) (1 μg ml^-1), were added directly to the reaction mixture and reduction of cytochrome c was recorded continuously at 550 nm in a Bausch & Lomb double-beam spectrophotometer (Spectronic 2000) for 20 min against a blank consisting of the reaction mixture without stimulants. Controls included HPMN in Hanks’ balanced salt solution (HBSS) containing cytochrome c without the addition of stimulant, and HBSS containing cytochrome c and stimulant but no HPMN. The concentration of cytochrome c reduced was calculated from the molar absorption coefficient ε_{550} = 2.1 × 10^4 M^-1 cm^-1. The specificity of cytochrome c reduction was controlled by the inclusion in some samples of 300 U superoxide dismutase (SOD) ml^-1 [EC 1.15.1.1; type I, 3000 U (mg protein)^-1; Sigma].

Assay for H2O2 release. This was done by the method of Pick & Keisari (1981). HPMN (2 × 10^6) in tubes (17.1 × 100 mm polystyrene; Falcon Labware) were incubated for 30 min in 1 ml prewarmed phenol red solution, containing 140 mM-NaCl, 10 mM-potassium phosphate buffer, pH 7.0, 5.5 mM-dextrose, 0.28 mM-phenol red (phenolsulphonphthalein, sodium salt; Sigma), and 8.5 units horseradish peroxidase ml^-1 (type I, 170 purpuragullin units per mg solid; Sigma). The stimulants were added and the tubes were incubated with shaking for 30 min at 37 °C. Controls included tubes with phenol red solution but no stimulant, tubes with phenol red solution but no cells, and tubes containing catalase (5000 U ml^-1) in addition to stimulant. At the completion of incubation, the tubes were centrifuged for 5 min at 2000 g. Cell-free supernates were made alkaline by the addition of 10 μl 1 M-NaOH per tube and the absorbance of the samples was read at 610 nm against a blank of phenol red solution to which 10 μl 1 M-NaOH was added. Standard curves were made, using the same batch of phenol red solution, with H2O2 solutions ranging in concentration from 1 to 60 μM. All stimulants and controls were tested in duplicate.

Induction of DNA strand scissions. HeLa cells (Flow Laboratories) were grown on cover slips (13 × 13 mm) and in 24-well plates (Nunc; 4 × 10^4 cells per well) in RPMI-1640 medium supplemented with 10% (v/v) foetal calf serum, 100 U penicillin ml^-1, 100 μg streptomycin ml^-1, 100 U mycostatin ml^-1 and 2 mM-l-glutamine (Biological Industries, Beth Haemek, Israel) in a humidified 5% (v/v) CO2/air mixture and were passaged every 4 d. During the exponential growth state (24 h after seeding), the HeLa cells were treated with one of the following: 0.5 ml HBSS containing 2 × 10^6 HPMN, HPMN activated by EB or PMA, EB alone, or PMA alone (PMA was dissolved in DMSO and stored at -70 °C until use). Bleomycin (Nippon Kayaku Co.) (500 μg ml^-1), used as a control, was added and incubated for 18 h with the HeLa cells. SOD and catalase (EC 1.11.1.6; Sigma) were dissolved in HBSS to the desired concentrations and added to the reaction mixture, which was then incubated for 2 h at 37 °C in a humidified 5% (v/v) CO2/air mixture. The cells were then rinsed three times with phosphate-buffered saline (0.13 M-NaCl, 0.014 M-sodium phosphate; PBS) pH 7.3. HeLa cells on cover slips were fixed for 5 min in acetone/ethanol (1:1, v/v) at room temperature and were further processed for visualization of nicks. HeLa cells in plates were fixed for 5 min in ethanol and further processed for quantification of the nicks induced by the different treatments described above.

In situ nick-translation assay. This was done by a modification of the method described by Iseki & Mori (1985). After fixation, the cells were incubated at room temperature with 30 μl nick-translation reaction mixture, containing 50 mM-Tris/HCl (pH 7.5), 5 mM-MgCl2, 240 U E. coli DNA polymerase I ml^-1 (Amersham), 30 μM each of dATP, dGTP, dCTP (Sigma) and either TTP or biotin-labelled dUTP (Enzo Biochemical Co.). For
monitoring the amount of reaction, 1.5 μM-[3H]dCTP (67 Ci mmol⁻¹, 2.48 TBq mmol⁻¹; Amersham) was added to the mixture. The specificity of the system was tested by doing control experiments, as illustrated in Fig. 1, in which the reaction mixture also contained 8 ng pancreatic DNAase I ml⁻¹ (Pharmacia). The reaction was terminated by rinsing the cells with PBS, to 50 mM-Tris/HCl (pH 7.5). To measure the incorporation of [3H]dCTP into macromolecules, the cells were treated with 5% (w/v) TCA (10 min), with 2.5% TCA (10 min) and finally with ethanol (5 min) at 4 °C. After drying, the acid-insoluble materials were dissolved with Hyamin 10-X (Packard), transferred into vials containing scintillation solution, and their radioactivity measured in a Packard Tri-Carb scintillation spectrophotometer, model 3310. Results were presented as relative units (RU) of incorporation (the amount of incorporation in the different treatments relative to the amount of incorporation in the HeLa cells alone).

Detection procedure. For visualization of the incorporated biotin-labelled dUTP the cells were treated with a blocking buffer solution (pH 7-3), comprising PBS and 0.1% Triton X-100, and then rinsed with PBS for 5 min. After removal of PBS, the cells were incubated for 30 min with a solution containing streptavidin-conjugated horseradish peroxidase in PBS, with 5 mM-EDTA and 0.1% BSA (Enzo Biochemical Co.). The cells were washed with PBS, treated for 2 min with blocking buffer and rinsed with PBS for an additional 5 min. Then a solution containing 0.5 mg aminoethylcarbazole ml⁻¹ (Savyon Diagnostics, Israel) in dimethylformamide, and 0.025% H₂O₂, was added. A positive reaction was indicated by a dark brown deposit in the nuclei of the HeLa cells. The damage in each treatment was evaluated by photographing four fields in each cover slip or well, under the light microscope, and counting the number of damaged nuclei (total of 400 cells in each treatment, in four experiments). Correlation between the percentage of peroxidase-positive nuclei in the HeLa cells (expressed as RU) and the absolute number of damaged nuclei was determined.

RESULTS

Superoxide anion production by EB- or PMA-stimulated HPMN

HPMN (2 × 10⁹) stimulated by 10⁷ IFU of C. trachomatis EB produced 23.1 ± 5.4 nmol (mean ± se) O₂⁻ compared to 104.9 ± 35 nmol O₂⁻ produced in the presence of 1 μg PMA ml⁻¹. Addition of SOD (308 U ml⁻¹) to the reaction mixture in the presence of 1 μg PMA ml⁻¹ reduced the O₂⁻ level to 68.1 ± 6.8 nmol whereas with SOD in the presence of 10⁷ IFU of C. trachomatis EB no O₂⁻ was detected. Addition of 616 U SOD ml⁻¹ completely inhibited O₂⁻ production by PMA-stimulated HPMN.

Hydrogen peroxide production by EB- or PMA-stimulated HPMN

HPMN (2 × 10⁹) stimulated by 10⁷ IFU of C. trachomatis EB produced 204 ± 6 nmol H₂O₂, and those stimulated by 1 μg PMA ml⁻¹ produced 265.8 ± 61 nmol H₂O₂. No H₂O₂ was detected after addition of 5000 U catalase ml⁻¹ to HPMN stimulated by 1 μg PMA ml⁻¹, but EB-stimulated HPMN produced 11.2 ± 2 nmol H₂O₂. This decrease in the amount of H₂O₂ produced may be due to the catalase reacting mainly with extracellular H₂O₂ and its inability to gain access to the phagosomes of the HPMN during phagocytosis and react with the intraphagosomal H₂O₂ formed. Since it is not likely that horseradish peroxidase enters the phagosome, the H₂O₂ measured is probably the result of the activity of myeloperoxidase released into EB-containing phagosomes. Stevens et al. (1978) have shown that catalase inhibited by only 50% the luminol-enhanced chemiluminescent response of PMN and related this to the inaccessibility of catalase to intracellular sites of H₂O₂ production.

Effect of EB- or PMA-stimulated HPMN on the formation of single-strand scissions (nicks) in the DNA of co-cultivated HeLa cells

The formation of nicks in the DNA of HeLa cells, induced by the different treatments, was quantified using [3H]dCTP and visualized using streptavidin–peroxidase complex to detect incorporated biotin-labelled dUTP in an in situ nuclear nick-translation reaction as described by Iseki & Mori (1985). The specificity of the in situ nick-translation reaction in our system was tested by performing the nick-translation reaction on fixed HeLa cells (Fig. 1). In the presence of E. coli DNA
polymerase I in the mixture, considerable incorporation was observed during the incubation. The inclusion of DNAase in the reaction mixture stimulated incorporation two- to threefold. When the reaction mixture contained neither DNA polymerase I nor DNAase, the incorporation was very low, indicating that non-specific adsorption of radioactive nucleotide in the fixed cell nuclei is negligible. The low but readily measurable incorporation in the absence of DNAase may be due to nicks naturally existing in the intact HeLa cell nuclei, but the possibility of nick formation in the process of fixation and during enzyme reaction cannot be ruled out. These results are in accordance with those obtained by Iseki & Mori (1985).

The incorporation of [3H]dCTP into the nicked DNA of 4 x 10⁵ HeLa cells exposed to HPMN (2 x 10⁴) activated by 10⁷ IFU of chlamydial EB (Fig. 2a) was 3-5 times greater than the incorporation for HPMN alone. Addition of SOD (308 U ml⁻¹) or catalase (5000 U ml⁻¹) to the EB-activated HPMN partially reduced (20% and 30% respectively) the incorporation. Addition of both SOD and catalase reduced the amount of strand scissions by 60%. Activation of the HPMN by 1 µg PMA ml⁻¹ similarly resulted in the formation of more nicks (6.9 RU) than obtained in the presence of HPMN alone (2.19 RU) (Fig. 2b). The presence of 5000 U catalase ml⁻¹ reduced the amount of nicks by 60% while SOD (308 U ml⁻¹) reduced it by only 25%.
DNA nicks induced by chlamydia-activated HPMN

Fig. 3. Visualization of in situ nuclear nick translation. HeLa cells (4 x 10^3) (a), and HeLa cells treated with HPMN (2 x 10^6) activated by 10^7 IFU EB (b), were fixed. The nick-translation reaction included biotinylated dUTP which was detected by the use of streptavidin–peroxidase, aminoethylcarbazole and H2O2 as the substrate. A positive reaction appeared as dark brown deposits in the HeLa cell nuclei (examples arrowed in b). Cells were counter-stained with 1% (w/v) naphthol blue-black. Bars, 50 μm.

The amount of free radicals released by EB-activated HPMN was lower than the amount produced by PMA-activated HPMN, yet the amount of measured damage was similar. HPMN activated by a lower concentration of PMA (0-1 μg ml⁻¹) gave 3-4 RU while HPMN activated by 2 x 10^6 EB ml⁻¹ gave 0-39 RU (data not shown). Increasing the concentrations of PMA and EB in the presence of HPMN resulted in amplification of the damage to the level of saturation.

Exposure of the HeLa cells to 10⁷ IFU of chlamydial EB alone (input multiplicity 25 IFU per cell) resulted in [³H]dCTP incorporation twice that in the control. This input multiplicity of EB was optimal in this system; higher concentrations were toxic.

Heat-inactivation of the EB preparation (56 °C, 20 min) caused a 50% reduction in its ability to induce DNA strand scissions in HeLa cells in the absence of HPMN (data not shown). This may be related to a reduced rate of association of heat-inactivated EB with the HeLa cells (Bose & Paul, 1982).

In order to locate and visualize the DNA strand scissions caused by the different treatments, we used biotin 11-dUTP in the nick-translation reaction and streptavidin–peroxidase in the detection system as described by Iseki & Mori (1985). The antibiotic bleomycin (500 μg ml⁻¹), thought to cause DNA break damage mediated by the action of oxygen-derived free radicals (Kuo & Haidle, 1973), was used as a control (as described by these latter authors); it induced DNA strand scissions which were localized specifically in the nucleus of the HeLa cells. Fig. 3 illustrates the damage induced by EB-activated HPMN, as visualized by the positive staining (a dark brown deposit in the nuclei of the HeLa cells). Similar results were obtained with HeLa cells treated with PMA-activated HPMN. EB alone also gave a positive reaction. The percentage of damaged nuclei was quantified by counting cells with a positive reaction in a total of 400 HeLa cells in four fields. The following results were obtained: HPMN alone (12.6% ± 1.6%), EB (10% ± 0.2%), PMA (3% ± 1.5%), HPMN activated by EB (30.5% ± 16%) or by PMA (60% ± 7.8%), and HeLa cells alone (1.62% ± 0.1%). The reason why only some of the cells were damaged in the presence of EB alone, although an input multiplicity of 25 was employed, may have been that the HeLa cells in the asychronous culture had different sensitivities. Correlation between the percentage of peroxidase-positive nuclei observed with the use of biotin-11 dUTP and the percentage of [³H]dCTP incorporation in the in situ nick-translation reaction was calculated with a correlation coefficient value (R) of 0.8.
DISCUSSION

The PMN leucocyte functions by protecting the body through its ability to phagocytose and destroy micro-organisms (Quie & Mills, 1978). Activation of HPMN with various stimuli results in the so-called 'respiratory burst' and production of oxygen free radicals (Allen et al., 1972). Increased generation of free radicals such as the superoxide anion and hydroxyl radical is a characteristic of activated inflammatory cells (Baggiolini, 1980). The reactive oxygen metabolites, in conjunction with myeloperoxidase, which oxidizes Cl− to hypochlorous acid (HOCl) (Prince & Gunson, 1987), are used by the HPMN to destroy bacteria, fungi, protozoa and some viruses (Allen et al., 1974; Belding & Klebanoff, 1970). Oxygen free radicals liberated from activated HPMN are also involved in damaging tumour cells and lung tissue (Clark & Klebanoff, 1975; Fanburg et al., 1986). They can additionally serve as effectors of cartilage destruction (Burkhardt et al., 1986), cause mutations in bacteria and mutations and sister chromatid exchange in mammalian cells (Nicotera et al., 1985; Weitzman & Stossel, 1981), and induce transformation (Weitzman et al., 1985). However, not much is known about PMN-mediated DNA damage other than that DNA strand breaks are formed in the DNA of activated PMNs themselves and in the DNA of cells co-cultivated with activated PMNs (Birnboim, 1981, 1983).

The model described here for the activation of HPMN by chlamydial EB, and the resulting induction of DNA strand scissions in co-cultivated HeLa cells, suggests a possible role of HPMN in the development of chronic and neoplastic diseases.

Previous studies by us and by other investigators have shown that EB of C. trachomatis (L2 and E serovars) can induce a chemiluminescent response (Soderlund et al., 1984; Zvillich & Sarov, 1985) and release myeloperoxidase from HPMN (Hammerschlage & Tosi, 1986). Also, peroxidase-positive phagolysosomes containing chlamydia (L2 serovar) have been observed in HPMN (Yong et al., 1986).

Relatively lower levels of O2·− and H2O2 were obtained when HPMN were activated by EB of chlamydia as opposed to PMA. Hammerschlage & Tosi (1986) also obtained low levels of O2·− from chlamydia-infected HPMN. It must be emphasized that the assay used for the detection of O2·− and H2O2 measures only the fraction of these products that is released into the medium. Consequently the values obtained represent lower values than the actual amounts of O2·− produced (Baggiolini, 1980).

Mello Filho & Meneghini (1984) showed that H2O2 at a concentration of 28 μM induced DNA single-strand scissions in cultured human fibroblasts. Thus, low doses of oxygen radicals liberated continuously by HPMN, at sites of inflammation, may exacerbate this condition. In this regard Weitzman et al. (1985) have shown that low doses of neutrophils caused more transformed foci in co-cultivated mouse fibroblasts than high doses of PMA-activated HPMN, which were thought to be lethal to the fibroblasts undergoing transformation.

The reduction in the amount of strand scissions induced in the DNA of the HeLa cells, by EB- or PMA-activated HPMN, was smaller in the presence of SOD (19% and 25% respectively), than in the presence of catalase (30% and 60% respectively). Birnboim (1981) showed that the formation of DNA strand breaks in HPMN can be inhibited by the addition of SOD or catalase; however, only catalase prevented the formation of nicks in DNA of cells co-cultivated with PMNs. Thus it appears that OH radicals arising from H2O2 are more active in inducing strand scissions in DNA than O2·− (Floyd, 1981). The mechanism by which EB-activated HPMN cause damage to the DNA of the co-cultivated HeLa cells needs further investigation. Frenkel et al. (1986) have shown that PMA-activated HPMN cause the formation of thymidine derivatives in co-incubated DNA, similar in yield and nature to those formed by high doses of ionizing radiation.

The fact that no synergistic effect was obtained in the amount of damage caused by EB-activated HPMN as compared to EB and HPMN alone can be explained by the fact that some of the EB are phagocytosed and inactivated by the HPMN (Zvillich & Sarov, 1985), which are in excess. Thus, the amount of damage caused directly by the EB is reduced, most of it being due to free radicals produced by the EB-activated HPMN.

The damage to the host DNA may be caused not only by invading activated HPMN but also by the chlamydiae themselves. Incubation of HeLa cells with EB alone also resulted in the
formation of DNA strand scissions (Fig. 2a). Recently it was found that infection of HeLa cells with *C. trachomatis* resulted in enhanced SOD activity whereas cytosolic catalase activity decreased in these infected cells (Weber et al., 1985). It was also found that in human endothelial cells 72 h post infection with *Rickettsia rickettsii* the amount of peroxides formed was four- to fivefold greater than in uninfected cells, suggesting a role for these peroxides in the endothelial damage caused by rickettsiae (Silverman et al., 1986). The elevated levels of SOD activity observed in the chlamydia-infected cells may be a secondary manifestation of an overall imbalance in oxygen metabolism (increase in $\mathrm{O}_2^-$ production), which may lead to elevated levels of chromosomal aberrations and sister-chromatid exchanges, as suggested for the development of Bloom's syndrome (Nicotera et al., 1985).

Birnboim (1983) suggested that DNA strand-break damage may be important in tumour promotion, and in this regard, certain chronic inflammatory conditions have been shown to be associated with leucocyte infiltration and a markedly increased risk of cancer (Dunham, 1972). Persistent chronic and recurrent chlamydial infections are common. They were recognized in various animal infections with *C. psittaci* (Kunimoto & Brunham, 1985) and were later observed in human *C. trachomatis* infections causing trachoma and lymphogranuloma venereum, as well as genital and respiratory tract infections (Ladany & Sarov, 1985).

In summary, the present study has demonstrated the ability of EB-activated HPMN, and EB alone, to cause DNA strand scissions (nicks) in co-cultivated HeLa cells. Whether this or other mechanisms play a role in chlamydia-induced damage to the host's DNA, and in the development of chronic and oncogenic diseases, requires further investigation.

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