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IN VITRO STIMULATION OF ALVEOLAR MACROPHAGE METABOLIC ACTIVITY BY POLYSTYRENE IN THE ABSENCE OF PHAGOCYTOSIS

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Summary.—The technique of lucigenin-dependent phagocytic chemiluminescence was used to investigate the stimulation of metabolic activity in human alveolar macrophages on contact with polystyrene. The results were similar to those obtained using a spectrophotometric assay of superoxide release based on ferricytochrome C reduction.

There was a marked stimulation of metabolic activity in the alveolar macrophages on incubation at 37° in polystyrene vials which was shown to be due to contact with and adherence to the polystyrene. This could be reduced by the addition of gelatin or foetal calf serum without preventing the ability of the cells to respond to opsonized particles. By using several metabolic inhibitors it was shown that lucigenin-dependent chemiluminescence was associated with the release of superoxide at the time of adherence.

The implications of these findings are discussed and it is suggested that the stimulation of alveolar macrophage metabolic activity by contact with polystyrene can contribute to the observed difference between alveolar macrophage and polymorphonuclear leucocyte oxygen consumption in the absence of phagocytosis.

PHAGOCYTIC CELLS undergo a burst of metabolic activity on contact with opsonized particles, during which oxygen (O_2) is consumed, superoxide (O_2^-) and hydrogen peroxide produced and hexose monophosphate shunt activity increased (Baldridge and Gerard, 1933; Iyer, Islam and Quastel, 1961; Karnovsky, 1968; Babior, Kipnes and Curnutte, 1973). It has been reported that alveolar macrophages (AM) consume more oxygen than polymorphonuclear leucocytes (PMN) in the absence of phagocytosis but that the latter show a greater increase in consumption on stimulation by opsonized particles (Oren *et al.*, 1963).

Allen, Stjernholm and Steele (1972) demonstrated low level chemiluminescence produced by PMN during phagocytosis. Other studies (Allen and Loose, 1976) have used the cyclic hydrazide luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) as a substrate for oxidation by reactive oxygen species produced by phagocytosing cells, thus increasing the amount of light generated. This has allowed the technique to be used readily for the investigation of serum opsonic activity and metabolic defects of peripheral blood leucocytes (Easmon *et al.*, 1980). However, some doubts exist about the production of luminol-dependent chemiluminescence by AM (Ziprin, 1978; Hatch, Gardner and Menzel, 1978) because of the difficulty in eliminating PMN contamination of bronchoalveolar lavage specimens.

Lucigenin (bisN-Methylacridinium nitrate) has recently been used as an alternative for the production of substrate-dependent chemiluminescence by PMN

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(Allen and Strong, 1980). In this study we investigate the production of lucigenin-dependent chemiluminescence by human AM both on contact with polystyrene reaction vials and on stimulation by opsonized zymosan. We examine the effect of medium constituents and suggest that metabolic stimulation as a result of adherence to foreign surfaces might contribute to the differences noted in resting and stimulated O_2 consumption in AM and PMN.

MATERIALS AND METHODS

Cell preparation and opsonization.—Alveolar macrophages were obtained at diagnostic bronchoscopy, by bronchoalveolar lavage with bicarbonate-buffered 0·9% saline, from patients with suspected diagnosis of sarcoidosis (n = 6), cryptogenic fibrosing alveolitis (n = 6), asbestosis (n = 1) and systemic lupus erythematosus (n = 1), as well as normal controls (n = 3). After washing twice at 4°, the cells were suspended in medium to a concentration of 4×10^5 nonspecific-esterase-positive (NSE^+) cells per ml (Yam, Li and Crosby, 1971) and kept in siliconized glass vials on melting ice until use. Cytocentrifuge preparations were made to quantitate contamination with PMN and lymphocytes by May–Grunewald Giemsa staining.

PMN were separated by a modification of the method described by Boyum (1968) from the peripheral blood of 6 healthy donors. After removal of the mononuclear cell layer following gradient centrifugation of defibrinated blood on Ficoll–Triosil (s.g. 1·077), the PMN were isolated by sedimenting erythrocytes with dextran (mol. wt 110,000), then treating with 0·83% Tris ammonium chloride. The cells were washed twice, resuspended in medium (4×10^5 PMN/ml) and kept at either 4° or 37° in siliconized glass containers until use.

Zymosan was opsonized by incubation with pooled normal human serum at 37° for 30 min. 400 μl serum was added to 20 mg zymosan suspended in 1·9 ml medium. After incubation the opsonized particles were washed twice and resuspended in phosphate-buffered saline (20 mg/ml).

Informed consent was obtained from all donors.

Chemiluminescence.—This was measured in a photometer (Luminometer 1250, LKB Wallac) the details of which have been previously described (Easmon *et al.*, 1980) using 2n l cylindrical polystyrene cuvettes (46 × 10 mm; LKB Wallac and Sterilin) at 37°. The reaction mixture consisted of 900 μl lucigenin (10^{-4}M) and 500 μl cell suspension (4×10^5 NSE^+ cells/ml). The light generated was recorded continuously on a chart recorder (LKB Wallac) and unless otherwise indicated evaluation was based upon the reading 12 min after addition to the reaction vial. Metabolic inhibitors, gelatin and foetal calf serum were added before the cell suspensions as indicated. Opsonized zymosan (2 mg) was added in suspension (100 μl). Preliminary experiments showed that the results obtained using cuvettes from LKB Wallac gave results approximately 25% higher than those from Sterilin. All results given are those obtained using Sterilin cuvettes.

Superoxide release.—This was determined by the superoxide dismutase-inhibitable reduction of cytochrome C (Babior *et al.*, 1973). AM (4×10^5 cells) or PMN (10^6 cells) were incubated for 15 min at 37° with 13 μM ferricytochrome C (horse heart Type VI) in the presence and absence of 50 μg superoxide dismutase. The amount of reduced cytochrome C in the cell free supernatants after centrifugation at 4000 *g* for 10 min was determined spectrophotometrically at 550 nm and the O_2^- generation calculated using the extinction coefficient of 21×10^3/cm/M (Massey, 1959).

Medium and chemicals.—Hanks' balanced salt solution (HBSS) and Medium 199 containing calcium and magnesium but without phenol red were obtained from Flow Laboratories. Gelatin was obtained from Difco Laboratories, sodium azide from BDH Chemicals Limited and zymosan A, antimycin A, catalase, lucigenin, superoxide dismutase and cytochrome C were obtained from Sigma Laboratories. Antimycin A was dissolved in dimethyl sulphoxide (25 mg/ml) and diluted to the required concentration with medium. Lucigenin was dissolved in HEPES-buffered HBSS, sodium azide in distilled water and other chemicals in phosphate-buffered saline. AM and PMN were suspended in Medium 199.

RESULTS

Stimulation of metabolic activity by contact with polystyrene

In Fig. 1 a typical chemiluminescence response on adding AM to polystyrene reaction vials containing lucigenin is shown. The immediate response produced when lucigenin was added to AM that have been incubated for 6 min at 37° in the reaction vial suggests that it was initiated by a reaction between the cells and the vial rather than by stimulation of the cells by lucigenin. Furthermore, on transferring the medium from the vial after incubation

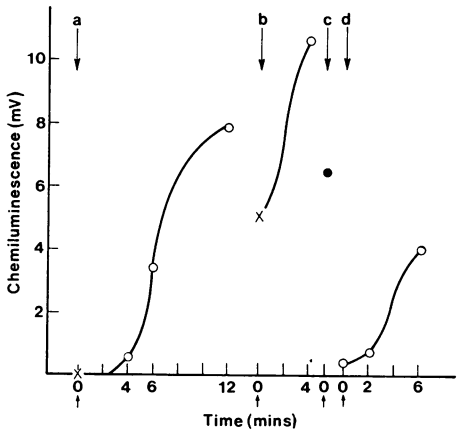

FIG. 1.—A typical example of lucigenin-dependent chemiluminescence from alveolar macrophages (AM) in the absence of opsonized particles. (a) Response on addition of 500 μl alveolar macrophage (AM) suspension (4°; 4×10^5 NSE$^+$ cells/ml) to reaction vial containing 900 μl lucigenin (10^{-4}M) at 37°. (b) Response obtained by incubating AM in vial at 37° for 6 min and then adding lucigenin at Time 0. (c) Response obtained by incubating AM in vial at 37° for 10 min, followed by removal of the medium and addition of lucigenin to the original vial at Time 0. (d) Response obtained by adding lucigenin to the medium that had been transferred to a second vial as in (c).

for 10 min at 37°, an immediate response was produced on the addition of lucigenin to the same vial but not on addition to the medium that had been transferred. A similar pattern was observed with PMN but the magnitude of the response was significantly different ($P < 0{\cdot}001$) when the 2 cell types were compared (Table I). The response with AM did not correlate with PMN contamination of the AM suspension (Table II; $r = 0{\cdot}19$). The addition of gelatin (0·1%) to the reaction vial before the addition of cells reduced the response of AM to approximately 20% but had less of an effect with PMN. Foetal calf serum (0·3%) reduced the response with both types of cell, although its effect on AM was less marked than gelatin (0·1%).

To further investigate the stimulation of phagocytic cells by contact with polystyrene, quantitative measurements of O_2^- release by AM and PMN were performed after incubation for 15 min in the presence or absence of gelatin (Table IV). As with chemiluminescence, it was found that AM released significantly more O_2^- ($P < 0{\cdot}001$) than PMN when incubated in polystyrene vials in the absence of particles but this could be reduced by the inclusion of gelatin in the medium.

Effect of metabolic inhibitors

The influence of various metabolic inhibitors on the lucigenin-dependent chemiluminescence produced when AM and PMN are added to polystyrene vials was investigated. Fig. 2 shows a typical example of the effects of antimycin A,

TABLE I.—*Comparisons between alveolar macrophage and polymorphonuclear leucocyte chemiluminescence*

	AM (Patients and controls)	PMN	AM (Controls)
Lucigenin + cells (4°)	6·67 (s.d. 5·7; n = 17)	0·64 (s.d. 0·17; n = 8)	7·83 (s.d. 3·1; n = 3)
Lucigenin + gelatin (0·1%) + cells (4°)	0·96 (s.d. 0·95; n = 15)	0·38 (s.d. 0·14; n = 8)	1·4 (s.d. 0·7; n = 3)
Lucigenin + cells (37°)	*	0·37 (s.d. 0·2; n = 6)	*
Lucigenin + FCS (0·3%) + cells (4°)	1·95 (s.d. 1·25; n = 9)	0 (n = 3)	ND
Lucigenin + gelatin (0·1%) + cells (4°) + zymosan	5·82 (s.d. 3·32; n = 17)	2·4 (s.d. 0·68; n = 6)	5·33 (s.d. 1·92; n = 3)

Chemiluminescent response (mV) of alveolar macrophages (2×10^5; AM) and polymorphonuclear leucocytes (2×10^5; PNM) 12 min after addition to reaction vials containing $0{\cdot}6 \times 10^4$M lucigenin in the presence or absence of zymosan. Effect of gelatin (0·1%), foetal calf serum (0·3%; FCS) and temperature at which the cells had previously been stored. AM (controls) were from Subjects 11, 15 and 16 (Table II).

* Because of adherence to the walls of the collection container before addition to the reaction vial this experiment was not possible.

TABLE II.—*Influence of PMN on chemiluminescence produced by AM*

Subject	AM ($\times 10^5$)	PMN ($\times 10^5$)	Lympho-cytes ($\times 10^5$)	CL Cells + Lucigenin (mV)	CL Cells + Lucigenin + gelatin (mV)	CL Cells + Lucigenin + antimycin (mV)
1	2·0	0·79	0·1	1·1	0	ND
2	2·0	1·95	0·26	0·6	0	3·4
3	2·0	0·08	0·12	6·8	1·6	0·1
4	2·0	3·69	0·38	6·8	3·5	5·3
5	2·0	0·14	0·92	3·9	0·8	0·4
6	2·0	0·72	0·86	7·8	0·7	ND
7	2·0	0·31	2·12	3·5	0·6	ND
8	2·0	1·43	0·62	2·5	0·7	ND
9	2·0	1·4	0·24	14·4	0·2	1·8
10	2·0	0·66	0·68	7·9	0·3	1·8
11	2·0	0·06	0·14	9·7	1·8	ND
12	2·0	0·76	1·08	4·3	0·3	1·3
13	2·0	0·74	0·11	2·1	ND	ND
14	2·0	1·92	0·08	24·5	1·6	ND
15	2·0	0·35	0·02	3·4	ND	ND
16	2·0	0·04	0·02	10·4	2·2	ND
17	2·0	0·02	0·02	3·6	0·2	ND

Chemiluminescence (CL) 12 min after addition of $2{\cdot}0 \times 10^5$ alveolar macrophages (AM) to polystyrene reaction vials at 37°, in the presence of lucigenin with and without antimycin A (0·34 mM) or gelatin (0·1%). The number of contaminating PMN and lymphocytes is shown.

TABLE III.—*Effect of inhibitors upon chemiluminescent response*

	Antimycin A	Catalase	SOD	Azide
PMN + lucigenin	970% (s.d. 700%; n = 7)	43% (s.d. 16%; n = 5)	1% (s.d. 0·5%; n = 7)	140% (s.d. 10%; n = 5)
AM + lucigenin	96%* (s.d. 170%; n = 7)	72% (s.d. 9%; n = 4)	1% (s.d. 0·5%; n = 7)	75% (s.d. 21%; n = 4)

Effect of antimycin A (0·34 mM), catalase (20 μg/ml), superoxide dismutase (SOD, 30 μg/ml) and azide (10^{-5}M) on the chemiluminescent response produced on addition of polymorphonuclear leucocytes (PMN) and alveolar macrophages (AM) to reaction vials containing $0{\cdot}6 \times 10^{-4}$M lucigenin at 37°. Results are expressed as the means of the percentages of the value obtained 12 min after addition of cells (except with antimycin when the peak value was taken) in the absence of inhibitor.

* See also Table II.

catalase, sodium azide and superoxide dismutase on the response obtained with AM and PMN. Antimycin A (0·34 mM) reduced the response of AM but increased the response of PMN (Table III). The residual response of the AM suspension correlated well with PMN contamination (Table II). Similar changes in O_2^- release by AM and PMN on addition of antimycin A were found using the ferricytochrome C reduction assay (Table IV). Azide (10^{-5}M) also reduced chemiluminescence with AM but the effect was not as marked and the residual response did not correlate with PMN contamination. Catalase (20 μg/ml) reduced the response of both cell types while superoxide dismutase (30 μg/ml) virtually abolished the chemiluminescence (Fig. 2; Table III).

Effect of gelatin upon zymosan-stimulated chemiluminescence

To help establish that the chemiluminescence-diminishing effects of gelatin and foetal calf serum were due to prevention of a reaction between the cells and the vial, rather than to either quenching of the light response or scavenging of the oxygen reactant species, their effects upon the response of AM and PMN to opsonized

TABLE IV.—*Superoxide release (O_2^-) by alveolar macrophages (AM) and polymorphonuclear leucocytes (PMN)*

Cell type	Antimycin	Gelatin	Zymosan	Superoxide anion (nmol/10^6 cells/15 min)
AM	−	−	−	5·08 (s.d. 1·74; n=5)
AM	−	+	−	1·38 (s.d. 0·8; n=5)
AM	−	+	+	9·32 (s.d. 3·6; n=5)
AM	+	−	−	0·10 (s.d. 0·1; n=2)
PMN	−	−	−	1·20 (s.d. 0·6; n=5)
PMN	−	+	−	0·38 (s.d. 0·1; n=5)
PMN	−	+	+	14·45 (s.d. 4·2; n=5)
PMN	+	−	−	18·43 (s.d. 2·6; n=2)

Superoxide release was determined spectrophotometrically by the method of Babior *et al.* (1973). AM (4×10^5 cells) or PMN (10^6 cells) were incubated in polystyrene vials for 15 min at 37° with 13 μM ferricytochrome C in the presence or absence of superoxide dismutase. Antimycin A (0·34 mM), gelatin (0·1%) or opsonized zymosan (100 μl) were included in the incubation mixture as indicated. AM were from Subjects 1, 3, 6, 11 and 12 (Table II).

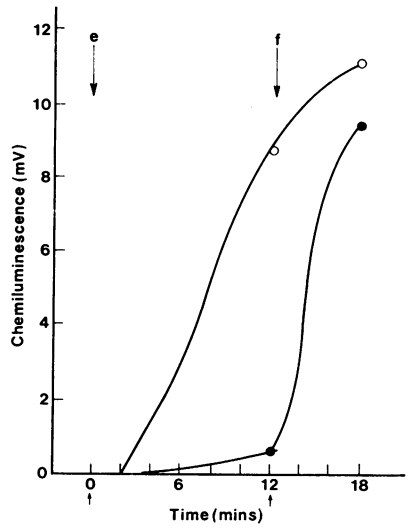

FIG. 3.—Effect of 0·1% gelatin, (i) ○—○ without (ii) ●——● with, on the chemiluminescence response of alveolar macrophages (AM). (e) Response on transference of 500 μl AM suspension (4°; 4×10^5 NSE+ cells/ml) to polystyrene vials at 37° and (f) on addition of opsonized zymosan (100 μl). Doubling the gelatin concentration did not influence the result.

zymosan were investigated. Because of the considerable response produced by addition of AM to reaction vials in the absence of gelatin and opsonized particles (Fig. 3) it was not possible to determine the influence of stimulation by opsonized zymosan in the absence of gelatin. However, in the presence of gelatin (0·1%) addition of opsonized zymosan produced a considerable response (Table I) which was of the same order of magnitude as that produced by cells in the absence of gelatin and particles. The response to opsonized zymosan added 12 min after the start of the incubation of AM in a reaction vial was less marked in the absence of gelatin than in its presence (Fig. 3). Increasing the lucigenin concentration did not influence this latter observation. Gelatin (0·1%) reduced the chemiluminescence produced by the addition of opsonized zymosan to a reaction vial containing lucigenin and PMN by less than 20%.

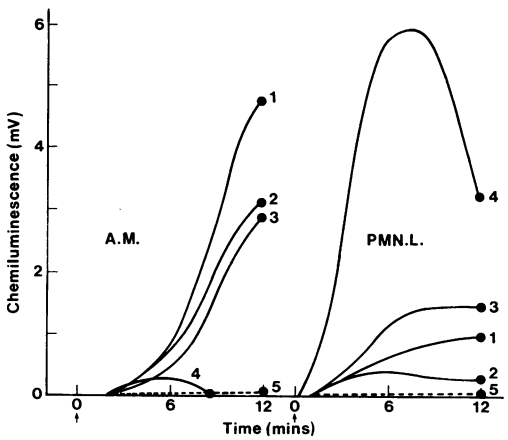

FIG. 2.—An example of the chemiluminescent response on adding 500 μl alveolar macrophage (AM) suspension (4°; 4×10^5 NSE+ cells/ml) or 500 μl polymorphonuclear leucocyte (PMN) suspension (4°; 4×10^5 cells/ml) to (1) reaction vials with 900 μl lucigenin (10^{-4}M). Effect of (2) catalase (20 μg/ml) (3) azide (10^{-5}M) (4) antimycin A (0·34 mM) and (5) superoxide dismutase (30 μg/ml) added to lucigenin before addition of cell suspension.

DISCUSSION

Alveolar macrophages are the resident phagocytic cells in the broncho-alveolar spaces and as such provide the first line of

defence against invading bacteria. Increasing evidence that these cells are intimately involved in the pathogenesis of a variety of other respiratory diseases (Hocking and Golde, 1979) has encouraged investigation of their metabolism. It is well recognized that the AM, in contrast to other phagocytic cells, depends on oxidative and glycolytic metabolic pathways for its energy supply both at rest and during phagocytosis. While PMN consume very little oxygen in the absence of phagocytosis, this is not the case with AM (Oren *et al.*, 1963). It has previously been shown (Kiyotaki *et al.*, 1978) that PMN release O_2^- during incubation in polystyrene dishes in the absence of phagocytosis and that this can be affected by the presence of protein in the medium. Furthermore O_2^- release by PMN following contact with opsonized particles correlates well with O_2 consumption (Markert, Allaz and Frei, 1980). The aim of this study was to investigate the metabolic activity of AM following contact with polystyrene reaction vials and to determine whether or not O_2^- is released into the reaction medium at the time of adherence.

It has been suggested (Allen and Strong, 1980) that phagocytic lucigenin-dependent chemiluminescence results from the extracellular interaction between O_2^- and lucigenin to form a dioxetane with subsequent cleavage to 2 molecules of N-methylacridone, one of which is in an excited state before relaxation with the release of a photon. Our findings using this technique are in agreement with the above observations on PMN and furthermore show that the magnitude of the response is significantly greater with AM than with PMN, which may be due to the involvement of only a subpopulation of the latter. The results obtained using chemiluminescence were confirmed by using an alternative method of measuring O_2^- release based on the reduction of cytochrome C.

The influence of medium constituents such as serum and gelatin is considerable and although their effect might involve binding to cellular fibronectin (Gold and Pearlstein, 1980) or other endogenous constituents of phagocytic cells that enhance adherence (Bockenstedt and Goetzl, 1980) the details of the mechanisms are unclear. While both markedly reduced the response obtained in the absence of opsonized particles, neither had the same effect when particles were added (Fig. 3). Furthermore, the minimal increase in light produced when particles were added to AM, after they had been in a reaction vial at 37° for a few minutes, clearly has important implications regarding the ability of these cells to mount a metabolic response to particles within a few minutes of adherence.

The results of experiments using metabolic inhibitors (Fig. 2, Table III) emphasized the dependence of lucigenin-dependent chemiluminescence on superoxide production. In the presence of superoxide dismutase no light was detected when using either AM or PMN. The response with AM, as would be expected in view of their dependence upon oxidative phosphorylation for energy production, was markedly suppressed by antimycin A, an inhibitor of terminal respiratory chain activity. Furthermore, the residual response correlated well with PMN contamination ($r = 0{\cdot}96$; Table II), which might explain the reported discrepancy between AM O_2 uptake and O_2^- release on the addition of antimycin A (Hoidal, White and Repine, 1979). In contrast, antimycin A increased the response obtained when using PMN and this was supported by the results obtained using the spectrophotometric assay based on ferricytochrome C reduction (Table IV). This effect of antimycin A upon PMN lucigenin-dependent chemiluminescence was also inhibitable by superoxide dismutase, decreased approximately 20% by catalase and increased by azide. The addition of catalase alone reduced the response with both AM and PMN probably by accelerating the breakdown of hydrogen peroxide. Sodium azide, an inhibitor of haem enzymes reduced the response with AM but increased it with PMN.

These findings raise the possibility that part of the observed difference in AM and PMN oxygen uptake in the absence of phagocytosis is the result of increased metabolic activity brought about by adherence of the macrophages to the container surface. The release of O_2^- at the time of adherence can readily be detected by lucigenin-dependent chemiluminescence, and the toxic effects of both this anion and related radicals may be of importance in the pathogenesis of pulmonary disease.

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