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Activation of neutrophils by a chemically separated but optically coupled neutrophil population undergoing respiratory burst

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Abstract. Neutrophils from pig blood were used as a model system to investigate the optical communication between cells. It was found that neutrophils stimulated to undergo respiratory burst can activate a second, chemically separated, but optically coupled population of neutrophils. The response of the latter was visualized as a temporary rising of their low-level chemiluminescence and an enhanced generation of superoxide radicals detected by both the reduction of ferricytochrome c and spin trapping. The results provide evidence that a long-range optical coupling of biological significance between living cells exists.
Key words. Neutrophil; intercellular communication; optical coupling; respiratory burst; low-level chemiluminescence.

The 'communication' between cells is commonly believed to be mediated by speical 'messenger' molecules such as hormones, antibodies, growth factors and neurotransmitters, as well as some ions[1]. It has also been speculated that the intercellular 'communication' may to some extent be mediated by optical interaction[2–5]. However, there has been little experimental evidence of the latter.

To investigate 'communication' between cells by means of optical interaction, two considerations are of critical importance. First, what kind of information passing from 'source cells' to 'target cells' can and will be observed? Second, what kind of response of the target cells can be measured with sufficient sensitivity? It is well established that neutrophils and other phagocytes can be metabolically activated either by phagocytosis itself or by chemical stimuli[6–8]. The activation is characterized by increased glucose utilization via the hexose monophosphate shunt, and increased non-mitochondrial O_2 consumption, known as respiratory burst[9,10], as well as a relatively high chemiluminescence (CL)[11]. It is also widely accepted that the low-level chemiluminescence (LLCL) is intrinsically associated with the redox metabolism of the cells and can be used as an indicator of their physiological state[12]. Assuming that the respiratory chemiluminescence burst of a cell population may carry information capable of inducing phagocytosis and that the LLCL may be a sensitive indicator of the response of the target cells, isolated neutrophils from pig blood were used as a model system to investigate possible intercellular communication by optical interaction.

Surprisingly, it was found that one population of neutrophils undergoing respiratory burst can activate a second, chemically separated, but optically coupled population of neutrophils. The response of the latter was visualized as a temporary rising of its LLCL as well as an enhanced generation of superoxide radicals detected by both the reduction of ferricytochrome c and spin trapping. The results suggest that a long-range optical coupling of biological significance between living cells exists.

Materials and methods

Neutrophils. The neutrophils were isolated from heparinized pig blood (50,000 I.U. heparin sodium salt in 100 ml blood) according to the following procedure: in each of the glass tubes, 20 ml blood was mixed with 5 ml 4.5% Dextran (MW = 515,000, Sigma) in 0.9% NaCl solution. The red cells were allowed to settle for 45 min at 4 °C and the leukocyte-rich plasma (30 ml each) was layered on top of Histopaque-1077 (6 ml, Sigma) in sterile tubes. The neutrophils were obtained as a pellet after centrifugation of the tubes at 400 *g* for 20 min. Contaminating erythrocytes were removed by hypotonic lysis (30 s in 3.4 ml ice-cold distilled water, then adding 0.6 ml of 1 M NaCl) and centrifugation. The cells were finally washed twice with saline and resuspended in either Dulbecco's modified medium (DMEM) (without serum) or Hank's balanced salt solution (HBSS) containing 5 mM D-glucose. Neutrophil viability was checked by trypen blue exclusion and always found to be greater than 90%. All the tubes, glassware and pipets used for the isolation of the cells were sterilized, and great caution was taken to avoid any activation of neutrophils during the isolation procedure.

The experiment. The experimental setup is shown in figure 1. The neutrophil suspension was placed in three identical quartz cuvettes ($24 \times 24 \times 40$ mm^3) as de-

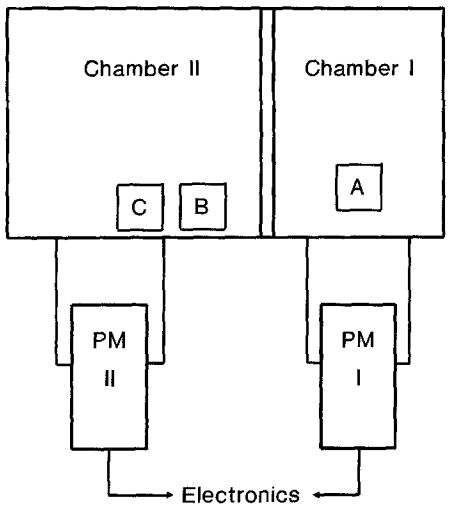

Figure 1. Diagram of the experimental setup for the investigation of optical coupling between two separated neutrophil populations. Two photomultipliers (PM I and II) continuously monitor the LLCL of the cells in cuvettes A and C.

scribed in the legend to figure 2. The cuvettes were placed in two combined dark chambers, each of them associated with a photomultiplier (EMI 9558QA) which was operated in single-photon counting mode and used to monitor the LLCL of the cell-containing cuvettes continuously. In order to reduce the dark counts, the photomultipliers (PM I and PM II) were cooled to −20 °C. The principle and technical details of each detecting system are similar to the measuring equipment reported by Ruth[13]. In the present investigation, the electric pulses corresponding to single photon incidents on each cathode of the PM were amplified, discriminated and registered by two PC computers through the multichannel scaler cards (Nucleus Inc., Oak Ridge). The cell suspension in each cuvette was stirred magnetically and kept at constant temperature of 34 °C by continuously low-power heating of the cuvette-holders. The two cuvettes B and C in chamber II were arranged so that the PM II detected the photons from B with an efficiency which was only 6.3% of that for detecting the photons from C. Therefore, it can be assumed that the photon counts registered by PM II originated almost exclusively from cuvette C. The cuvette B and C, separated by a distance of 5 mm, could 'see' each other, whereas cuvette A was completely isolated from B and C. Cuvette A serves as a control for C.

Throughout the investigation, we stimulated only the cells in cuvette B by the addition of phorbol myristate

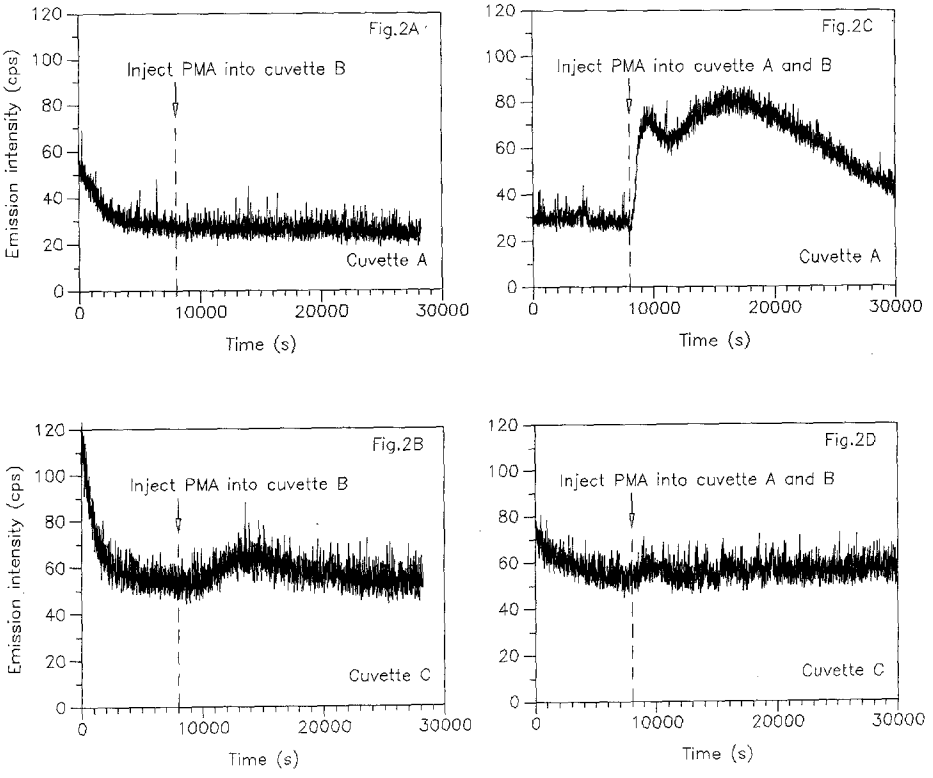

Figure 2. The temporary increase of low-level chemiluminescence of neutrophils induced by a separate neutrophil population undergoing respiratory burst. Two types of experiments were performed: 1) 10 ml of neutrophil suspension (1.1×10^7 cells/ml) were added to three identical quartz cuvettes A, B and C, which were then placed in the dark chambers for 8000 s to reach thermal equilibrium (34 °C) and eliminate any delayed luminescence. Then 1 ml of PMA solution was injected into cuvette B without opening the dark chamber. The LLCL of cuvettes A and C before and after the injection were recorded as shown in figure 2A and figure 2B respectively. 2) 10 ml of cell suspension (1.4×10^7 cells/ml) were added to each of three cuvettes, but the cells in cuvette C had been inactivated (50 °C for 30 min). 1 ml PMA was injected into A after the cuvettes had been in dark chambers for 8000 s. The LLCL of cuvettes A and C before and after the injection were recorded as shown in figure 2C and figure 2D respectively.

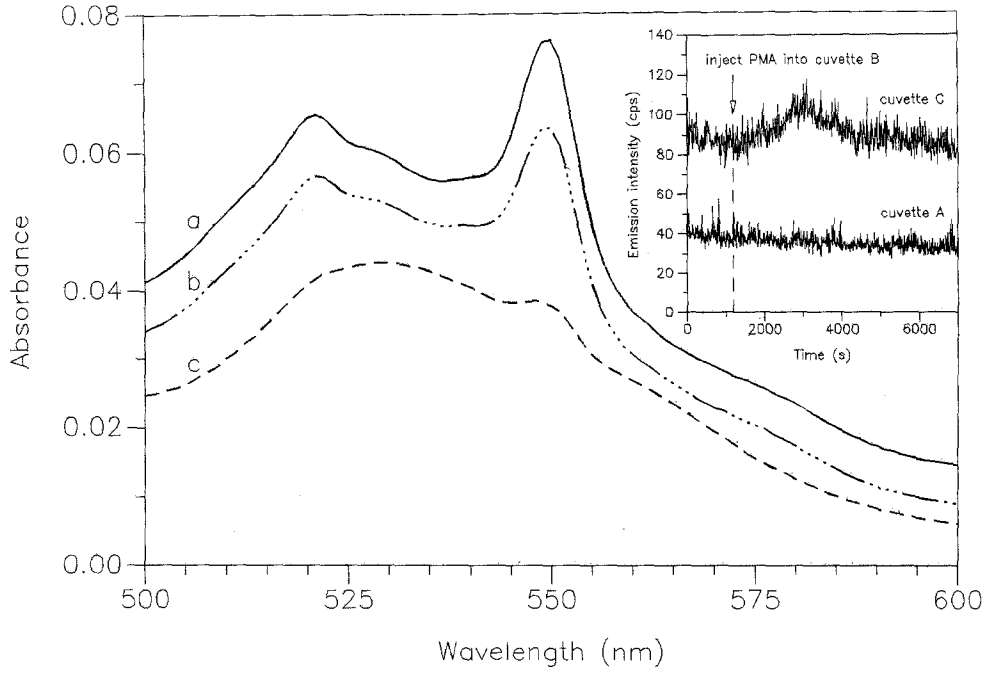

Figure 3. The enhanced reduction of ferricytochrome c in the neutrophil suspension optically coupled to a separate population of neutrophils undergoing respiratory burst. 11 ml of neutrophil suspension (2.1×10^7 cells/ml, in HBSS) containing 5.0×10^{-6} mole dm^{-3} ferricytochrome c were added to cuvettes A and C respectively, but 10 ml of neutrophil suspension (2.3×10^7 cells/ml, in HBSS) to cuvette B. The LLCL measurement started 2 h after the cuvettes were placed in the dark chambers. 1 ml of PMA was injected into cuvette B 1200 s after the LLCL measurement started. The cell suspensions from cuvettes A and C were taken for centrifugation 5800 s after the addition of PMA. The absorption spectra of the supernatants from cuvettes C and A, as well as the 5×10^{-6} mole · dm^{-3} of ferricytochrome c in cell-free HBSS, are shown as curve a, b and c respectively. The inset shows the registered chemiluminescence from cuvettes C and A before the cell suspension was subjected to the ferrocytochrome c assay.

acetate (PMA, 1 μg/ml in 10% dimethyl sulfoxide and 90% HBSS) and observed the changes in cell suspension in cuvette C using the cell suspension in cuvette A as control.

Ferricytochrome. The reduction of ferricytochrome c was assayed by spectrophotometry according to ref. 14. Neutrophils were suspended in HBSS as described below in the legend to figure 3, and 5×10^{-6} M ferricytochrome c was added to the cell suspension of cuvette C and A. A certain period of time (usually 2 h) after stimulation of the cells in cuvette B, the cell suspensions in C and A were centrifuged at 8000 *g* for 60 s. The supernatants were immediatley measured with a spectrophotometer (Lamda 2, Perkin Elmer) using HBSS as blanks.

Superoxide radicals. The generation of superoxide radicals in the cell suspension was detected by the spin-trapping method[14]. 10 ml of neutrophil suspension (2.2×10^7 cells/ml in DMEM) were added to cuvettes A, B and C. In addition, 0.2 ml of a 50 mM solution of chelating agent diethylenetriaminepentaacetic acid (DETAPAC) (Sigma, in 0.15 M phosphate buffer of pH 7.0) were added to cuvette A and C. A certain period of time after the PMA-stimulation of cells in cuvette B (see legend to fig. 4), 0.4 ml of the cell suspension was taken from each cuvette and mixed with 0.1 ml of 0.5 M DMPO (Sigma, in 0.15 M phosphate buffer of pH 7.0). Each mixture was then transferred to three separate quartz capillaries (0.8 mm i.d.) in a quartz tube (3 mm i.d.) and measured with Bruker ESP 300 ESR spectrometer at room temperature. The measurement conditions were: microwave 10 mW at 9602 MHz, modulation 1.6 G at 100 kHz, receiving gain 2.0×10^6, central magnetic field 3420 G, scan 200 G with speed of 10 G/min, time constant 5 s. The DMPO was purified with active charcoal (Sigma, untreated powder, 250–350 mesh) before use.

Results and discussion

Temperary increase of LLCL from cuvette C. An equal volume of neutrophil suspension ($10^6 - 10^7$ cells/ml) was added to each cuvette. When PMA was injected into cuvette B, the respiratory burst of the cells was triggered by the stimulation of protein kinase C (ref. 15,16), followed by a CL burst. As a result, we regularly observed a temporary increase of LLCL from cuvette C (fig. 2B). In contrast, no LLCL change in the control cuvette A could be detected (fig. 2A). The characteristics of the CL burst of the cells in cuvette B, which should be 'seen' by the cells in cuvette C but could not be recorded well by a third photomultiplier for purely technical reasons, were imitated by injecting the same amount of PMA in both cuvettes A and B simulta-

neously and by recording the CL burst in cuvette A. In order to estimate the possible contribution of the CL burst from B to the registered CL of PM II, cells which had lost their phagocytotic ability after heating at 50 °C for 30 min, were put into cuvette C. As is shown in figure 2D, the change of CL-intensity registered by PM II was then much smaller than when viable neutrophils were in C. This indicates that the CL rising registered by PM II (fig. 2B) is really due to events taking place in viable cells in cuvette C. It was also noted that the CL rising of the viable cells in cuvette C (fig. 2B) was much slower than the onset of the CL burst from the PMA-stimulated neutrophils (fig. 2C). This further excludes the possibility that the temporary CL increase detected by PM II could be due to scattering of the photons originating from the stimulated cells in cuvette B. These three types of experiments were repeated nine times. Although the magnitude of the response of the neutrophils in cuvette C to the stimulation of the cells in B varied from measurement to measurement, the essential characteristic (i.e. a temporary increase of LLCL) shown in figure 2 was quite reproducible.

The table summarizes nine measurements of the response of the cells in cuvette C to the stimulated cells in cuvette B. The maximal increase of the low-level CL from the traget cells varies from 6.5 ± 2.4 to 20.3 ± 1.9 counts/s and the time to the maximum response varies from 1400 to 6500 seconds. The table also shows that the 'additional reagents' present in some of these experiments do not interfere with the reproducible increase of LLCL in the target cells.

Enhanced reduction of ferricytochrome c in cuvette C. The response of the cells in cuvette C to the PMA-stimulated cells in cuvette B means obviously that some 'message' is transferred from the latter to the former. It is logical to ask whether the message is to 'call' the cells in cuvette C to start a respiratory burst too. If some cells in cuvette C were really undergoing respiratory oxidative burst after receiving the information from the activated cells in a separate cuvette B, one should also expect more generation of O_2^- and/or $\dot{O}H$ in cuvette C. Therefore, the reduction of ferricytochrome c was used to detect O_2^-, since ferricytochrome c is reduced by O_2^- and the reduced form of cytochrome c (ferrocytochrome c) has a strong absorption peak at 550 nm. This set of experiments was again carried out in the same dark chambers as described above in order to prevent any possible interference from ambient light. Cuvettes A and C contained the same amount of neutrophils suspended in HBSS and exactly the same amount of added ferricytochrome c. PMA was injected only into cuvette B to simulate its cells, and the LLCL of cuvettes A and C was monitored continuously until the cell suspensions were taken for assay of ferrocytochrome c, i.e. one and a half hours after injection of PMA into cuvette B, both cell suspensions from A and C were centrifuged, and the supernatants were measured spectrophotometrically. A typical result from such a biochemical assay is shown in figure 3. It shows that more ferric ions of ferricytochrome c was reduced (giving rise to higher absorption at 550 nm) in cuvette C than in cuvette A. In comparison to the fairly low absorption of ferricytochrome c in cell-free HBSS, a considerable reduction of ferricytochrome c in cuvette A was also observed. This may be due to the activity of cytochrome c reductase of the cells or the O_2^- generated during normal cellular metabolism in the cells of cuvette A. The inset of figure 3 shows the detected LLCL from

A summary of nine measurements of the response of the neutrophils in cuvette C to the PMA-stimulated neutrophils in cuvette B.

Date	Cell concentration ($\times 10^7$/ml)	CL before stimulation (counts/s $\pm$ SD)[a]	CL at maximum response (counts/s $\pm$ SD)[a]	Rising time[b] (s)	Additional reagent in cell suspension
21.1.94	1.1	53.3 ± 1.3	64.2 ± 1.8	5500	0.1 mM DETAPAC[c]
24.1.94	7.0	50.3 ± 1.6	56.8 ± 1.8	5300	5×10^{-6} M cytochrome c[d]
28.1.94	2.1	45.1 ± 0.9	53.5 ± 1.0	3600	none[c]
31.1.94	2.1	50.9 ± 0.7	61.3 ± 0.8	3500	none[c]
1.2.94	2.3	50.3 ± 0.6	69.4 ± 2.4	1400	5×10^{-6} M cytochrome c[d]
9.2.94	2.3	53.7 ± 1.6	74.0 ± 1.0	2000	5×10^{-6} M cytochrome c[d]
21.2.94	0.69	58.9 ± 1.2	68.0 ± 2.0	4100	5×10^{-6} M cytochrome c[d]
28.2.94	2.2	56.5 ± 1.2	67.9 ± 1.4	>2000[e]	0.1 mM DETAPAC[c]
28.3.94	2.6	59.6 ± 0.8	76.7 ± 1.0	6500	none[c]

[a]The standard deviation is calculated based on 10 consecutive values of CL count rate; each of them were measured in 10 s interval.
[b]The time length for the induced CL of the cells in cuvette C to reach to its maximum after injection of PMA in cuvette B.
[c]The cells were suspended in DMEM medium.
[d]The cells were suspended in HBSS containing 5 mM D-glucose.
[e]Since the cell suspension was taken for ESR assay, the measurement of CL from the cells in cuvette C was stopped before the CL reached to its maximum.

C and A measured over the entire duration of the experiment until the cell suspensions were removed for assay. With the exception of a little earlier increase in LLCL, a similar pattern as that of figure 2A and 2B was observed.

Detection of O_2^- by spin-trapping. Increased generation of O_2^- in the cells of cuvette C which previously received information from the PMA- stimulated neutrophils in cuvette B can also be checked by the spin-trapping method. This has the advantage over the measurement of ferricytochrome c reduction of being direct and less ambiguous[14]. Speroxide reacts with the spin trap 5,5-dimethyl-1-pyrroline N-oxide (DMPO) to form an unstable superoxide adduct (DMPO-OOH) which is then quickly converted to the more stable nitroxide 5,5-dimethyl-2-hydroxylpyrrolidoxyl (DMPO-OH). DMPO-OH can be readily measured by electron spin resonance (ESR) spectroscopy. As in the experiment of ferricytochrome c reduction, the spin-trapping was also carried out in dark chambers; however, DETAPAC was added to the cell suspension of cuvettes A and C to a final concentration of 1 mM (see legend to fig. 4). The DETAPAC was added to chelate iron ions in the cell suspension and reduce the conversion of O_2^- to $\dot{O}H$ by the iron-catalyzed Haber-Weiss reaction[17]. When the LLCL from cuvette C reached its maximum after injection of PMA into cuvette B, 0.4 ml cell suspension taken from each cuvette was mixed with 0.1 ml of 0.5 M DMPO. The ESR spectra of the mixtures were then measured.

Typical results are shown in figure 4. The spectrum of the mixture of DMPO with the cell suspension from cuvettes B and C (shown as spectrum 1 an 2 in fig. 4A) are very typical for DMPO-OH ($g = 2.0053$, $a_N = a_H = 14.9$ G). The spectrum of DMPO with the cell suspension from the control cuvette A is almost at background level (spectrum 3 in fig. 4A). This shows quite clearly that more O_2^- was spin-trapped in the cell suspension of cuvette C than in the control cuvette A. The observation of a slightly higher ESR signal from cuvette C in comparison to the signal from cuvette B may be explained by the fact that the 'spin trap' DPMO was added to the cell suspension when the respiratory burst of the PMA-stimulated cells in cuvette B had already declined to a considerable extent, while the 'induced' burst of the cells in cuvette C was just at its peak.

The increase in the low-level chemiluminescence and both of the biochemical assays, namely reduction of ferricytochrome c and the ESR signal of spin-trapped radicals, strongly suggest that an interaction between the cells of B and C exists. The experimental observation presented here cannot be understood in terms of purely 'chemical' communication. They point to an

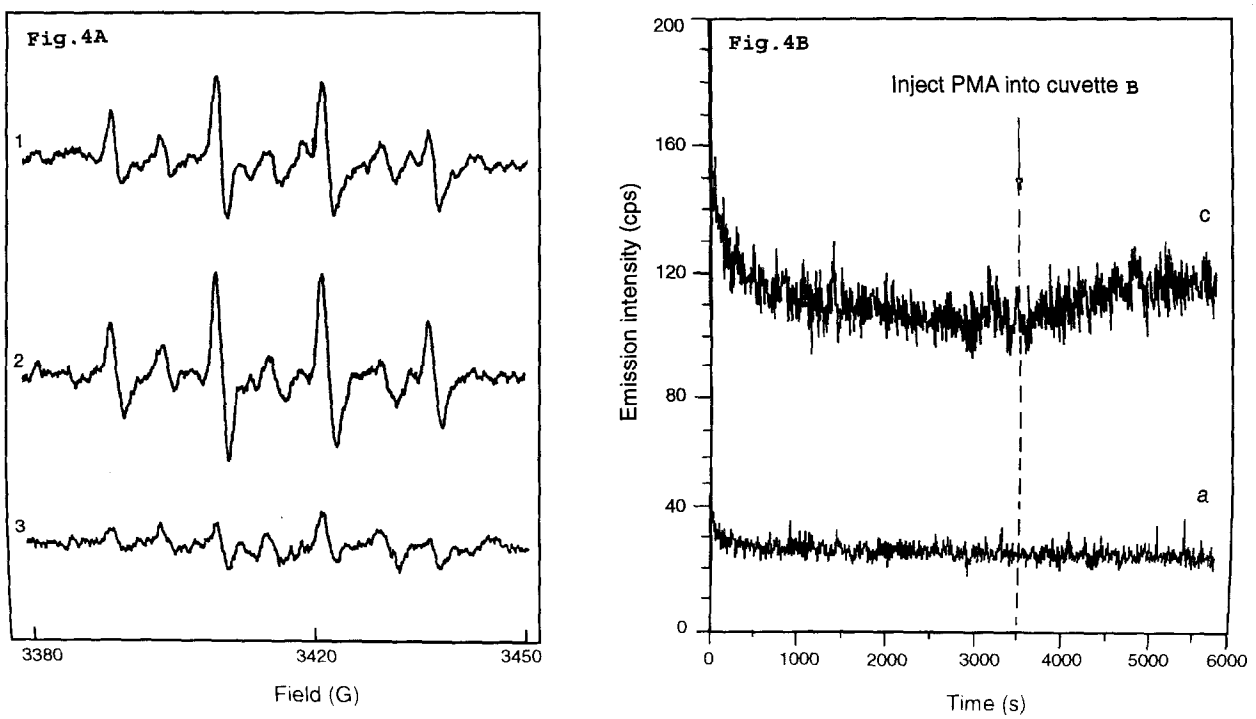

Figure 4. The ESR spectra of DMPO-trapped radicals in a neutrophil population which is either optically coupled to or isolated from a separate population of neutrophils undergoing respiratory burst. 10 ml of neutrophil suspension (2.2×10^7 cells/ml in DMEM) were added to cuvettes A, B and C. An additional 0.2 ml of 50 mM DETAPAC solution were added to cuvettes A and C. 1 ml of PMA was injected into cuvette B after 3500 s of adaptation of the cells in the dark chambers. 2200 s after the PMA-injection, 0.4 ml of cell suspension were taken from each cuvette and mixed with 0.1 ml of 0.5 M DMPO. The ESR signal of the mixture of DMPO with cell suspensions from cuvettes B, C and A are shown as 1, 2 and 3 in figure 4A. The LLCL of the cells in cuvettes C and A, shown as curves c and a in figure 4B, were recorded before the cell suspensions were taken for ESR assay.

electromagnetic (optical) communication channel in biological systems. Some explanations for such phenomena, based on Dicke's theory[18], have been suggested[19]. A recent report to the regulation of the secretion activity in one mammary gland by the lipid peroxidation-chemiluminescence from a second separate mammary gland[20] is somewhat similar in nature to the present findings. However, there is still a lot of work to be done in order to understand these effects.

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