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Two distinct mechanisms for stimulation of oxygen-radical production by polymorphonuclear leucocytes

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1. Oxygen-radical production stimulated from rat polymorphonuclear leucocytes by either unopsonized latex particles (diameter = 1.01 μm) or chemotactic peptide (*N*-formyl-Met-Leu-Phe) was monitored by using luminol-dependent chemiluminescence. 2. Azide inhibited by more than 80% the luminescence response induced by chemotactic peptide whether added before or after stimulation. However, the luminescence response to latex particles was progressively less susceptible to azide inhibition if the azide was added after the stimulus. 3. Cytochalasin B, which was shown to abolish phagocytosis of the latex beads, also abolished the chemiluminescence response. However, the same cells showed a greatly enhanced response to chemotactic peptide. 4. Cytochalasin B-treated cells secreted approx. 45% of total cellular myeloperoxidase in response to chemotactic peptide, but there was no detectable secretion in response to unopsonized latex particles. 5. Microperoxidase equivalent to 20% of cellular peroxidase activity added to the cells before addition of the stimulus had no effect on the response to latex particles but increased approx. 2-fold the peak rate of chemiluminescence induced by chemotactic peptide. 6. It was concluded that the unopsonized latex particles stimulated oxygen-radical production by the mechanism that involved endocytosis, whereas chemotactic peptide stimulated production by a mechanism that involved exocytosis of myeloperoxidase, the latter mechanism requiring an increase in intracellular free [Ca^{2+}].

It has been known for 50 years that phagocytic stimulation of PMN results in a burst of oxygen consumption (Baldridge & Gerald, 1933; Ado, 1933). This 'oxygen burst' is not due to increased mitochondrial respiration, as it is resistant to cyanide poisoning (Becker *et al.*, 1958; Sbarra & Karnovsky, 1959), but the result of the production of oxygen radicals such as $O_2^{-\bullet}$, $OH^\bullet$, $^1O_2$, H_2O_2 and OCl^-. These highly toxic species of oxygen play a key role in the killing of endocytosed bacteria (Babior *et al.*, 1973; Weening *et al.*, 1975). It has also been proposed that they may play a role in the pathogenesis of several diseases, including rheumatoid arthritis. Although the production of oxygen radicals by PMN is thought to involve a membrane-bound NADPH oxidase and a unique cytochrome *b* (Segal & Jones, 1979), the precise mechanism of activation of this pathway remains to be established.

Direct measurement of intracellular free [Ca^{2+}] by using the photoprotein obelin in PMN and macrophages had led to the proposal that the natural and experimental stimuli can be divided into two distinct classes (Campbell & Hallett, 1983). One, exemplified by the chemotactic peptide fMet-Leu-Phe, causes a rise in cytoplasmic free [Ca^{2+}], which is necessary for activation of oxygen-radical production, whereas the other, exemplified by unopsonized latex particles, activates the radical response by a mechanism independent of intracellular [Ca^{2+}], though it can be dependent on extracellular [Ca^{2+}] (Hallett *et al.*, 1981). The aim of the present study was to examine further this hypothesis, and in particular to establish a relationship between the two types of stimuli and the role of endo- and exo-cytosis in the production of the radicals.

Abbreviations used: PMN, polymorphonuclear leucocytes; fMet-Leu-Phe, *N*-formylmethionyl-leucyl-phenylalanine.

Experimental

Preparation of rat peritoneal polymorphonuclear leucocytes

PMN were isolated from the peritoneal exudate of Wistar rats (200–300g) 12–14h after intraperitoneal injection with 10–15ml of a sterile solution of sodium caseinate (12%, w/v)/NaCl (0.9%, w/v) (Hallett *et al.*, 1981). Erythrocytes were removed by selective lysis with iso-osmotic NH_4Cl and PMN were separated by centrifugation through Ficoll–Paque. The cells were washed and resuspended in Hepes [4-(2-hydroxyethyl-1-piperazine-ethanesulphonic acid]-buffered Krebs medium containing 120mM-NaCl, 4.8mM-KCl, 1.2mM-$MgSO_4$, 1.2mM-KH_2PO_4, 1.3mM-$CaCl_2$ and 25mM-Hepes, adjusted to pH 7.4 with NaOH. One rat yielded approx. 10^8 cells, which were 98% PMN after purification.

Monitoring of oxygen-radical production

Oxygen-radical production was monitored by measuring the luminol-dependent chemiluminescence from the cells (Hallett *et al.*, 1981). Luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) was dissolved in dimethyl sulphoxide and added to the cell suspension to give final concentrations of 11 μM-luminol and 0.1% (v/v) dimethyl sulphoxide. Luminescence was measured with a purpose-built apparatus as previously described (Campbell *et al.*, 1979). The sample (500 μl) was placed in a thermostatically-controlled light-tight housing positioned in front of a photomultiplier tube (Centronics; model P4232B). Additions were made to the sample by a syringe whilst in front of the photomultiplier tube. The output from the photomultiplier tube is shown as a continuous recording on a chart recorder that is calibrated in luminescence counts per second (c.p.s.).

Measurement of secretion

Myeloperoxidase activity was measured spectrophotometrically, by determining the rate of oxidation of guaiacol (*o*-methoxyphenol) in the presence of H_2O_2, in sodium phosphate buffer, pH 7.0. The appearance of oxidized product was measured spectrophotometrically at 436nm (Chance & Maehly, 1955). Hexosaminidase activity was measured by determining the production of fluorescent product from the substrate, 4-methylumbelliferyl 2-acetamido-2-deoxy-β-D-glucopyranoside. The reaction was carried out in citrate buffer, pH 5, at 37°C for 20min and stopped by the addition of Na_2CO_3 (0.89M final concentration). The concentration of substrate in the assay was 125 μM and the product was measured fluorimetrically with an excitation wavelength of 360nm and emission wavelength of 448nm (Barrett & Heath, 1977).

After the incubation period with the stimulus, the cells were separated from the supernatant after centrifugation at 8000 g for 5s in a Beckman Microfuge. The pellet was solubilized by the addition of 0.1% (v/v) Triton X-100 and the detergent was also added to the supernatant to give a final concentration of 0.1% (v/v). The enzyme activity was determined in both the pellet and supernatant, and the percentage secretion calculated from the following equation:

$$\text{Secretion (\%)} = \frac{E_s}{E_s + E_p} \times 100$$

where E_s is the enzyme activity in the supernatant and E_p is the enzyme activity in the pellet. The sum $E_s + E_p$ was equal to the total activity assayed before stimulation and provided an internal check of the assumptions made that (i) no enzyme was synthesized or activated during the experiment and (ii) agents added to the cells did not produce spurious absorption or fluorescence. In all experiments the coefficient of variation of the sum $E_s + E_p$ was less than 10%.

Chemicals

Sodium caseinate (Nutrose) was purchased from Difco Laboratories, Detroit, MI, U.S.A.; Ficoll–Paque was from Pharmacia Fine Chemicals, Uppsala, Sweden; luminol was from BDH Chemicals, Poole, Dorset, U.K.; and bovine serum albumin (fraction V) was from Armour Pharmaceuticals Co., Eastbourne, Sussex, U.K. Latex beads (diameter 1.01 μm) were purchased from Dow Chemicals, Indianapolis, IN, U.S.A., and microperoxidase, MP11 and chemotactic peptide (fMet-Leu-Phe) from Sigma Chemical Co., Poole, Dorset, U.K. Methylumbelliferyl 2-acetamido-2-deoxy-β-D-glycopyranoside was purchased from Koch–Light, Colnbrook, Bucks., U.K. All other chemicals were AnalaR grade and purchased from BDH.

Results

Inhibition by azide of PMN chemiluminescence

It has yet to be established how much of the oxygen-radical production by PMN occurs within endocytosed vesicles or extracellularly. The initial approach to the investigation of this problem was to search for an inhibitor of radical production, monitored by chemiluminescence, which could block the cell response completely when added with the stimulus but which would not inhibit if chemiluminescence was already occurring within endocytosed vesicles.

Azide is a potent inhibitor of many haem-containing enzymes, including peroxidases (Klebanoff, 1970). It may also quench singlet oxygen (Hasty *et*

al., 1972). Azide completely abolished luminol-dependent chemiluminescence of PMN when added with latex beads or chemotactic peptide. This contrasted with a maximum inhibition by superoxide dismutase of 75–80%, and by catalase of only 10–50%. When azide was added at different times after stimulation of the cells by latex beads, its inhibitory effect was reduced, being only 20% when added after 100 s, close to the peak in light intensity (Fig. 1). In contrast, when azide was added at different times after stimulation of the cells by chemotactic peptide, it was still able to inhibit the response by more than 80% (Fig. 1).

This suggested that most of the radical production induced by latex beads was occurring intracellularly, in a compartment impermeable to azide, whereas most of that induced by chemotactic peptide was extracellular or in a compartment readily permeable to azide.

Effect of cytochalasin B on PMN chemiluminescence

In order to test whether chemiluminescence was originating from endocytotic vesicles, the effect of an inhibitor of phagocytosis, cytochalasin B (Allison, 1973), was investigated. Pre-incubation of cells with cytochalasin B totally abolished the chemiluminescence induced by latex beads. However, there was an approx. 20-fold increase in chemiluminescence induced by chemotactic peptide (Fig. 2). Furthermore, the characteristic monophasic chemiluminescence response was transformed into biphasic response. The concentration of cytochalasin B used was shown to inhibit fluid-phase uptake accompanying phagocytosis of latex beads (i) by fluorescence microscopy of 6-carboxyfluorescein added extracellularly with the latex beads and (ii) by measurement of the fluorescent product of an extracellularly added substrate (4-methylumbelliferyl acetamido-2-deoxy-β-D-glucopyranoside) of hexosaminidase, a lysosomal enzyme. The possibility that inhibition was the result of a non-specific toxic effect was excluded by showing that the same population of cytochalasin B-treated cells that failed to respond to latex beads was still able to produce oxygen radicals in response to chemotactic peptide. These results showed that (i) cytochalasin B inhibited phagocytosis and (ii) phagocytosis was an essential step in the chemiluminescence-generating mechanism of the cells only when stimulated by unopsonized latex beads.

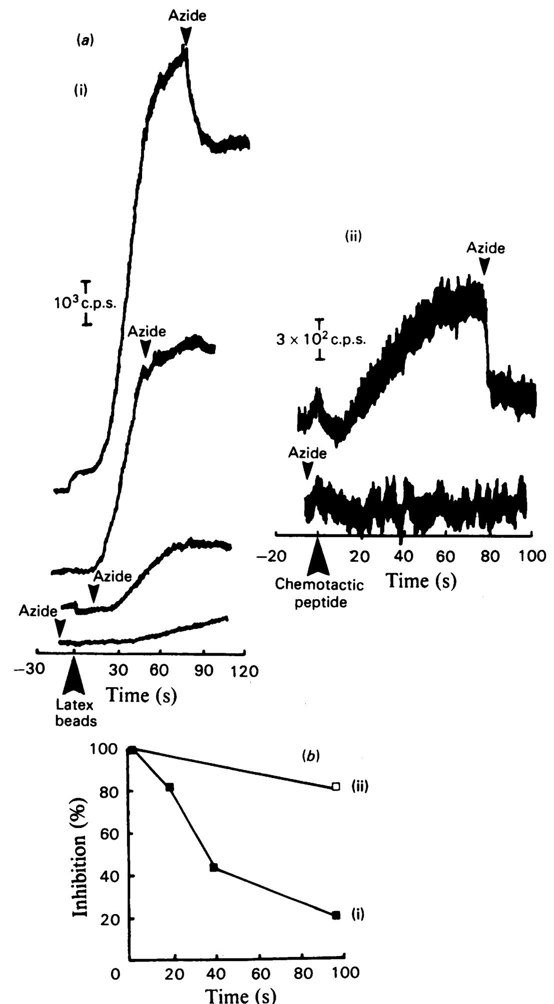

Fig. 1. *Effect of azide on luminol chemiluminescence in response to latex particles or chemotactic peptide*

(*a*) The traces show the luminol chemiluminescence response from approximately 10^6 PMN when stimulated (i) with latex beads (4×10^9/ml) or (ii) with chemotactic peptide (1 μM) added as indicated by the large arrow head. At the time indicated on the traces by the small arrow head, azide (1 mM) was added to the cell suspension while chemiluminescence was being monitored. Luminescence is shown on the calibration marker as luminescence counts per second (c.p.s.). (*b*) The graph shows the percentage inhibition caused by azide (1 mM), at various times, in response to (i) latex beads and (ii) chemotactic peptide.

Secretion of myeloperoxidase

It has been reported that PMN treated with cytochalasin B secrete myeloperoxidase in response to a number of physiological and experimental stimuli (Davies *et al.*, 1971; Malawista *et al.*, 1971; Zigmond & Hirsch, 1972). Since peroxidases catalyse luminol chemiluminescence the possibility existed that the stimulation of chemiluminescence by chemotactic peptide and its enhancement by cytochalasin B was dependent upon the release of myeloperoxidase. In the absence of cytochalasin B

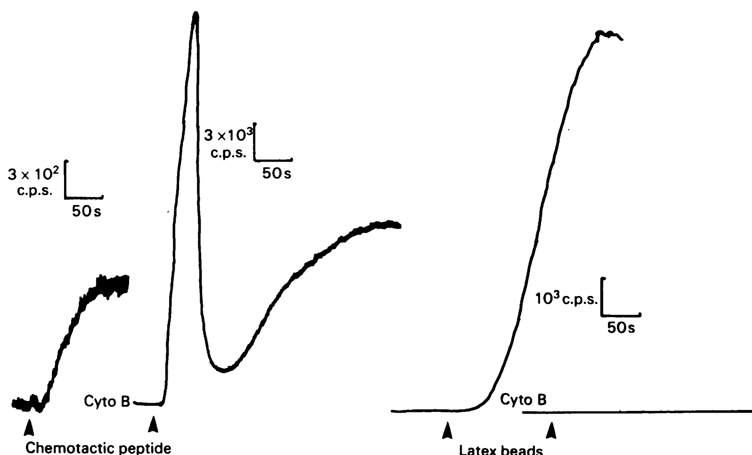

Fig. 2. *Effect of cytochalasin B (Cyto B) on luminol chemiluminescence response*
The chemiluminescent traces show the effect of pre-incubating the cells for 5 min at 37°C with cytochalasin B (5 μg/ml) before addition of the stimulus at the point indicated on the trace by the arrow head (chemotactic peptide at 1 μM; unopsonized latex particles at 4 x 10^9/ml; cytochalasin B at 5 μg/ml). The calibration marker shows scale of the luminescence trace in luminescence counts per second (c.p.s.), and it should be noted that the first trace is amplified with respect to the other three.

there was no detectable release of myeloperoxidase from PMN into the medium in response to chemotactic peptide (Table 1). However, after pre-incubation with cytochalasin B, chemotactic peptide evoked a large release of myeloperoxidase. The secretion was dependent upon external Ca^{2+}, being inhibited by approx. 80% in the absence of external Ca^{2+} (i.e. in the presence of 1 mM-EGTA). Secretion was also stimulated by the Ca^{2+} ionophore A23187. In contrast with chemotactic peptide, the addition of latex particles to either untreated cells or cells pre-treated with cytochalasin B did not evoke any detectable release of myeloperoxidase (Table 1).

The time course of myeloperoxidase secretion was compared with the time course of oxygen-radical production (Fig. 3). Secretion of myeloperoxidase was initiated during the first phase of oxygen-radical production and was complete within approx. 100 s, when the second phase in chemiluminescence was observed.

Table 1. *Secretion of myeloperoxidase*
Results are means ± S.E.M. for three determinations. Cytochalasin B (5 μg/ml) dissolved in dimethyl sulphoxide (final dimethyl sulphoxide concn. 0.1%, v/v) or dimethyl sulphoxide alone was added to cells 5 min before the stimulus. After a further 10 min incubation at 37°C the cells were centrifuged and the secretion of myeloperoxidase was determined.

	Enzyme secretion (%)	
	Stimulus	Stimulus + cytochalasin B
No stimulus	0.5 ± 0.25	1.4 ± 0.21
Latex beads (4 x 10^9/ml)	0.5 ± 0.25	1.7 ± 0.10
Chemotactic peptide (1 μM)	0.6 ± 0.31	46.2 ± 3.8
Ionophore A23187 (1 μM)	7.5 ± 0.10	24 ± 0.25

Effect of external peroxidase in the chemiluminescent response

The possibility existed (i) that the enhancement by cytochalasin B of the response to chemotactic peptide was due to externalization of myeloperoxidase, and (ii) that the biphasic shape of the response correlated with the time of its release. In order to test this, the effect of external peroxidase activity on the PMN chemiluminescent response was investigated.

Microperoxidase, a proteolytic product of cytochrome *c* (Harbury & Loach, 1960), was added to cells before the addition of stimulus. The concentration of microperoxidase used had a peroxidase activity approximately equivalent to 20% secretion of myeloperoxidase when compared by the spectrophotometric assay. This concentration of microperoxidase had no effect on the chemiluminescent response stimulated by latex beads. However, the response stimulated by chemotactic peptide was enhanced by external peroxidase (Fig. 4). In the absence of external peroxidase, the chemiluminescence rose after a lag of approx. 40 s, reaching a

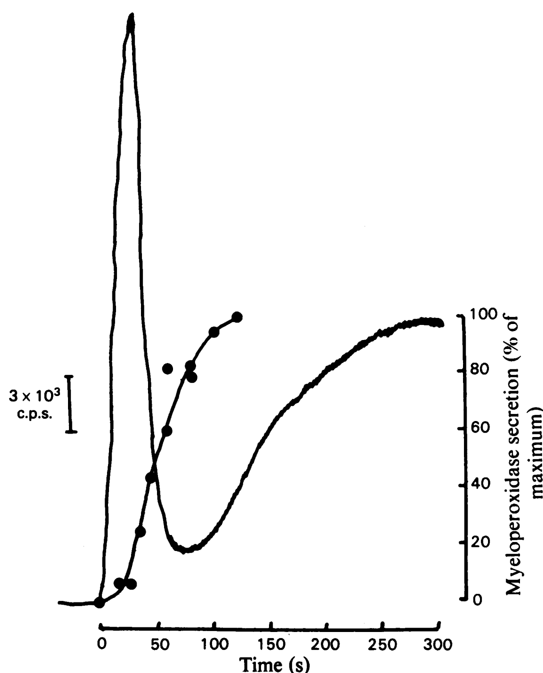

Fig. 3. *Time course of myeloperoxidase secretion in relationship to chemiluminescence response*

Cells (10^6/ml) were pre-incubated for 5 min with cytochalasin B (5 μg/ml) at 37°C before addition of chemotactic peptide (1 μM) at time zero. The chemiluminescence trace is shown. In a parallel set of tubes, the reaction was stopped by rapid centrifugation and separation of supernatant from the cells. The secretion of myeloperoxidase is shown (●) as a percentage of the maximum.

plateau within approx. 2 min, whereas in the presence of external peroxidase there was a very rapid luminescence spike occurring after a lag of only 10 s. This result was therefore consistent with chemiluminescence observed with chemotactic peptide being limited by the rate of peroxidase externalization.

Discussion

Oxygen-radical production by PMN and macrophages plays a crucial role in the killing of endocytosed micro-organisms (Babior *et al.*, 1973; Weening *et al.*, 1975). If these highly reactive species of oxygen are to be established as major pathogens in diseases such as rheumatoid arthritis, then it is essential that the molecular basis of stimuli that provoke external production is characterized. It is well established that intracellular Ca^{2+} is the trigger of many vesicular exocytotic cells, whereas the role of intracellular Ca^{2+} in endocytosis is confused (Rubin, 1982; Campbell, 1983). The possibility therefore existed that radical production provoked by stimuli, such as chemotactic peptide, dependent on an increase in intracellular free [Ca^{2+}], might cause release of radicals, whereas phagocytic stimuli might produce radicals mainly intracellularly. Three approaches were adopted to investigate this. First, the time-dependence of inhibition by azide of PMN chemiluminescence was investigated. Secondly, the effect of an inhibitor of phagocytosis, cytochalasin B, on chemiluminescence of PMN was investigated.

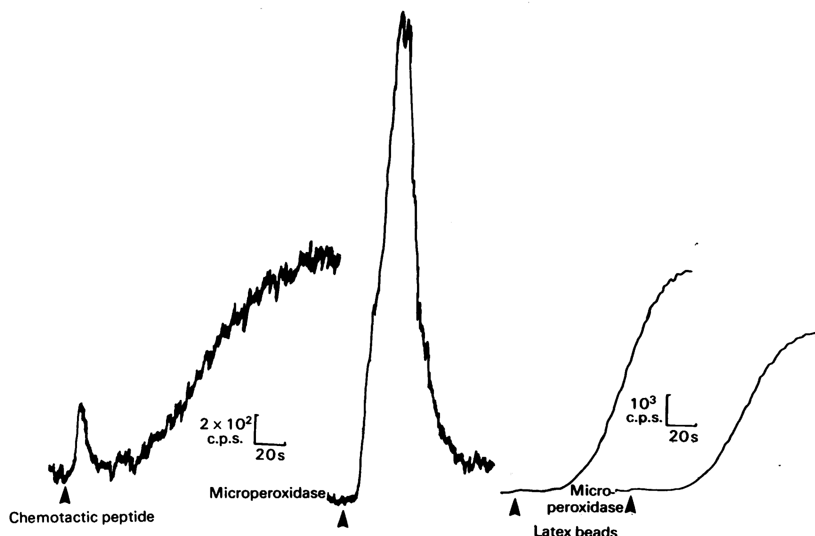

Fig. 4. *Effect of microperoxidase on luminol chemiluminescence response*

The chemiluminescence traces labelled 'Microperoxidase' show that responses from cells in Hepes-buffered Krebs medium with microperoxidase (10 μg/ml) but no bovine serum albumin. The unlabelled traces are from cells in similar medium but without microperoxidase added. At the arrows, the stimuli were added (chemotactic peptide at 1 μM; latex beads at 4 × 10^9/ml.

Thirdly, chemiluminescence was correlated with enzyme secretion, and in particular myeloperoxidase.

It was concluded that oxygen-radical production as monitored by luminol chemiluminescence could be stimulated by one of at least two mechanisms. Unopsonized latex particles stimulate chemiluminescence at a site inaccessible to azide that is dependent upon the ability of the cells to phagocytose. Furthermore, the chemiluminescence generated at this site is not limited to peroxidase activity. This is consistent with the site of oxygen-radical production being within peroxidase-containing phagosomes. In contrast, chemotactic peptide stimulated chemiluminescence from a site that was accessible to azide inhibition, and was not dependent on the ability of the cells to phagocytose. Furthermore, the chemiluminescence could be enhanced either by inducing secretion of myeloperoxidase or by adding external peroxidase. This is consistent with the site of oxygen-radical production being external to the cell and dependent upon exocytosis. The two proposed routes by which chemiluminescence is generated are shown diagrammatically (Fig. 5).

The existence of two mechanisms of oxygen-radical production was predicted from measurements of intracellular free [Ca^{2+}] in oxygen-radical-producing hybrid cells (Hallett & Campbell, 1982). It was found that, whereas both chemotactic peptide and unopsonized particles stimulated oxygen-radical production, only chemotactic peptide stimulated a rise in intracellular [Ca^{2+}] (Campbell & Hallett, 1983). The rise in intracellular [Ca^{2+}] was shown to be essential for chemotactic peptide to stimulate both the 'oxygen burst' and oxygen-radical production monitored by chemiluminescence. It is now proposed that this requirement for intracellular Ca^{2+} leads to extracellular production of oxygen radicals through an activation of the radical-generating pathway. In contrast with chemotactic peptide, unopsonized particles stimulate oxygen-radical production into intracellular vesicles by a mechanism that is independent of a rise in intracellular [Ca^{2+}], and therefore it must now be concluded that both phagocytosis and fusion of phagosomes with lysosomes are also independent of a rise in intracellular [Ca^{2+}]. The finding that unopsonized latex particles failed to stimulate secretion from cytochalasin B-treated cells provides further evidence that the interaction of unopsonized particles with the cell surface does not cause an increase in intracellular free [Ca^{2+}], since agents that do stimulate a rise in intracellular free [Ca^{2+}], chemotactic peptide or ionophore A23187, stimulated secretion. A similar lack of secretory stimulation has been reported for unopsonized zymosan particles on human PMN (Goldstein *et al.*, 1975).

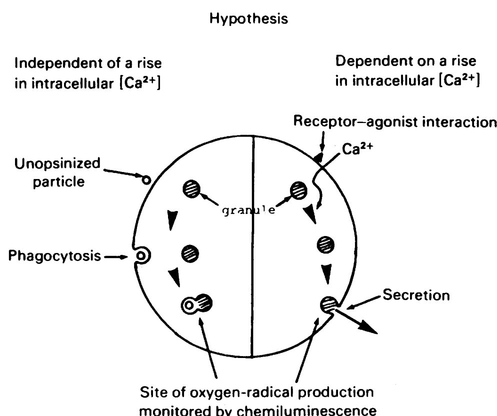

Fig. 5. *Proposed two mechanisms of oxygen-radical production*

The existence of the two mechanisms of oxygen-radical production has important implications for the pathogenesis of some inflammatory diseases, such as rheumatoid arthritis. It has been proposed that the external production of toxic radicals of oxygen may cause damage to the extracellular matrix and to nearby cells (Fridovitch, 1979). The endocytosis-dependent mechanism of production would represent a physiological event whereby endocytosed bacteria were destroyed without the release of toxic oxygen radicals, whereas the intracellular Ca^{2+}-dependent route would represent a pathological event whereby damage to adjacent tissue would result. In view of the enhancement of extracellular oxygen-radical production induced by cytochalasin B (Fig. 2; Williams & Cole, 1981), it is now necessary to investigate whether such fungal products occur in man under pathological conditions. Possible naturally occurring stimuli of PMN are C3b, C5a, the membrane attack complex of complement, immune complexes (Fc) and viruses. These now require classification into the two classes of stimuli in order to identify which are likely to be involved in the pathogenesis of inflammatory diseases such as rheumatoid arthritis through an oxygen-radical-mediated mechanism.

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