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# Stimulation of $Ca^{2+}$-dependent chemiluminescence in rat polymorphonuclear leucocytes by polystyrene beads and the non-lytic action of complement

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**Summary.** (1) Chemiluminescence of rat polymorphonuclear leucocytes was stimulated by a phagocytic stimulus, latex beads (diameter = 1·01 $\mu$m). The maximum chemiluminescent intensity increased with bead concentration in the range 0·2–20 $\times 10^9$ beads/ml. This response was abolished in the absence of extracellular $Ca^{2+}$ (1 mM EGTA).

(2) Chemiluminescence could also be stimulated by the $Ca^{2+}$ ionophore A23187 in the presence of extracellular calcium.

(3) Addition of human serum, as a source of complement, to rat polymorphonuclear leucocytes preincubated with anti-5′-nucleotidase serum resulted in a rapid stimulation of chemiluminescence, after a lag of about 40 s.

(4) The stimulation of chemiluminescence by antibody plus complement was not the result of cell lysis because (i) no significant release of lactate dehydrogenase was detected at the time of the chemiluminescent response (ii) chemiluminescence was associated with the cells and not the surrounding media (iii) cell lysis did not produce chemiluminescence.

(5) Chemiluminescence stimulated by antibody plus complement or by beads was inhibited by the 'calmodulin-blocker', trifluoperazine (50% inhibition with approximately 20–30 $\mu$M).

(6) $Cu^{2+}$ ($10^{-4}$ M), which can inhibit C9 action, inhibited the rapid rise in chemiluminescence induced by antibody plus complement, but not the bead-induced chemiluminescence.

(7) Depletion of C9 from human serum markedly inhibited the complement induced chemiluminescence response. Addition of purified C9 restored the response.

(8) It was concluded that formation of the terminal complement attack complex at the surface of rat polymorphonuclear leucocytes induces a $Ca^{2+}$-dependent chemiluminescence in the cells, in the absence of cell lysis.

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## INTRODUCTION

It has been known for many years that the activation of complement by antibody bound to cell surface antigens can cause lysis of the cell. However, it has also been reported that the activation of complement can lead to disturbances in cell function without lysis (Fell, Coombs & Dingle, 1966; Griffiths, 1971; Raisz, Sandberg, Goodson, Simmons & Mergenhayer, 1974; Ito, Miledi & Vincent, 1974; Cines & Schreiber, 1979;

Stevens and Henkart, 1979). The intracellular chemical mechanisms involved in these non-lytic effects are unknown.

Changes in intracellular free $Ca^{2+}$ concentration mediate the effects of many cell stimuli and drugs, and are involved in cell injury caused by toxins and anoxia (Rasmussen & Goodman, 1977; Duncan, 1978; Ashley & Campbell, 1979). Using erythrocyte 'ghosts' we have shown that formation of the terminal complement attack complex $\overline{C5b6789}$, causes a rapid increase in intracellular free $Ca^{2+}$ from less than 0·3 $\mu$M to 1–30 $\mu$M (Campbell, Daw & Luzio, 1979; Campbell, Daw, Hallett & Luzio, 1981).

The aim of the work reported in this paper was to show that this rapid increase in intracellular $Ca^{2+}$ could cause non-lytic changes in cell function. The chemiluminescence of rat polymorphonuclear leucocytes was investigated since this response can be detected very rapidly with intact cells, and is thought to be $Ca^{2+}$-dependent (Allen, Stjernholm & Steele, 1972; Wilson, Trush, Van Dyke & Neal, 1978).

A preliminary report of some of the findings has been presented (Hallett, Newby, Luzio and Campbell, 1980).

## MATERIALS AND METHODS

*Chemicals*

Sodium caseinate (Nutrose) was purchased from Difco Laboratories, Detroit, U.S.A. and Ficoll-Paque from Pharmacia Fine Chemicals, Uppsala, Sweden. Luminol (5-amino-2,3-dihydro-1,4 phthalazinedione) were purchased from B.D.H. Chemicals, Poole, Dorset and the calcium ionophore A23187 from Calbiochem. Behring Corp., La Jolla, U.S.A. Bovine serum albumin (fraction V) was obtained from Armour Pharmaceuticals Co., Eastbourne, Sussex. Latex beads ($d = 1{\cdot}01$ $\mu$m) were purchased from Dow Chemical Co., Indianapolis, U.S.A. All other chemicals were of 'AnalaR' grade and purchased from B.D.H. Chemicals. Trifluoperazine dihydrochloride (Stelazine) was a kind gift from Smith, Kline and French Laboratories, Herts.

*Preparation of rat polymorphonuclear leucocytes*

Rat polymorphonuclear leucocytes (PMNs) were prepared by the method described by Newby (1980). Twelve to fourteen hours after intraperitoneal injection of Wistar rats (200–300 g) with sterile sodium caseinate (Nutrose), the peritoneal exudate was collected. PMNs were separated from red blood cells and other white cells by treatment with isotonic $NH_4Cl$ and centrifuged through Ficoll-Paque (Pharmacia). The cells were then suspended in Krebs-Ringer-Hepes medium, containing 120 mM NaCl, 4·8 mM KCl, 1·2 mM $MgSO_4$, 1·2 mM $KH_2PO_4$, 1·3 mM $CaCl_2$, 0·1% (w/v) bovine serum albumin and 25 mM Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] adjusted to pH 7·4 with NaOH. One rat yielded approximately $10^8$ cells which were more than 98% PMNs after purification.

*Preparation of serum as a source of complement*

Human blood was taken from normal volunteers and, after clotting, serum to be used as a source of complement was separated and stored at −70°.

*Preparation of purified C9*

Human complement component C9 was purified by the method of Biesecker & Müller-Eberhard (1980). From the hydroxylapatite column a fraction was collected which yielded only one main band on polyacrylamide electrophoresis and was between 50%–75% pure C9. It was approximately five times more concentrated compared with plasma used in the following experiments. There was no detectable C1q, C3, C4, C5, C1 inhibitor or factor B present, and contamination from complement components was calculated to be less than 1%. Only one rocket was detected in immunoelectrophoresis. The main band eluted from 7% polyacrylamide gels was C9 as shown by reconstitution of the terminal attack complex with $\overline{C5b678}$ (kind gift of Professor P. J. Lachmann) and used for generation of antibodies to C9 and for further experiments.

*Preparation of antisera*

Rabbit anti (rat liver 5′-nucleotidase) serum was prepared as previously described (Stanley, Edwards & Luzio, 1981), was heat treated at 56° for 30 min in order to inactivate complement, and stored at −20°. This antiserum inhibited rat PMN 5′-nucleotidase (Newby, 1980), and after addition of human serum as a source of complement caused lysis of PMNs within 30–60 min at 37°, as shown by release of lactate dehydrogenase.

Antiserum to C9 was prepared by injecting rabbits intradermally with 20–50 $\mu$g C9 in an emulsion of Freund's complete adjuvant. After 4 weeks the rabbits were given a second injection, using Freund's incomplete adjuvant. The rabbits were bled after a further 10

days, the serum collected, heated at 56° for 30 min and stored at −20°.

*Preparation of C9 depleted serum*

Antiserum to C9 was used to remove C9 from whole human serum by immunoprecipitation and shown by rocket immunoelectrophoresis. Four hundred microlitres of antiserum were added to 1 ml of human serum containing EDTA (5·4 mM) and incubated at room temperature for 1 hr. The immunoprecipitate was removed by centrifugation at 12,000 *g* for 2 min. Control serum was prepared using non-immune rabbit serum in place of anti-C9 antiserum. There was no detectable loss of C3 or C5, as shown by immunoelectrophoresis.

*Measurement of PMN chemiluminescence*

Luminescence was measured using a purpose built apparatus as previously described (Campbell, 1974; Campbell, Lea & Ashley, 1979). The sample (0·5 ml) was placed in a thermostatically-controlled light-tight housing in front of a photomultiplier tube (Centronics, P4232B). Additions could be made with a syringe to the sample whilst in front of the photomultiplier tube. Luminescence was recorded using a chart recorder or scalar as luminescence counts per second (c.p.s.). When luminol was added to the cells to potentiate the chemiluminescence it was first dissolved in dimethyl sulphoxide (DMSO) and added to the cell suspension to give a final concentration of 11 μM luminol and 0·1% v/v DMSO.

## RESULTS

### The chemiluminescence of rat polymorphonuclear leucocytes

Addition of polystyrene beads, as a phagocytic stimulus, to rat PMNs resulted in a natural chemiluminescence (Fig. 1a). The light emission was increased approximately 1400-fold by the addition of 1·1 μM luminol (Fig. 1b). The chemiluminescence increased a further five-fold with luminol concentration up to about 5 μM when a plateau was reached. Chemiluminescence was linearly related to concentration of polystyrene beads over the range $0{\cdot}2–20 \times 10^9$ beads/ml (Fig. 2), and to the cell concentration over the range $10^4–10^8$ cells/ml. All further experiments were done using 11 μM luminol with a cell concentration of approximately $10^6$ cells/ml.

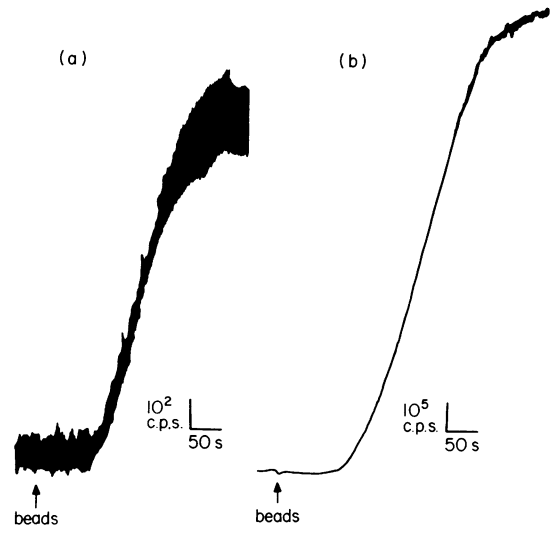


**Figure 1.** (a) Rat polymorphonuclear leucocytes ($1{\cdot}2 \times 10^7$ cells/ml) were incubated for 2 min before addition of beads ($2 \times 10^{10}$ beads/ml) at the arrow. (b) Rat polymorphonuclear leucocytes ($1{\cdot}2 \times 10^7$ cells/ml) were incubated for 2 min with luminol, 1·1 μM dissolved in dimethylsulphoxide (final concentration 0·1%, v/v), before addition of latex beads ($2 \times 10^{10}$ beads/ml). Luminescence was monitored at 37°, and luminescence rate measured in luminescence counts per second (c.p.s.).

### Dependence of rat polymorphonuclear leucocyte chemiluminescence on $Ca^{2+}$

In order to test that the chemiluminescence of rat PMNs was dependent on $Ca^{2+}$ the effect of the $Ca^{2+}$ chelator EGTA and the $Ca^{2+}$ ionophore A23187 were studied.

Addition of beads in the absence of extracellular $Ca^{2+}$ and presence of EGTA (1 mM) resulted in abolition of the chemiluminescence (Fig. 3a). Addition of the calcium-ionophore A23187 (1·9 μM) in the absence of beads stimulated chemiluminescence, in the presence of extracellular $Ca^{2+}$ (Fig. 3b).

### Non-lytic effect of anti-5′-nucleotidase antibody plus complement on polymorphonuclear leucocyte chemiluminescence

Incubation of PMNs with antiserum raised against the surface antigen ectoenzyme 5′-nucleotidase, caused an increase in chemiluminescence after addition of human serum as a source of complement (Fig. 4). This increase occurred approximately 40 s after addition of

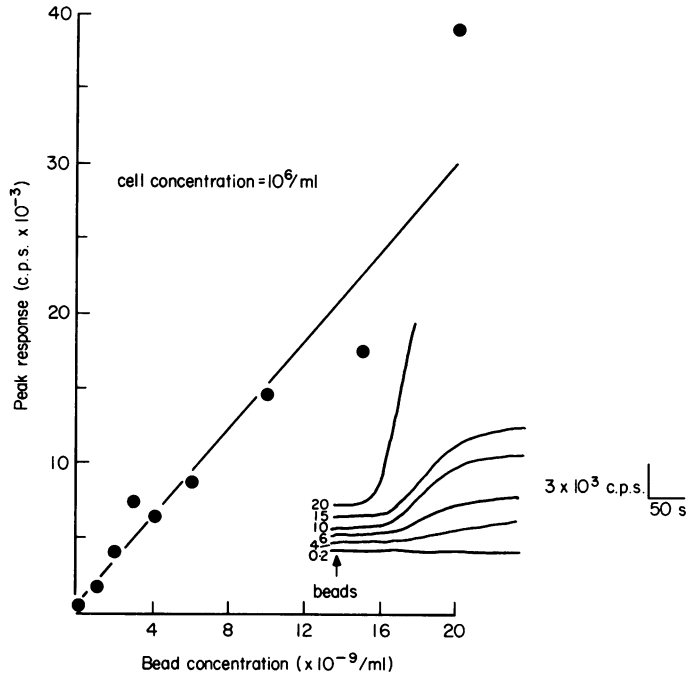


**Figure 2.** Rat polymorphonuclear leucocytes ($10^6$/ml) were incubated with luminol (11 $\mu$M); DMSO (0·1%) for 2 min before addition of latex beads in the final concentration shown. Monitoring of luminescence was at 37°, and the responses are shown in the insert. Peak luminescence rate (c.p.s.) is shown against bead concentration.

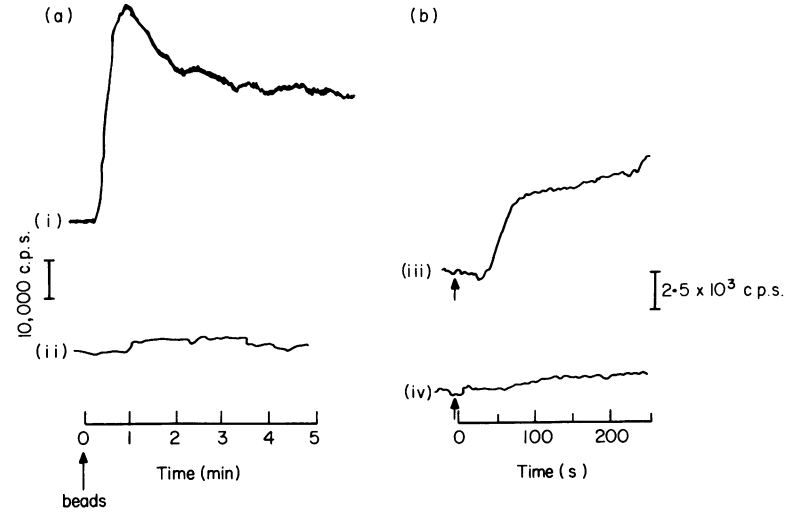


**Figure 3.** (a) Cells were suspended in Krebs-Ringer-Hepes buffer containing either: (i) $Ca^{2+}$, 1 mM or (ii) EGTA, 1 mM with no added $Ca^{2+}$. The external $Ca^{2+}$ concentration in (ii) was less than $10^{-9}$ M. Beads ($4 \times 10^9$/ml) were added and luminescence recorded. (b) Cells were suspended in Krebs-Ringer-Hepes buffer containing $Ca^{2+}$, 1·3 mM. Luminescent trace (iii) shows the effect of adding the $Ca^{2+}$ ionophore A23187 (1·9 $\mu$M) dissolved in DMSO (final concentration = 0·1%) and (iv) shows the effect of DMSO (0·1%) alone.

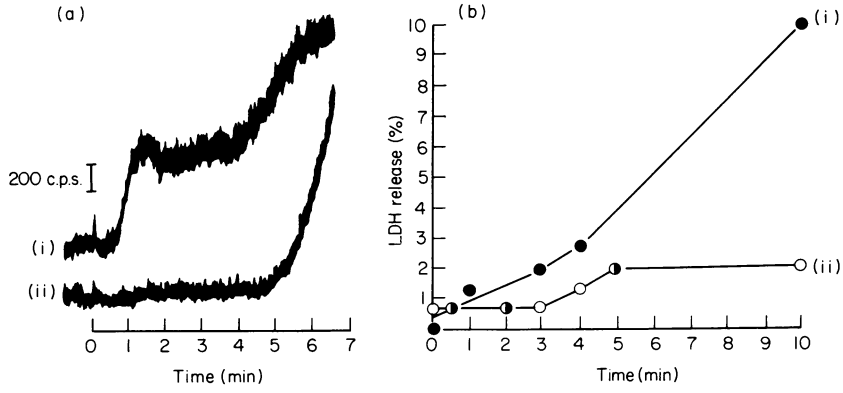


**Figure 4.** (a) Luminescence trace (i) shows the effect of preincubating cells ($10^6$/ml) with rabbit anti-5′-nucleotidase antiserum (1/50 dilution) for 2 min at 37°. At zero time, human serum (1/20 dilution) was added to the cells. Trace (ii) shows the effect of preincubating the cells with non-immune rabbit serum (1/50 dilution) before addition of human serum (1/20 dilution). (b) Lactate dehydrogenase (LDH) activity was measured spectrophotometrically. LDH released into the incubating medium was measured at timed intervals and expressed as a percentage of the total LDH in the cell suspension. Graphs (i) and (ii) refer to the same conditions as shown in Fig. 1a, and half-closed symbols are points common to both (i) and (ii).

the human serum. The antisera used in these experiments caused no increase in chemiluminescence in the absence of human serum or in the presence of heat-treated (56°, 30 min) human serum.

The chemiluminescence observed with antibody plus complement was biphasic. The first phase was dependent on antibody and reached a maximum intensity within 2–3 min, like the bead and ionophore responses (Fig. 4a). The second phase began approximately 5 min after the addition of human serum and continued to rise for at least 15–30 min. A slow response also occurred in the absence of antibody, but not when heated (56°, 30 min) human serum was used (Fig. 4a).

The effect of antibody plus complement was not the result of cell lysis. This was shown as follows: (i) less than 1% of total lactate dehydrogenase was released by antibody plus complement during the time course of PMN chemiluminescence (Fig. 4b); (ii) lysis of the cells by hypo-osmotic shock or detergent (scintillation grade triton X-100) did not stimulate PMN chemiluminescence, nor was chemiluminescence observed after subsequent addition of beads. The lysis conditions did not inhibit the reaction of luminol with $H_2O_2$ *in vitro*; (iii) centrifugation of the cells (1000 ***g*** for 1 min) showed that chemiluminescence was associated with the cells and not the extracellular medium. Homogenization of cells stimulated by antibody plus complement or beads released the chemiluminescence. The chemiluminescence was found in the supernatant after centrifugation of the homogenate at 1000 ***g*** for 2 min, but in the pellet after centrifugation at 10,000 ***g*** for 15 min.

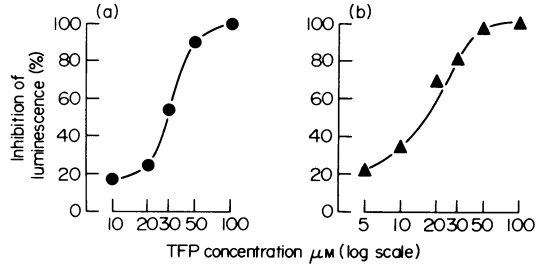


**Figure 5.** The effect of preincubation of cells with trifluoperazine (TFP) for 2 min before stimulation either with (a) rabbit anti-5′-nucleotidase serum (1/50 dilution) plus human serum (1/20 dilution) or (b) latex beads ($4 \times 10^9$/ml). The ordinate shows the percentage inhibition of peak luminescence calculated from the formula: % inhibition = (1 − (Li)/(Lo)) 100, where Li = peak luminescence in the presence of inhibitor, and Lo = peak luminescence in the absence of inhibitor.

### Effect of trifluoperazine

Trifluoperazine (1–100 μM), a compound shown to block the action of calmodulin *in vitro* (Levin & Weiss, 1977), inhibited PMN chemiluminescence induced by beads and antibody plus complement (Fig. 5). The concentration of trifluoperazine that caused 50% inhi-

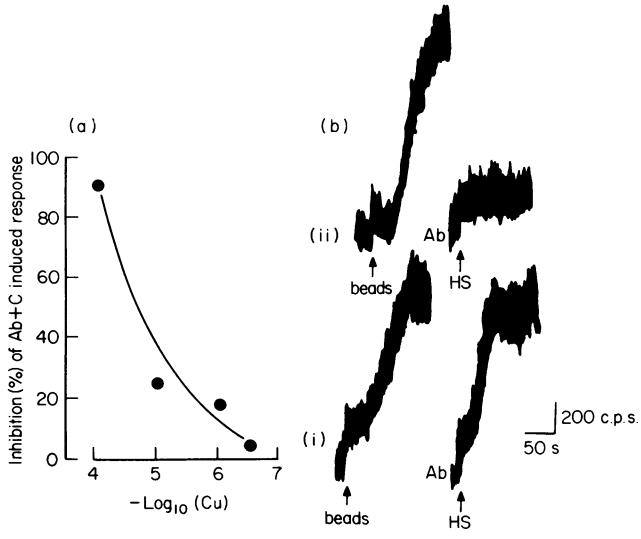


**Figure 6.** (a) Polymorphonuclear leucocytes ($2 \times 10^6$/ml) were incubated for 2 min with rabbit anti-5′-nucleotidase serum (1/50 dilution) before addition of human serum (1/20 dilution) containing $CuSO_4$ to give the final concentration shown on the abscissa. The ordinate shows the percentage inhibition with respect to the uninhibited control. (b) Comparison of effect of $Cu^{2+}$ on bead-induced and antibody plus complement-induced responses. (i) Latex beads ($4 \times 10^8$ beads/ml) were added to PMNs ($2 \times 10^6$/ml) to give a response of comparable magnitude to that produced by antibody (1/50 dilution) and human serum (1/20 dilution). (ii) Stimulation of PMNs was repeated in the presence of $CuSO_4$, $10^{-4}$ M.

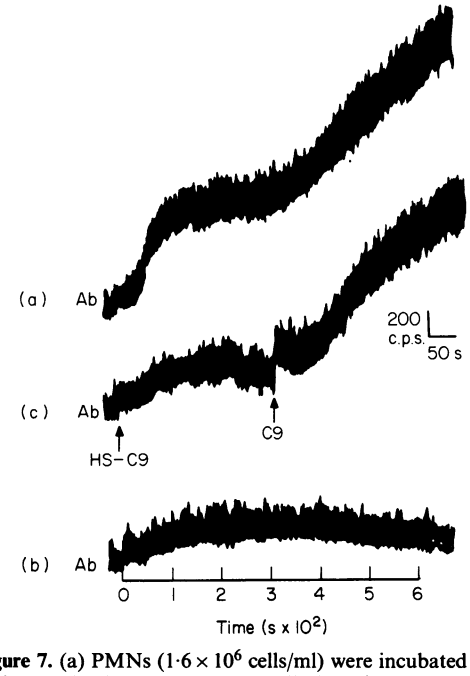


**Figure 7.** (a) PMNs ($1{\cdot}6 \times 10^6$ cells/ml) were incubated with anti-5′-nucleotidase serum (1/20 dilution) for 2 min before addition at time zero of human serum (1/8 dilution) pretreated with non-immune serum as described in the Materials and Methods section. (b) PMNs were incubated with antiserum as in (a) before addition at time zero of human serum (1/8 dilution) pretreated with anti-C9 antiserum (see Materials and Methods section) to produce C9-depleted serum. (c) After incubation of PMNs with antiserum, C9-depleted serum were added as in (b). After 300 s, purified C9 (10 $\mu$l) was added.

bition of PMN chemiluminescence was approximately 20–30 $\mu$M for either stimulus.

### Requirement for C9 in stimulation of PMN chemiluminescence by complement

From studies on the erythrocyte 'ghost' model system, it was found that the increase in intracellular calcium induced by antibody plus complement depended upon binding of C9 to the terminal complex (Campbell *et al.*, 1981). The possibility that PMN stimulation was the result of increased intracellular calcium induced by terminal complex formation and C9 binding was therefore investigated.

The ability of the terminal complement attack complex to cause cell lysis can be inhibited by $Cu^{2+}$ over the range $10^{-4}$–$10^{-5}$ M (Yamamoto & Takahashi, 1975; Boyle, Langone & Borsos, 1979). $CuSO_4$

($10^{-6}$–$10^{-4}$ M) inhibited the rapid PMN chemiluminescence caused by antibody plus complement (Fig. 6a). However, the chemiluminescence induced by beads was not inhibited by $10^{-4}$ M $CuSO_4$ (Fig. 6b).

Depletion of C9 from serum markedly inhibited the chemiluminescence response stimulated by antibody plus complement (Fig. 7a). However, if purified C9 was added back to cells treated with antibody and C9-depleted serum, a rapid stimulation of chemiluminescence was observed (Fig. 7b). This stimulation by the addition of C9 was dependent upon the presence of calcium in the extracellular medium and was completely abolished by EGTA (12 mM) sufficient to chelate the $Ca^{2+}$ present. $Ca^{2+}$ was present initially to allow C1 binding to antibody and formation of the $\overline{C5b678}$ complex on the PMN membrane, EGTA was then added before addition of C9.

## DISCUSSION

The chemiluminescence of PMNs due to the production of peroxide moieties (Klebanoff & Clark, 1978) requires extracellular $Ca^{2+}$, can be induced by $Ca^{2+}$ ionophore A23187 and is inhibited by trifluoperazine, a known inhibitor of calmodulin action (Figs 4 and 7). Activation of the complement pathway at the cell surface induced PMN chemiluminescence over a similar time scale to polystyrene beads or $Ca^{2+}$ ionophore A23187. The effect on chemiluminescence of complement activation was inhibited by concentrations of trifluoperazine similar to those required to inhibit the effect of polystyrene beads. These data are consistent with the hypothesis that a rise in intracellular free $Ca^{2+}$ is required for induction of PMN chemiluminescence.

Polymorphonuclear leucocytes could respond to the activation of complement in any of four ways: (i) lysis caused by the terminal attack complex $\overline{C5b6789}$; (ii) phagocytosis of particles opsonized by C3b (Easmon, Cole, Williams & Hastings, 1980); (iii) chemotaxis caused by receptors to the complement components C3a, C5a and $\overline{C5b67}$ (Klebanoff & Clark, 1978); and (iv) a rise in intracellular $Ca^{2+}$ induced by C9 binding to the terminal complex (Campbell *et al.*, 1981).

PMN chemiluminescence caused by anti-cell antibody plus complement could not be explained by cell lysis (Fig. 4a, b).

Although it is possible to stimulate PMN chemiluminescence with antibody-coated cell fragments this could not account for the effects reported in the present paper. The PMNs used were >98% intact as shown by lactate dehydrogenase in the medium and did not respond to antibody alone. Opsonization of particles by complement could also be ruled out as a stimulus of PMN chemiluminescence in these experiments since addition of antibody-coated human erythrocytes plus complement did not activate the PMN response.

The possibility that stimulation was the result of a diffusible factor generated by the antibody–complement interaction was discounted by two observations: (i) extracellular fluid from PMNs stimulated by antibody plus complement, did not induce rapid stimulation of chemiluminescence in fresh PMNs; (ii) in a mixed population of rat PMNs and antibody-coated human erythrocytes, addition of human serum did not stimulate rapid chemiluminescence from the rat PMNs.

Stimulation of PMN chemiluminescence by antibody plus complement was dependent upon the presence of C9 (Fig. 7), and stimulation on replacing C9 was dependent upon extracellular calcium. Both of these observations are consistent with PMN stimulation resulting from terminal complex formation in the cell membrane, causing an increase in intracellular calcium.

The observation that $Cu^{2+}$ inhibited anti-cell antibody plus complement-induced PMN chemiluminescence but not the bead response (Fig. 6) was consistent with a requirement for C9 (Boyle, Langone & Borsos, 1979) in the terminal complex.

It was concluded that activation of complement causes a non-lytic production of peroxide moieties inside PMNs. The data are consistent with the hypothesis that formation of the complete terminal complex including C9 can cause a rise in intracellular $Ca^{2+}$ which then stimulates the cell (Hallett *et al.*, 1980; Campbell *et al.*, 1981). Preliminary results with fused PMN-erythrocyte 'ghosts' hybrids containing the $Ca^{2+}$-activated photoprotein obelin (Ashley & Campbell, 1979) indicate that it is now possible to measure directly an increase in intracellular free $Ca^{2+}$ in PMNs and that the $Ca^{2+}$ concentration rises after addition of anti-cell antibody plus complement.

The results described in this paper therefore provide a basis for understanding the chemical mechanisms involved in activating cells in immune mediated diseases, such as the secretion of lysosomal enzymes which occurs in rheumatoid arthritis.

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