

The effect of increasing amounts of synovial fluid isolated from patients with rheumatoid arthritis on the respiratory burst of human neutrophils

Wpływ wzrastających ilości płynu stawowego izolowanego od pacjentów z reumatoidalnym zapaleniem stawów na intensywność „wybuchu tlenowego” ludzkich neutrofilów

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Słowa kluczowe: reumatoidalne zapalenie stawów, neutrofile, chemiluminescencja, płyn stawowy.

Summary

It has been proposed that the inappropriate release of oxidants and proteolytic enzymes from activated neutrophils is responsible for joint damage observed in rheumatoid arthritis (RA). The present study examines the effect of increasing concentrations of synovial fluid [SF] (0–80%) on the oxidative response of neutrophils isolated from SF of RA patients and the blood of healthy subjects. To reflect better the real conditions of the inflamed joint we used in our experiments higher concentrations of SF than was previously reported. We found that increasing the concentrations of SF has a different effect on lucigenin-dependent and luminol-dependent CL response.

Streszczenie

Sugeruje się, że to neutrofile poprzez wydzielane przez nie oksydanty i enzymy proteolityczne są odpowiedzialne za uszkodzenia stawów, obserwowane podczas reumatoidalnego zapalenia stawów. W niniejszej pracy neutrofile, izolowane zarówno od zdrowych dawców, jak i pacjentów chorych na reumatoidalne zapalenie stawów, ekspozowano na zwiększające się stężenia płynu stawowego (0–80%). Warunki te uznano za zbliżone do warunków panujących *in vivo* w jamie stawu kolanowego. Wykazano, że zwiększające się stężenia płynu stawowego znacząco odmiennie wpływały na badane typy chemiluminescencji: luminolo- i lucigeninozależne.

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Introduction

The phenomenon of chemiluminescence (CL) generated by polymorphonuclear neutrophils (PMN) was initially reported by Allen *et al.* [1]; it is associated with increased glucose oxidation via the hexose monophosphate shunt and the formation of reactive oxygen intermediates (ROI) by activated neutrophils, during the respiratory burst (RB). This activation can be demonstrated by measuring the energy released as light (CL).

Since the realization that during the respiratory burst, apart from the rapid release of ROI, activated neutrophils can also generate light, the technique of chemiluminescence (CL) has been widely used to measure the molecular controls that regulate reactive oxidant generation [2, 3]. We have used two CL assays reported as useful for the measurement of ROI release.

Two enzyme systems are responsible for the generation of oxidants. The first represents a plasma membrane-bound nicotinamide dinucleotide phosphate (NADPH) oxidase which generates superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) during the RB when activated during phagocytosis. These ROI may react with suitable transition metals to form hydroxyl radicals (OH^\cdot). The second system is represented by a myeloperoxidase (MPO), which is a haemoprotein located in azurophilic granules, this being discharged into the phagocytic vesicles by degranulation. MPO reacts with H_2O_2 to generate hypochlorous acid (HOCl) and related chloramines [2-4].

Luminol-dependent CL is particularly useful for studying the neutrophil functions as it measures the activity of NADPH oxidase and of MPO. It is also capable of monitoring both the intracellular and the extracellular oxidant generation as luminol freely penetrates these cells.

Lucigenin-dependent CL only measures the rate of oxidant secretion, independently of the amount of degranulation or of extracellular MPO. Lucigenin does not penetrate neutrophils and the light emission detected is independent of the activity of MPO and dependent on the NADPH oxidase activity [4,5].

Large numbers of neutrophils are present in the synovial fluids (SF) of patients with rheumatoid arthritis (RA). It has been proposed that inappropriate release of oxidants and proteolytic enzymes from activated neutrophils is responsible for the joint damage observed in RA.

There are studies showing the release of oxidants from neutrophils incubated with SF in concentrations up to 25% [3-10]. The present study examines the effect of higher concentrations of SF (up to 80%) on the

RB of neutrophils isolated from the blood of RA patients and from healthy subjects. We believe that the concentrations of SF used in our experiments better reflect the real conditions of the inflamed joint.

Material and methods

Fifty patients attending the outpatient clinics of the Warsaw Institute of Rheumatology were studied. All had definite seropositive RA according to the American Rheumatology Association criteria. Mean age was 59 years and mean duration of time since diagnosis, 12.3 years. All patients were on non-steroidal anti-inflammatory treatment and none had received steroids during the 6 months prior to enrolment.

Chemicals

Zymosan A, PMA, albumin and lucigenin, luminol and fMLP were obtained from Sigma Chemical Co, St Louis, USA. Phosphate buffered saline (PBS), Gradisol G and glucose were obtained from Polfa, Poland. RPMI 1640 medium was obtained from BioWhittaker, Belgium.

Preparation of SF

Synovial fluid samples obtained from RA patients were centrifuged at 10,000 g for 30 minutes and pooled ($n = 50$). This heterologous SF (h-SF) was then stored in 1 ml aliquots at a temperature of $-20^\circ C$.

Preparation of human SF neutrophils

Neutrophil count in the SF varied from 1×10^7 to 1×10^8 /ml and the percentage varied from 75% to 98% (mean 87%). Each SF was immediately diluted in RPMI 1640 (1 : 5) and centrifuged. The cells were washed twice in PBS, supplemented with glucose plus albumin, and neutrophils were separated from the SF cells by Gradisol G gradient centrifuge, followed by hypotonic lysis of red blood cells (RBCs). SF neutrophils stained by May-Grunwald-Giemsa technique were morphologically normal and the viability was $> 80\%$ as judged by the trypan blue exclusion test. The cells were used within 2 hours of preparation.

Preparation of human blood neutrophils

The cells were isolated from blood samples of healthy volunteers by using Gradisol G sedimentation followed by centrifugation and hypotonic lysis of contaminating RBCs. Neutrophils were morphologically normal and their viability was $> 90\%$ as judged by the trypan blue exclusion test. Cells were used within 3 h of preparation.

Neutrophil stimulating agents

Oposonized zymosan (OZ) an indicator of phagocytosis activity was prepared from pooled human serum. N-formyl-methionyl-leucyl-phenylalanine (fMLP) was used to measure receptor mediate. Phorbol myristate acetate (PMA) activates neutrophils independently of plasma receptor occupancy by activation of NADPH oxidase through stimulation of protein kinase C.

Chemiluminescence (CL) assay

The CL response was measured with a BioOrbit 1251 Luminometer at 37°C using a water jacket sample holder. Neutrophils (2×10^5) in 100 μ l of PBS were kept at 37°C for 10 min and mixed with 100 μ l of OZ (final concentration 1 mg/ml) and with 15 μ l of luminol solution (final concentration 150 μ M) or 20 μ l of lucigenin (final concentration 200 μ M). The samples were adjusted to 1 ml PBS with glucose and albumin (final concentration 1g/1000ml). In one subgroup, neutrophils from healthy subjects were incubated with 0-80% SF at 37°C. In another subgroup neutrophils from healthy subjects were stimulated by OZ, PMA and fMLP and exposed to 0-80% SF, as above.

Results

The generation of oxidants by neutrophils, following exposure to stimulating agents, was detected by measuring luminol and lucigenin-dependent CL. Luminol-dependent CL measures both intracellular and extracellular activity of the myeloperoxidase-hydrogen peroxide system, whereas lucigenin-dependent CL measures the rate of extracellular O_2^- secretion.

The present study examines the effect of higher concentrations of SF (up to 80%) on the RB of neutrophils isolated from the blood of RA patients and from healthy subjects. We believe that the concentrations of SF used in our experiments better reflect the real conditions of the inflamed joint.

The effect of opsonised zymosan (OZ) on the oxidative response of neutrophils and the increased amounts of h-SF

The h-SF isolated from patients with RA decreased the luminol-dependent CL response of both OZ stimulated neutrophil preparations obtained from healthy subjects and RA SF. The response is shown diagrammatically in figure 1.

The oxidative response of SF-N isolated from 17 autologous SF was also tested. In all but six cases there was no measurable release of oxidants by the

neutrophils; these six cases showed variable levels of decreased CL responses; the response of the SF-N to their own SF (10-80%), in one of the 6 cases, is also shown in figure 1.

Elevated CL activity was detected when blood-N stimulated with OZ were incubated with h-SF in the range 10-30% SF. A major and unexpected elevation of the CL response was measured when blood-N were incubated with 10% to 20% heterologous synovial fluid concentrations, but at higher concentrations (30% to 80%) the CL response was decreased. Such responses were similar in all cases and typical only for a combination of blood-N and h-SF. In contrast the CL response was at the lowest level when SF-N were incubated with h-SF (range 50-80% SF).

Increased concentrations of RA-h-SF stimulated solid the lucigenin-dependent CL response (fig. 2). The maximal CL response was obtained when cells were

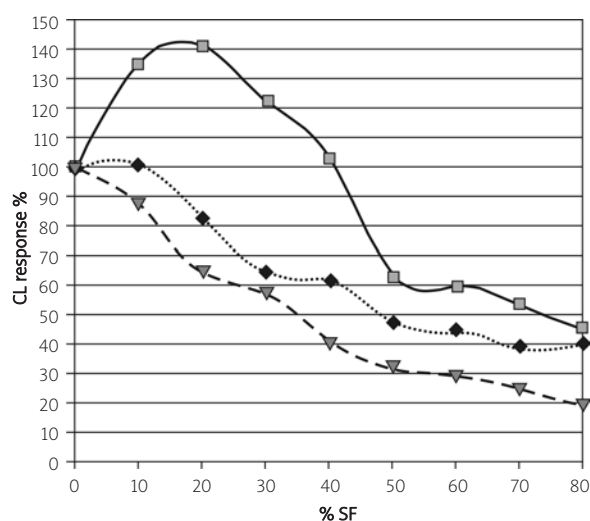


Fig. 1. The luminol-dependent CL response (intra- and extracellular oxidant production) of neutrophils isolated from normal blood ($n = 50$) or SF from patients with RA ($n = 35$) stimulated by opsonised zymosan (OZ) and exposed to increased amount of SF (0-80%). (■) blood-derived-N + heterologous SF, (▼) SF-derived-N + heterologous SF, (◆) SF-derived-N + autologous SF. **Ryc. 1.** Poziom luminolozależnej chemiluminescencji (wewnątrz- i zewnątrzkomórkowa produkcja oksydantów) neutrofilów izolowanych z krwi zdrowych dawców ($n = 50$) lub płynu stawowego (PS) pacjentów z RZS ($n = 35$), stymulowanych opsonizowanym zymozanem (OZ) i eksponowanych na wzrastające stężenia PS (0-80%). (■) N z krwi + heterologiczny PS, (▼) N izolowane z PS + heterologiczny PS, (◆) N izolowane z PS + autologiczny PS.

incubated with 80% SF. The CL activity of blood-derived-N at a concentration of 80% SF was approx. 17 times higher than control values. Similar results were observed for at least 49 other experiments using h-SF with RA- and blood-derived-neutrophils; the CL activity of SF-derived-N at 80% h-SF was approx. 17 times higher compared to control (0% h-SF). The CL response of SF-derived-N exposed to their own autologous SF was at medium levels (in 6 cases from 17 experiments).

The effect of PMA on the oxidative response of neutrophils isolated from blood or SF and exposed to increasing amounts of h-SF

Increased concentrations of h-SF decreased the luminol-dependent CL response of RA synovial fluid neutrophils stimulated by PMA (fig. 3). In contrast, PMA-stimulated blood neutrophils were CL-activated

by lower concentrations of h-SF, but inhibited with higher concentrations of h-SF (60-80%).

Luminol-dependent CL activity was lowest when SF-N were incubated with h-SF (CL activity was at a medium level when SF-N were incubated with SF of the same donor) and it was in the upper range when blood-N were incubated with h-SF. A major elevation of CL response was measured when blood-N were incubated in 10% h-SF; average result of CL response was almost 160% when the SF concentration was 10%, compared to incubation without SF. Similar results were obtained in every experiment.

Increased concentrations of h-SF as well as autologous SF stimulated the lucigenin-dependent CL response of RA SF-N, with values at 80% concentration of h-SF being about 3500% higher compared to control (0% SF). In contrast, similar incubations with human blood neutrophils had relatively little effect, with approx. 200% increase at 80% h-SF (fig. 4).

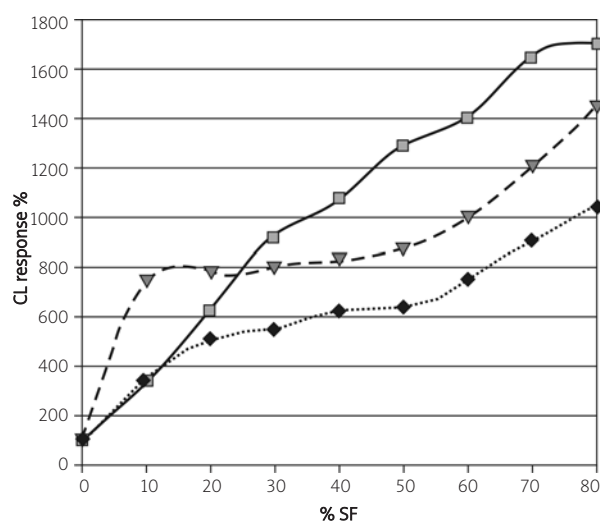


Fig. 2. The lucigenin-dependent CL response (extracellular O_2^- secretion) of neutrophils isolated from normal blood ($n = 50$) and SF from patients with RA ($n = 35$) stimulated by opsonised zymosan (OZ) and exposed to increasing amounts of SF (0-80%). (■) blood-derived-N + heterologous SF, (▼) SF-derived-N + heterologous SF, (◆) SF-derived-N + autologous SF.

Ryc. 2. Poziom lucigeninozależnej chemiluminescencji (zewnątrzkomórkowa produkcja O_2^-) neutrofilów izolowanych z krwi zdrowych dawców ($n = 50$) lub płynu stawowego (PS) pacjentów z RZS ($n = 35$), stymulowanych opsonizowanym zymozanem (OZ) i eksponowanych na wzrastające stężenia PS (0–80%). (■) N z krwi + heterologiczny PS, (▼) N izolowane z PS + heterologiczny PS, (◆) N izolowane z PS + autologiczny PS.

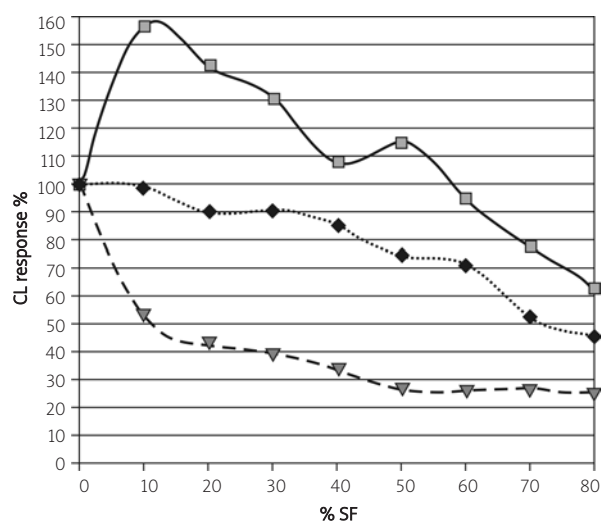


Fig. 3. The luminol-dependent CL response (intra- and extracellular oxidant production) of PMA-stimulated neutrophils isolated from normal blood ($n = 50$) or SF from patients with RA ($n = 35$) and exposed to increased amounts of SF (0-80%). (■) blood-derived-N + heterologous SF, (▼) SF-derived-N + heterologous SF, (◆) SF-derived-N + autologous SF.

Ryc. 3. Poziom luminolozależnej chemiluminescencji (wewnątrz- i zewnątrzkomórkowa produkcja oksydantów) neutrofilów izolowanych z krwi zdrowych dawców ($n = 50$) lub płynu stawowego (PS) pacjentów z RZS ($n = 35$), stymulowanych PMA i eksponowanych na wzrastające stężenia PS (0–80%). (■) N z krwi + heterologiczny PS, (▼) N izolowane z PS + heterologiczny PS, (◆) N izolowane z PS + autologiczny PS.

The CL response to stimulation of autologous SF (80% SF) was also very high, exceeding 3000%, compared to control (0% SF). The response to the stimulation of blood-N (blood-N + heterologous SF) was rather low, compared to that of SF-N, but still about 200% higher at 80% concentration of SF compared to control (0% SF).

The effect of fMLP on the oxidative response of neutrophils isolated from blood or synovial fluid and exposed to increased concentrations of h-SF

Increased concentrations of h-SF decreased the luminol-dependent CL response of fMLP-stimulated human blood neutrophils (blood-N), and RA synovial fluid neutrophils (SF-N) (fig. 5). In general the exposure to increasing concentrations of h-SF led to a similar

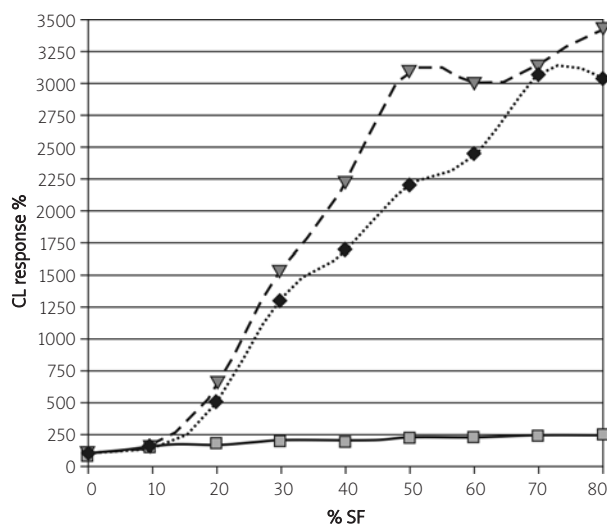


Fig. 4. The lucigenin-dependent CL response (extracellular O_2^- secretion) of PMA-stimulated neutrophils isolated from normal blood ($n = 50$) or SF from patients with RA ($n = 35$) and exposed to increased amounts of SF (0–80%). (■), blood-derived-N + heterologous SF, (▼) SF-derived-N + heterologous SF, (◆) SF-derived-N + autologous SF.

Ryc. 4. Poziom lucigeninozależnej chemiluminescencji (zewnątrzkomórkowa produkcja O_2^-) neutrofilów izolowanych z krwi zdrowych dawców ($n = 50$) lub płynu stawowego [PS] pacjentów z RZS ($n = 35$), stymulowanych PMA i eksponowanych na wzrastające stężenia PS (0–80%). (■) N z krwi + heterologiczny PS, (▼) N izolowane z PS + heterologiczny PS, (◆) N izolowane z PS + autologiczny PS.

drop of luminol-dependent CL in all experiments. In all 3 separations of neutrophils the CL response was about 50% lower in the presence of 80% SF, than in the absence of SF.

The lucigenin-dependent CL response after stimulation with fMLP was similar to that with PMA. Increased concentrations of h-SF stimulated the lucigenin-dependent CL response of fMLP-stimulated RA synovial fluid neutrophils (SF-N) (fig. 6). The CL activity of SF-N at 80% concentration of h-SF was approx. 27 times higher, compared to control (0% SF). In contrast, the CL activity of blood-N under similar conditions produced a relatively modest 3-fold increase.

Moreover, the CL response of SF-N (autologous-SF) at 80% concentration of SF was 2500% higher than in the absence of SF.

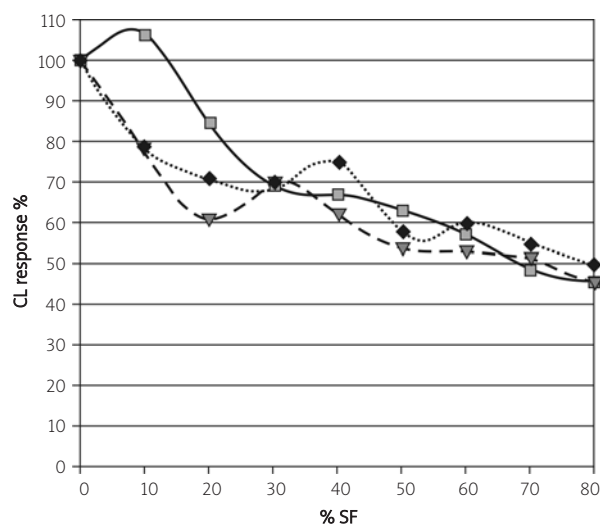


Fig. 5. The luminol-dependent CL response (extracellular oxidant production) of fMLP-stimulated neutrophils isolated from normal blood ($n = 50$) or SF from patients with RA ($n = 35$) and exposed to increased amounts of SF (0–80%). (■) blood-derived-N + heterologous SF, (▼) SF-derived-N + heterologous SF, (◆) SF-derived-N + autologous-SF.

Ryc. 5. Poziom luminolozależnej chemiluminescencji (wewnątrz-i zewnątrzkomórkowa produkcja oksydantów) neutrofilów izolowanych z krwi zdrowych dawców ($n = 50$) lub płynu stawowego (PS) pacjentów z RZS ($n = 35$), stymulowanych fMLP i eksponowanych na wzrastające stężenia PS (0–80%). (■) N z krwi + heterologiczny PS, (▼) N izolowane z PS + heterologiczny PS, (◆) N izolowane z PS + autologiczny PS.

The effect of PMA on the oxidative response of neutrophils isolated from blood and incubated in 80% heterologous synovial fluid for 24/48 hours

Normal blood neutrophils exposed to 80% h-SF for 24/48 hours increased the extracellular superoxide secretion (measured by addition of lucigenin) compared to the total free radical secretion (measured by addition of luminol) (fig. 7, 8).

After 48 hours of incubation in h-SF, the extracellular secretion of free radicals represents over 90% of total free radical production, compared to 70% after 24 h of incubation and to immediate incubation (40%). During incubation (0 to 48 hours) the maximal value of CL response, both luminal and lucigenin-dependent, significantly decreases.

Discussion

Rheumatoid arthritis (RA) is a chronic autoimmune disease in which certain, not yet discovered, factors

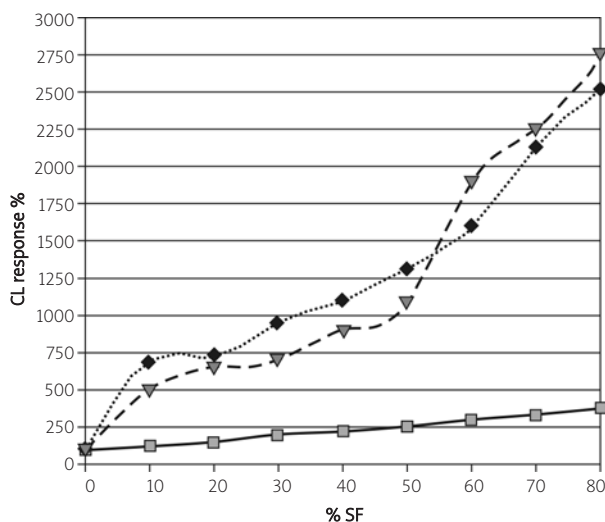


Fig. 6. The lucigenin-dependent CL response (extracellular O_2^- secretion) of fMLP-stimulated neutrophils isolated from normal blood ($n = 50$) or SF from patients with RA ($n = 35$) and exposed to increased amounts of SF (0-80%). (■) blood-derived-N + heterologous-SF, (▼) SF-derived-N + heterologous-SF, (◆) SF-derived-N + autologous-SF.

Ryc. 6. Poziom lucigeninozależnej chemiluminescencji (zewnątrzkomórkowa produkcja O_2^-) neutrofilów izolowanych z krwi zdrowych dawców ($n = 50$) lub płynu stawowego (PS) pacjentów z RZS ($n = 35$), stymulowanych fMLP i eksponowanych na wzrastające stężenia PS (0-80%). (■) N z krwi + heterologiczny PS, (▼) N izolowane z PS + heterologiczny PS, (◆) N izolowane z PS + autologiczny PS.

trigger the activation of leukocytes, which attack the body's own tissues, resulting primarily in inflammation of peripheral joints and the destruction of joint tissues. The leukocyte with the greatest capacity to inflict damage within diseased joints via the inappropriate release of oxidants and proteolytic enzymes is the neutrophil. Neutrophils possess a range of potent proteinases and hydrolases, and have the ability to generate a series of reactive oxygen intermediates (ROI) via the combined activities of NADPH (reduced form) oxidase and myeloperoxidase (MPO) [5-7].

Considering: 1) the enormous number of neutrophils present in the diseased joint during active RA (up to 5×10^9 cells); 2) the large quantities of proteolytic enzymes secreted; and 3) their ability to act synergistically to evoke tissue damage, neutrophils are considered the major source of tissue damage inside the diseased joint [11].

Luminol-dependent CL, which is capable of monitoring both intracellular and extracellular oxidant generation (NADPH oxidase and MPO) as luminal, freely penetrates neutrophil cell membranes. Highly reactive radicals generated by phagocytosing neutrophils oxidize luminol to the electronically excited aminophthalate ion, which is relaxed to ground state by photon emission. The luminol-amplified CL assay is a simple, rapid, reproducible, and useful method for the study of neutrophil metabolism [9, 10, 11].

Lucigenin dependent CL, on the other hand, only measures the rate of oxidant secretion, independently of the amount of degranulation or extracellular MPO, because lucigenin does not penetrate neutrophils and the light emission detected is independent of the activity of MPO [7].

Several lines of independent experimentation have shown that neutrophils isolated from the blood of healthy donors, or from SF RA patients, and exposed to h-SF have been activated in vitro [3-10].

Bender *et al.* [3] observed that pre-incubation of normal blood neutrophils in 10% SF enhanced the luminol CL, and O_2^- release, suggesting that both MPO and NADPH oxidase activity were activated in parallel during exposure to 10% SF from RA patients. Activation of neutrophils by soluble and insoluble aggregates of immunoglobulins from SF of patients with RA was observed by Robinson *et al.* [5]. In that study activated neutrophils, primed or not, were exposed to 10% SF and luminol CL and O_2^- release were measured. Primed neutrophil suspensions activated by 10% SF showed a biphasic luminol CL response with a rapid, transient burst of O_2^- secretion.

Synovial fluid (20% vol.) isolated from RA patients activated luminol CL in blood neutrophils, and the

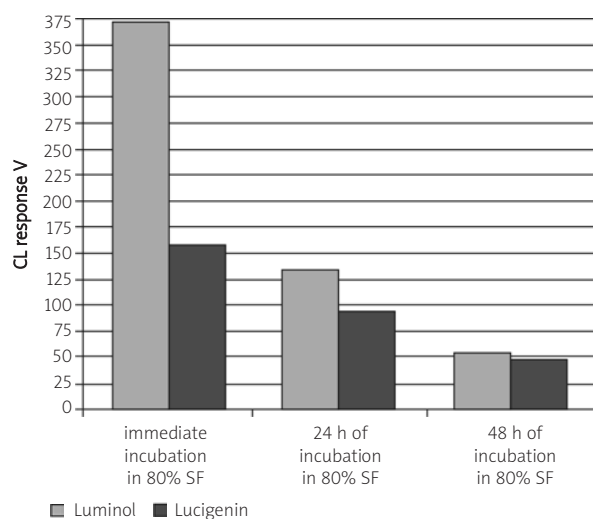


Fig. 7. The luminol and lucigenin-dependent CL response of neutrophils isolated from normal blood and stimulated by PMA after 24/48 h of incubation in 80% SF.

Ryc. 7. Poziom luminolo- i lucigeninozależnej chemiluminescencji neutrofilów izolowanych z krwi zdrowych dawców inkubowanych przez 24/48 godz. z 80-procentowym PS i poddanych stymulacji przez PMA.

maximal activity stimulated varied over a 50-range in the study of Nurcombe *et al.* [7]. In contrast, the same fluids only activated a much lower range (two- or three-fold) of maximal rates of lucigenin CL and cytochrome c reduction, two assays which only measure O_2^- secretion, being independent of MPO. However, according to Duluray *et al.* [6] only a minority (7/35) of SF from patients with RA stimulated the oxidative response of neutrophils.

All the above-mentioned reports were performed with SF in concentrations that did not exceed 20%. Other studies confirmed that SF (used in concentrations of 0-25%) produced a rapid CL response (luminol- and lucigenin-dependent) when incubated with neutrophils [5-8].

It is known that for proper conclusions to be drawn, during *in vitro* experiments in laboratory conditions, these should imitate as much as possible the *in vivo* environment.

With this in mind, our experiments have examined the oxidative response of neutrophils, using concentrations close to the *in vivo* concentrations of SF. We observed that these higher concentrations of SF have a quite different effect on lucigenin-dependent and luminol-dependent CL response.

Irrespective of the stimulator used (OZ, fMLP or PMA) the increased concentrations of SF resulted in

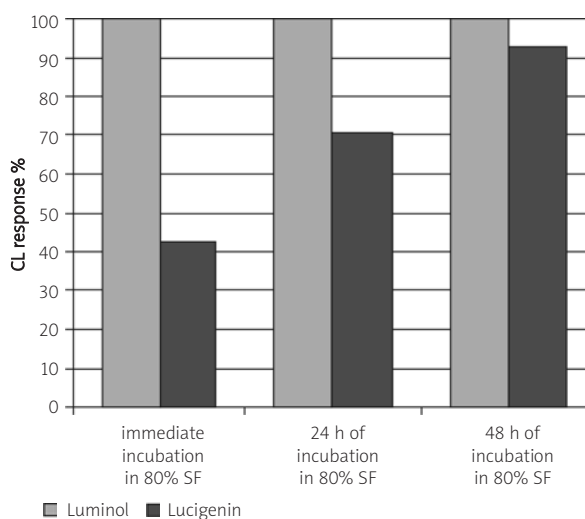


Fig. 8. Comparison of the lucigenin- and luminol-dependent CL response of neutrophils isolated from normal blood and subjected to PMA stimulation immediately and after 24/48h of incubation in 80% SF.

Ryc. 8. Porównanie poziomów luminolo- i lucigeninozależnej chemiluminescencji neutrofilów poddanych bezpośredniej stymulacji przez PMA, z neutrofilami inkubowanymi przez 24 lub 48 godz. w 80-procentowym PS i stymulowanymi PMA po tym czasie.

a reduction of luminol-dependent CL response and an increase of lucigenin-dependent CL. This effect was observed irrespective of whether neutrophils were isolated from SF or blood of RA patients or healthy subjects.

This indicates that the increase of SF concentration results in higher extracellular O_2^- secretion and in lower NADPH oxidase- and MPO-dependent oxidant production.

The reason for the drop of the luminol-dependent CL observed may be a result of inhibition of MPO found in the h-SF and sera of patients with RA and normal subjects, which acts by binding to MPO and thereby prevents MPO-releasing oxidative products in serum, as described previously [8]. The MPO inhibitor may be one of an array of antioxidant mechanisms present in serum preventing MPO catalyzing reactions which would release ROI under inappropriate conditions. A similar effect was observed by Davies *et al.* [12], who demonstrated that neutrophils isolated from SF caused a reduction in both MPO and NADPH oxidase activity.

Moreover, SF from RA patients, and its components hyaluronic acid (HA) and D-glucuronic acid, markedly decreased the generation of O_2^- , hydrogen peroxide,

hydroxyl radical and CL measured in both systems [13]. These investigators suggested that D-glucuronic acid or HA may react with both O_2^- and hydrogen peroxide and that SF may contain other scavenging enzymes, such as superoxide dismutase, catalase, and peroxidase, and/or other undefined antioxidants, such as albumin, flavonoids, α -tocopherol and ascorbic acid. In contrast, activation of the neutrophil-MPO-hydrogen peroxide system by SF was reported by Nurcombe *et al.* [14].

SF from RA patients is a complex mixture containing soluble and modulatory factors released by various activated synovial cells which may have stimulatory or inhibitory effects on neutrophils [15, 16].

The unexpected increase of luminol CL when exposing neutrophils to 10% SF, observed in our study, was countered by a decrease of CL with higher concentrations of SF. Similar observations were made by Sugisawa *et al.* [17]. In that study the luminol CL stimulated with *S. aureus* was measured in the presence of colostrum, normal milk or serum. When neutrophils were incubated with colostrum, the oxidative burst was enhanced in comparison to the control with concentrations up to 10%, but it was inhibited at 50%. This suggests that bovine colostrum includes both CL inhibitory and activating factors that are attributable to the oxidative burst. The oxidative burst promoting activity in the colostrums declined as their concentrations increased. When the concentration of colostrum was high, antioxidant activity was stronger than the oxidative burst promoting activity. On the other hand, in the case of low concentrations, the oxidative burst promoting activity behaves as a superior factor. IL-1 and IL-6 have been identified as oxidative burst promoting substances in bovine milk [17].

This may be partially explained in our experiments. It cannot however explain the potent increase of lucigenin CL.

In chronic inflammatory arthritis, such as RA, large numbers of neutrophils are attracted across the synovial membrane, and become activated. If the neutrophils are not efficiently depleted, their production of inflammatory mediators, such as ROI, could prolong the inflammatory reaction, leading to further tissue destruction. The fate of neutrophils in the inflamed joint is unclear; there is no evidence that they re-enter the circulation, and it has often been assumed that they disappear by a non-specific process of necrosis or a specific process of apoptosis. The addition of autologous or heterologous inflammatory SF to blood or joint cavity (SF) neutrophils cultured *in vitro* caused a significant dose-dependent increase in the percentage of cells becoming apoptotic with time as measured

morphologically and confirmed by DNA degradation. It should be noted that these effects were observed only at relatively high concentrations of SF (50 and 75%) [18].

Additional support for the increasing relevance of lucigenin-dependent chemiluminescence during incubation is an increase of extracellular activity of ROI release compared to total free radical secretion represented by luminol CL. After 48 hours of incubation lucigenin-dependent CL represented over 90 percent of the luminol CL, whereas when immediately incubated and measured, it represented slightly over 40 percent of luminol CL.

Although the presence of activated neutrophils in SF is well documented, it is unknown how the neutrophils are activated and how long they persist at the site of inflammation. Apoptosis is considered to be a prerequisite for the clearance of an inflamed site, and for limitation of the inflammatory response. A failure to undergo apoptosis is thought to result in an unrestrained release of cytotoxic activity of neutrophils, and thus in damage of the surrounding tissue [19].

As was shown by Icking-Kunert *et al.* [20] SF neutrophils from patients with RA undergo major alterations, including transdifferentiation to cells with dendritic-like characteristics. To mimic the *in vivo* situation, co-culture experiments were carried out using 50% SF. These authors suggested that the prolonged lifespan of activated neutrophils may contribute to the progression of the inflammatory process to chronicity.

Promotion of extracellular release of ROI, observed in our experiments, is likely to be associated with the high concentrations of SF used, and raises the possibility that extracellular activity of neutrophils may be a general characteristic which prolongs the inflammatory process.

Our findings confirm the proposal that the use of high concentrations of SF, as close as possible to the physiological, are strongly recommended for *in vitro* experiments.

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