Molecular and Cellular Biochemistry **103**: 51–62, 1991.

Oxygen-derived free radicals producing activity and survival of activated polymorphonuclear leukocytes

Kailash Prasad, Ajai K. Chaudhary and Jawahar Kalra
Departments of Physiology and Pathology, College of Medicine, University of Saskatchewan and Royal University Hospital, Saskatoon, Saskatchewan, Canada

Received 6 June 1990; accepted 15 October 1990

Key words: oxygen free radicals, chemiluminescence, polymorphonuclear leukocytes, phagocytosis

Summary

Activation of polymorphonuclear (PMN) leukocytes is known to generate oxygen free radicals (OFR). However the fate of activated PMN leukocytes is not known. We investigated the OFR producing (chemiluminescence) activity and the survival of the activated PMN leukocytes. The study was divided into two groups. Group I, *In vivo* study (n = 7): zymosan (8.4 mg/kg) was administered intravenously in the anesthetized dogs and the blood samples were collected before and after 5, 15, 30, 60 and 120 min of zymosan administration. This group represents the *in vivo* pre-stimulated PMN leukocytes; Group II, *In vitro* study (n = 7): the blood were collected from dogs and further divided into two groups. Group A (n = 7): non-stimulated, without any added zymosan and group B (n = 7): zymosan was added to stimulate PMN leukocytes. Blood samples from group A and B were also collected at various time intervals similar to *in vivo* studies. Oxygen free radical producing activity of PMN leukocytes was monitored by measuring luminol-dependent chemiluminescence (CL). Opsonized zymosan was used to activate PMN leukocytes. The studies in which the PMN leukocytes were stimulated in *in vivo,* both oxygen derived free radicals and superoxide dismutase (SOD) inhibitable oxygen free radical CL decreased significantly for 60 min and tended to reach thereafter to the pre-stimulated values. The resting chemiluminescence (chemiluminescence without zymosan stimulation in the assay medium) increased significantly for 15 min reaching to pre-stimulated values at 30 min and thereafter. In *in vitro* studies, oxygen derived free radicals CL of pre-stimulated PMN leukocytes (Group B) was depressed for the whole duration of investigation while SOD inhibitable CL was depressed for only 60 min. There was approximately a two-fold increase in the resting CL within 5 min of PMN leukocyte activation and it remained high for the whole duration of study. The chemiluminescence of non-stimulated PMN leukocytes *in vitro* (group A) remained practically normal throughout the period of observation. In *in vivo* studies, total white blood cells (WBC) and PMN leukocyte counts decreased initially and tended to approach towards pre-stimulated values at the end of the protocol. There were no changes in these counts in *in vitro* studies. These results indicate that the capacity to generate OFR is decreased in the *in vivo* and *in vitro* pre-stimulated PMN leukocytes. However this activity recovers with time. This study also suggests that the activated PMN leukocytes are not destroyed.

Introduction

Oxygen-derived free radicals, superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$), have been implicated as mediators of tissue injury in several pathological conditions such

as heart failure, inflammation, irradiation, ischemia/reperfusion, circulatory shock injury and respiratory distress syndrome [1–7]. There are various sources of oxygen free radicals including xanthine-xanthine oxidase system [7, 8], polymorphonuclear leukocytes [6, 9] and arachidonic acid metabolism [10]. Activation of polymorphonuclear (PMN) leukocytes initiates a 'respiratory burst' with a sudden and large increase in oxygen-derived free radicals (OFR) [11] and hypochlorous acid [9] which would have deleterious effects on the various organ systems in the body [1, 2, 4, 7]. The questions regarding the fate of the activated PMN leukocytes remain unanswered. These questions include: a) what happens to the OFR releasing activity of the pre-stimulated PMN leukocytes? It is possible that activated PMN leukocytes would have less capacity to produce and release oxygen free radicals with subsequent stimulation. b) Are these PMN leukocytes destroyed after activation? Luminol-dependent chemiluminescence would provide a continuous method for monitoring the rate of production of oxygen free radicals by PMN leukocytes and the PMN leukocyte counts will determine if they are destroyed when activated.

We, therefore, studied 1) the effects of stimulation of PMN leukocyte on the PMN leukocyte counts. However, the decrease in the PMN leukocyte count would not necessarily reflect destruction of the PMN leukocytes. A decrease in the circulating PMN leukocyte may be due to aggregation of PMN leukocytes in the pulmonary and other vasculature and 2) the chemiluminescence activity of pre-stimulated PMN leukocytes. Opsonized zymosan was used to activate PMN leukocytes. Oxygen free radical producing activity and survival of activated PMN leukocytes may differ in *in vivo* from *in vitro*. Therefore, the studies were conducted both in *in vivo* and *in vitro*.

Materials and methods

Two series of experiments were conducted. In *in vivo* series, mongrel dogs of either sex weighing between 16 to 20 kg were anaesthetized with sodium pentobarbital (30 mg/kg) given intravenously. The animals were ventilated with room air through endotracheal tube using a Harvard respirator with a volume of 20 ml/kg and a respiratory rate of 20/min. An indwelling venae-catheter was positioned in the forearm vein for administration of zymosan and collection of blood samples for chemiluminescence and total white blood cells (WBC) and PMN leukocyte counts. In *in vitro* series, the blood samples were collected from unanaesthetized dogs for studies.

Preparation of opsonized zymosan

The method for preparation of opsonized zymosan was essentially similar to that of Kato *et al.* [12] and as described in the manual of LKB Wallac for luminescence [13]. In short, zymosan A from saccharomyces cerevisiae obtained from Sigma Chemical Company was opsonized by addition of 1 ml of zymosan suspension (50 mg/ml) in Hank's balanced salt solution (HBSS) to 3 ml of serum. The HBSS solution of the following composition (pH 7.4): $CaCl_2$, 0.14 g; KCl, 0.4 g; KH_2PO_4, 0.06 g; $MgCl_2$. $6H_2O$, 0.1 g; $MgSO_4$. $7H_2O$, 0.1 g; NaCl, 8.0 g; $NaHCO_3$, 0.35 g; Na_2HPO_4. $7H_2O$, 0.09 g; distilled water, 1 liter was used. The mixture was incubated for 40 min at 37° C with continuous agitation and then centrifuged at 3000 rpm for 10 min at ambient temperature (18–20° C). The supernatant was removed and the pellet resuspended in 4 ml of HBSS. This pellet suspension was centrifuged at 3000 rpm for 10 min at ambient temperature. The pellet was, then, resuspended in 5 ml of HBSS to give a final concentration of 10 mg/ml of zymosan.

PMN leukocyte counts and chemiluminescence

The venous blood was collected in EDTA (ethylenediaminetetraacetic acid) containing tubes for measurement of chemiluminescent activity of PMN leukocytes. Total white blood cell and PMN leukocyte counts were made using a Technicon H6000™ system. The method for chemiluminescence measurement was essentially similar to that

of Tono-oko *et al.* [14] and Prasad *et al.* [6] and as described in the manual of LKB Wallac for luminescence [13]. In short, for the measurement of luminol-dependent chemiluminescence, 0.05 ml (approximately 2.5×10^5 PMN leukocytes) of blood were added to a counting vial containing 0.25 ml of Hank's balanced salt solution, pH 7.4 and 0.1 ml of luminol (10^{-4} M). Samples were placed in a luminometer and the background chemiluminescence were recorded for 10 min. Phagocytosis was initiated by the addition of 0.1 ml (10 mg/ml) of opsonized zymosan. The final volume of the mixture was 0.5 ml. In those tubes where zymosan or SOD were not used, the final volume (0.5 ml) of the test mixture were made up by adding appropriate amount of HBSS. The chemiluminescence was monitored using a LKB-Wallac 1251 luminometer. The counts were made for four seconds every four minutes for a period of sixty minutes. Luminol-dependent chemiluminescence (CL) for each sample was determined a) without activation by zymosan (resting), b) with activation by zymosan, and c) with activation by zymosan in the presence of 0.1 ml superoxide dismutase (SOD) (1 mg/ml; 3050 U/mg protein). The area under each curve was integrated to give total CL response during the period of monitoring. The difference in areas under zymosan activated and resting curves is designated as 'oxygen-derived free radicals' CL while that under zymosan activated in the absence and in the presence of SOD is designated as SOD inhibitable oxygen free radicals CL. The integrated area under the curve is in mV · min. The unit for chemiluminescence is mV · min per 10^6 PMN leukocytes. The results are expressed in absolute values (mV · min/10^6 PMN) or as percent change in CL activity.

Experimental protocol

I. PMN leukocytes stimulation in in vivo

Seven anaesthetized dogs in this group received opsonized zymosan in the dose of 8.4 mg/kg intravenously to stimulate PMN leukocytes in *in vivo*. Blood samples for total WBC and PMN leukocyte counts, and PMN leukocyte chemiluminescence were collected before and after 5, 15, 30, 60, 90 and 120 min of zymosan administration in *in vivo*. The chemiluminescence of pre-stimulated PMN leukocytes in the blood samples were measured without activation by zymosan, with activation by zymosan (0.1 ml of 10 mg/ml) and with activation by zymosan in the presence of SOD (0.1 ml of 1 mg/ml).

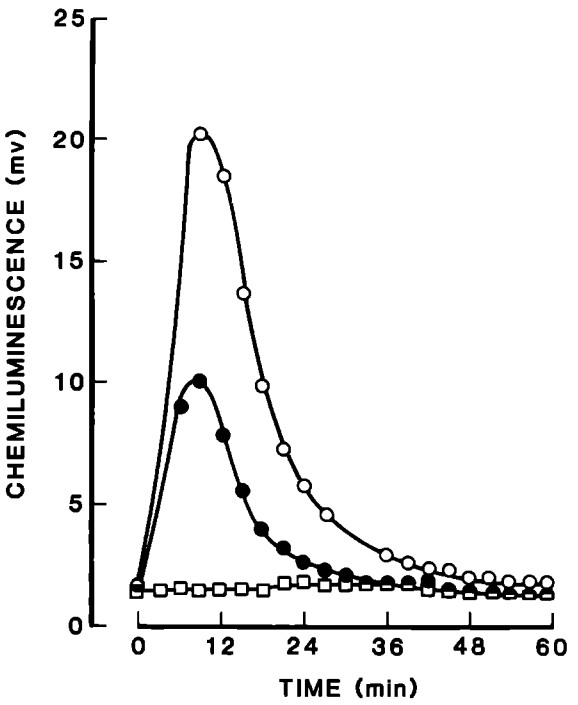

Fig. 1. Typical tracing of zymosan induced chemiluminescence of PMN leukocytes of blood from dogs in the presence and absence of superoxide dismutase (SOD). □, resting chemiluminescence (without zymosan activation); ○, zymosan-induced chemiluminescence in the absence of SOD; ●, zymosan-induced chemiluminescence in the presence of SOD.

II. PMN leukocytes stimulation in vitro

This group comprised of 7 unanaesthetized dogs. Thirty ml of venous blood from each dog was collected in EDTA containing tube. This blood was divided into two groups. Group A: non-stimulated, without any added zymosan; and Group B: Zymosan-stimulated, zymosan in the concentration of 150 μg/ml was added to the blood of this group. The blood of these two groups were kept at room temperature (18–20° C). Samples of blood for

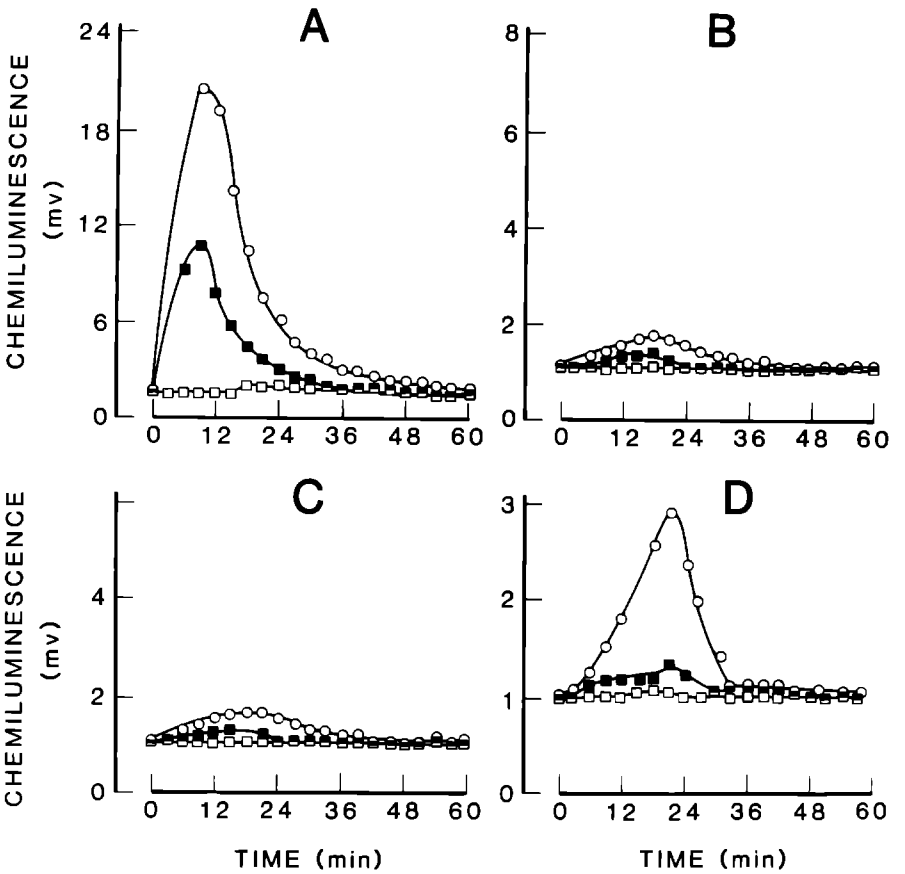

Fig. 2. Changes in chemiluminescence of pre-stimulated polymorphonuclear (PMN) leukocytes of blood from dogs treated with zymosan (*in vivo*). The chemiluminescence was determined before (A), and after 5 (B), 15 (C), and 120 (D) min of zymosan administration. Abscissa shows the duration of chemiluminescence measurement for each sample while ordinate indicates the chemiluminescence (mV). □, resting chemiluminescence (without additional zymosan activation); ○, zymosan-induced PMN chemiluminescence; ■, zymosan-induced chemiluminescence in the presence of SOD. Note the decrease in the chemiluminescence induced by added zymosan at 5 and 15 min and a recovery towards control values at 120 min in the pre-stimulated PMN leukocytes (zymosan administered *in vivo*) of blood.

WBC and PMN counts and PMN leukocyte chemiluminescence from group B were collected at 0, 5, 15, 30, 60, 90 and 120 min after zymosan addition. Blood samples from group A were also collected at similar time intervals. The chemiluminescence of non-stimulated (group A) and pre-stimulated (group B) PMN leukocytes in the blood samples were measured without any further addition of zymosan, with activation by zymosan (0.1 ml of 10 mg/ml) and with activation by zymosan in the presence of SOD (0.1 ml of 1 mg/ml).

Statistical analysis

Statistical analysis of the results was made using paired and unpaired student 't' test and non-parametric statistical test (Mann-Whitney Rank Sum Test, BMDP Statistical Software). Mann-Whitney Rank Sum Test was used for percent change analysis only. A 'P' value < 0.05 was considered significant. All results were presented as mean ± SEM.

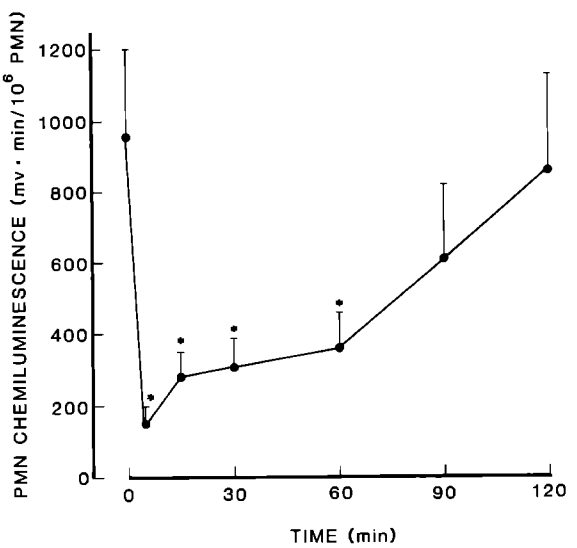

Fig. 3. Changes in the SOD inhibitable chemiluminescence of *in vivo* pre-stimulated PMN leukocytes of blood from dogs. The values at zero time represent the chemiluminescence of the non-stimulated PMN leukocytes and the values at subsequent intervals are from *in vivo* stimulated PMN leukocytes. Each point on the curve represents the mean ± SEM from seven dogs. The results are expressed in absolute values (mV · min/10^6 PMN). * $P < 0.05$, comparison of the values at different time intervals with respect to the values before zymosan treatment ('0' min). Note a marked decrease in the oxygen derived free radical chemiluminescence in pre-stimulated PMN leukocytes.

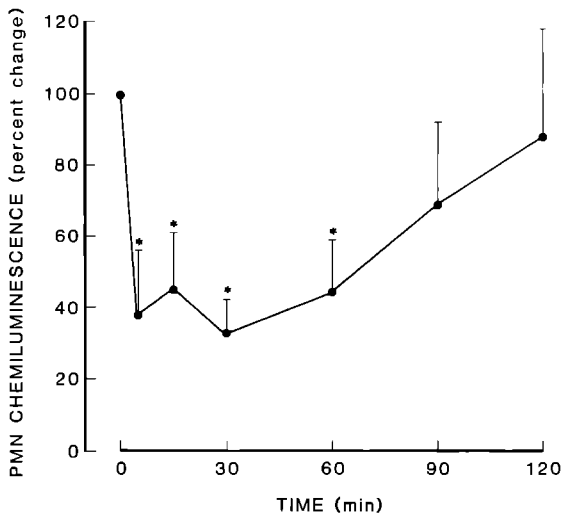

Fig. 4. Changes in the chemiluminescence due to oxygen-derived free radical of *in vivo* stimulated PMN leukocytes of blood from seven dogs. The results are expressed as percent of the values before zymosan treatment ('0' time) taken as 100 percent. Note a marked decrease in the oxygen derived free radical chemiluminescence in pre-stimulated PMN leukocytes. * $P < 0.05$, comparison of the values at different time intervals with respect to the values before zymosan treatment ('0' min).

Results

Chemiluminescence studies

Luminol-dependent chemiluminescence provides a highly sensitive and continuous method for monitoring the rate of production of oxygen free radicals by PMN leukocytes. A typical tracing of the zymosan-induced chemiluminescence of PMN leukocytes in blood in the presence and in absence of superoxide dismutase (SOD) is shown in Fig. 1. The chemiluminescence was determined a) without activation by zymosan (resting), b) with activation by zymosan and c) with activation by zymosan in the presence of SOD. The area under each curve was integrated to give chemiluminescence (CL) response during the period of monitoring. The chemiluminescence increased rapidly with the addition of zymosan and reached a peak value within 8–12 min after which it decreased slowly for the duration of the observation period. The integrated area for chemiluminescence was high in the presence of zymosan as compared to that in its absence. The zymosan-induced CL was lower in the presence of SOD than in its absence.

Chemiluminescence studies in pre-stimulated PMN leukocytes in vivo

Chemiluminescent activities of PMN leukocytes of blood before and after 5, 15, 30, 60 and 120 min of zymosan administration *in vivo* were studied in 7 dogs to determine if oxygen free radical-producing activity of pre-stimulated PMN leukocytes are affected. Typical tracings of one such study before and after 5, 15 and 120 min of zymosan administration in dog are shown in Fig. 2 and the results of these studies are summarized in Figs 3–5. Before zymosan administration (0 min), the resting curve, which represents the oxygen free radicals in the blood in the absence of PMN stimulation by zymosan, shows very low chemiluminescence. There was a marked increase in the zymosan induced

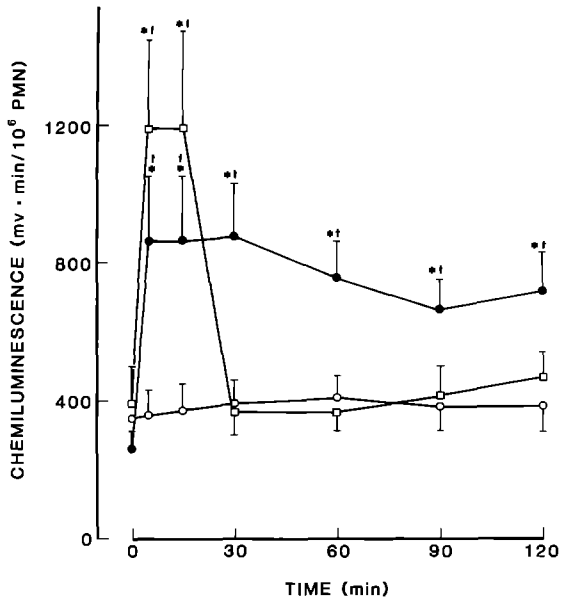

Fig. 5. Changes in the resting chemiluminescence of non-stimulated PMN leukocytes in *in vitro* blood; and *in vivo* and *in vitro* pre-stimulated PMN leukocytes. The PMN leukocytes *in vivo* and *in vitro* were stimulated by administering (adding) zymosan. The results are expressed as mean ± SEM of absolute values. ○, non-stimulated PMN leukocytes in *in vitro;* ●, *in vitro* pre-stimulated PMN leukocytes; □, *in vivo* pre-stimulated PMN leukocytes. * $P < 0.05$, comparison of the values at various time course with respect to the values before zymosan treatment ('0' min) in the respective groups. † $P < 0.05$, non-stimulated vs pre-stimulated PMN leukocytes in *in vitro* or *in vivo*.

chemiluminescence. PMN leukocyte activation by zymosan in the presence of SOD had lower CL as compared to that in the absence of SOD. In pre-stimulated PMN leukocytes, the resting CL remained low throughout the 2 hour period of observation except at 5 and 15 min where there was a significant increase in CL (Fig. 5). The SOD inhibitable chemiluminescence activity of pre-stimulated PMN leukocytes (Fig. 3) decreased significantly at 5 min. The activity thereafter began to increase to reach almost towards values at 0 min but still significantly lower till 60 min when compared the values at '0' min. Oxygen derived free radical chemiluminescence (Fig. 4) decreased significantly in the pre-stimulated PMN leukocytes within 5 min and remained decreased till 60 min. The activity tended to reach towards the values at '0 min' after 90 min of pre-stimulation. These results suggest that there is a decrease in the capacity of the pre-stimulated PMN leukocytes to release oxygen free radicals. The activity of PMN leukocytes to release oxygen free radicals tends to return to near normal values at the end of 2 hours of observation period.

II. Chemiluminescence studies in pre-stimulated PMN leukocytes in in vitro

As given in the experimental protocol, this study consisted of two groups of blood: A) Non-stimulated, without zymosan stimulation and B) zymosan stimulated (pre-stimulated). Typical tracings of chemiluminescence of non-stimulated and stimulated PMN leukocytes in blood at 0, 15 and 120 min are shown in Fig. 6 and the results are summarized in Figs 5, 7 and 8. While the resting CL in blood of non-stimulated PMN leukocytes was low, the resting CL in blood of pre-stimulated PMN leukocytes was high throughout the period of observation. Zymosan activated PMN chemiluminescence activity in the absence and in the presence of SOD was normal and was almost unchanged throughout the period of studies. Activation of pre-stimulated PMN leukocytes by zymosan produced a lower chemiluminescence as compared to that of non-stimulated PMN leukocytes. Superoxide dismutase (SOD) inhibitable chemiluminescence (Fig. 7) in the pre-stimulated PMN leukocytes decreased significantly up to 60 min. There was no significant change except at 30 min in the SOD inhibitable chemiluminescence of the PMN leukocytes that were not pre-stimulated. Superoxide dismutase inhibitable chemiluminescence of pre-stimulated PMN was significantly lower than that of non-stimulated PMN at 5 and 15 min only. The oxygen derived free radical chemiluminescence (Fig. 8) of pre-stimulated PMN leukocytes decreased significantly within 5 min and remained depressed throughout the period of observation. Oxygen derived free radical chemiluminescence of non-stimulated PMN leukocytes did not change throughout the experiment except at 30 min period where there was a decrease in the CL activity when compared to the values at '0' min. Oxygen derived free radical chemiluminescence activity of pre-stimulated

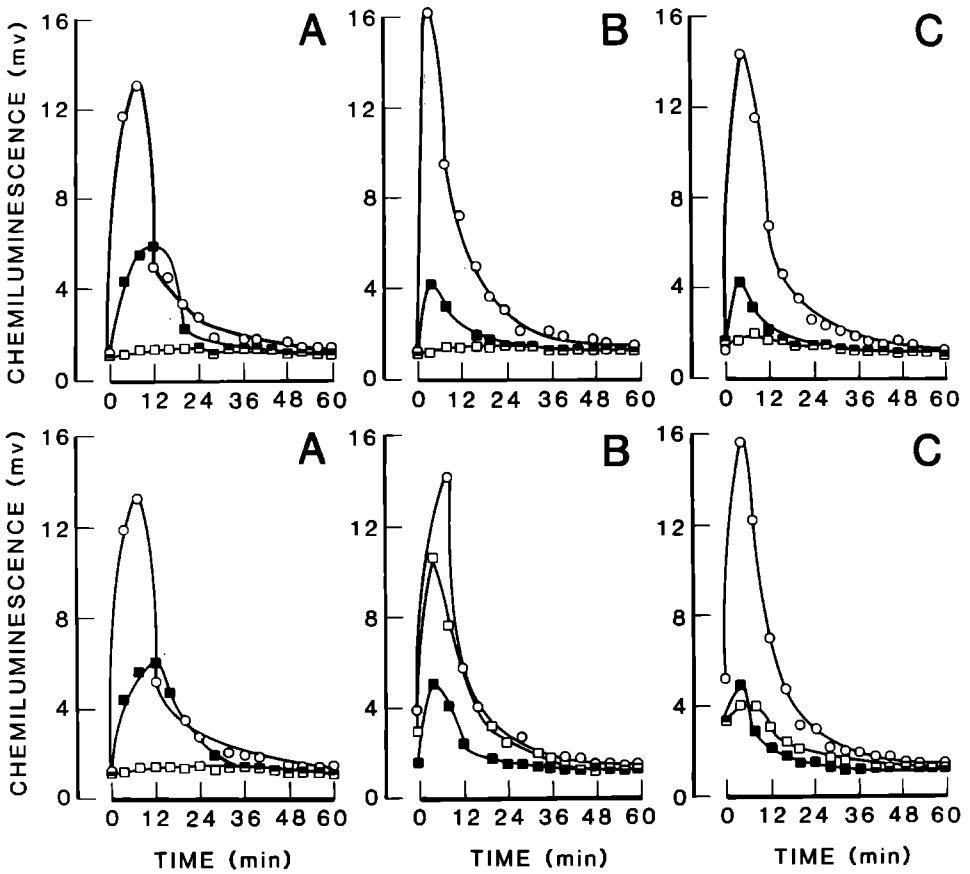

Fig. 6. Changes in chemiluminescence of non-stimulated (upper pannel) and in *in vitro* pre-stimulated (lower pannel) PMN leukocytes. The chemiluminescence was determined before (A), and after 15 (B) and 120 (C) min of zymosan treatment (lower pannel). Chemiluminescence of the non-stimulated PMN leukocytes (upper pannel) was determined at time intervals similar to that of stimulated PMN leukocytes. □, resting chemiluminescence; ○, zymosan induced chemiluminescence; ■, zymosan-induced chemiluminescence in the presence of SOD. Note the decrease in the chemiluminescent activity of the in *in vitro* pre-stimulated PMN leukocytes. Also note a marked increase in the resting chemiluminescence of the blood of *in vitro* pre-stimulated PMN leukocytes.

PMN leukocytes was significantly lower than that of the non-stimulated PMN leukocytes throughout the 2 hour period of observation.

Resting chemiluminescence of pre-stimulated blood

The resting chemiluminescence of blood from pre-stimulated PMN leukocytes was high throughout the 2 hour period of observation. Chemiluminescence of such blood in the absence and in the presence of SOD without activation of PMN leukocytes by zymosan *in vitro* was measured to see if this resting chemiluminescence was due to superoxide anions. In all seven experiments SOD markedly inhibited the resting chemiluminescence suggesting that the major component of resting CL is due to superoxide anion. A typical tracing from one such study is shown in Fig. 9.

Total WBC and PMN leukocyte count

The total WBC and PMN leukocytes for *in vitro* and *in vivo* studies are shown in Table 1. Total WBC and PMN leukocyte counts of blood with and without zymosan stimulation in *in vitro* studies remained unchanged throughout the period of obser-

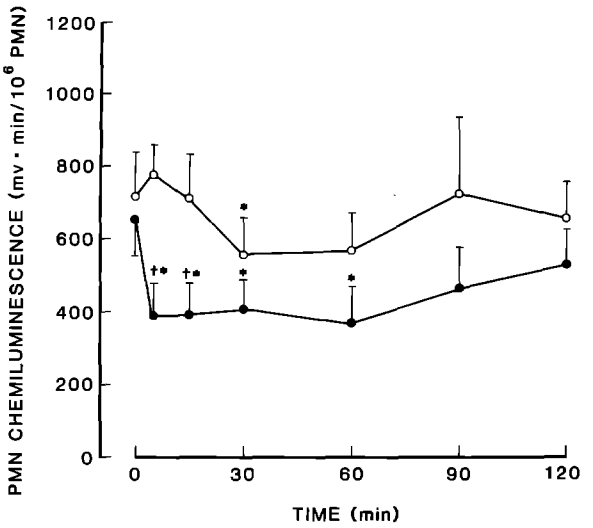

Fig. 7. Changes in the SOD inhibitable chemiluminescence of *in vitro* non-stimulated and stimulated PMN leukocytes. The results are expressed as mean ± SEM in absolute values from 7 experiments. The values at '0' min represent the chemiluminescence of non-stimulated PMN leukocytes in both groups. ○, non-stimulated PMN leukocytes; ●, stimulated PMN leukocytes. * $P < 0.05$, comparison of the values at various time course with respect to the values at '0' minute in the respective groups. † $P < 0.05$, stimulated vs non-stimulated group.

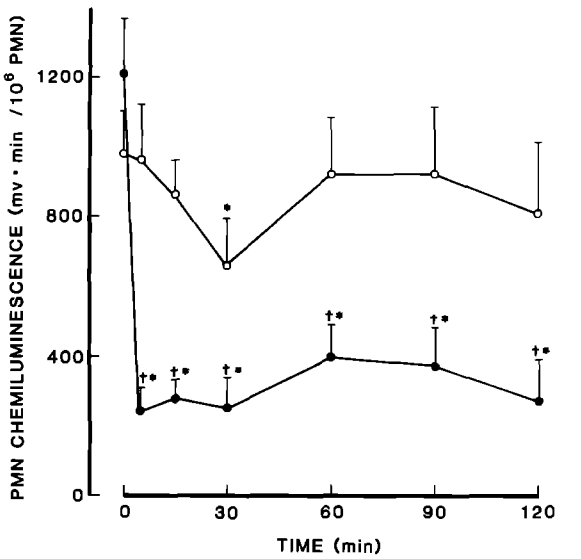

Fig. 8. Changes in the chemiluminescence due to oxygen derived free radicals of *in vitro* non-stimulated and stimulated PMN leukocytes of blood. Results are expressed as mean ± SEM in absolute values from seven experiments. ○, non-stimulated PMN leukocytes; ●, stimulated PMN leukocytes. * $P < 0.05$, comparison of the values at various time course with respect to the values at '0' minute in the respective groups. † $P < 0.05$, stimulated vs non-stimulated group.

vation. The PMN leukocyte and WBC counts decreased in *in vivo* studies where PMN leukocytes were stimulated by administration of zymosan. However the decrease in PMN leukocyte counts were significant only at 5 and 15 minutes after zymosan administration. These results of *in vivo* and *in vitro* studies indicate that PMN leukocytes when activated by zymosan are not destroyed. The lower values in *in vivo* groups may be due to some other reason.

Discussion

Opsonized zymosan was used to stimulate PMN leukocytes in the present studies. Opsonized zymosan has been used by other investigators to stimulate PMN leukocytes [6, 7, 15–17]. It is to be noted that whole blood was used to investigate the PMN chemiluminescence in this study. No attempt was made to isolate PMN leukocytes. However, the chemiluminescence of the blood was expressed in terms of PMN leukocyte content of the blood. Whole blood has been used by various investigators in the past for chemiluminescence studies [6, 7, 14, 18]. PMN leukocyte stimulation is accompanied by increased oxygen consumption [19] leading to the production of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) which may react to form hydroxyl radical ($\cdot OH$) and singlet oxygen ($^1O_2$) [1].

The chemiluminescent activity (the SOD inhibitable oxygen free radicals and oxygen derived free radical) of the *in vivo* stimulated PMN decreased significantly for 60 min and the activities thereafter tended to return towards the values at 0 min (pre-stimulated). This type of response in stimulated PMN leukocytes might be expected if zymosan administration in dogs had already stimulated PMN leukocytes to release oxygen free radicals and hence additional zymosan (for stimulation of PMN leukocytes for luminometer study) would release less oxygen free radicals. The activity of PMN leukocytes presumably recovers with time to provide

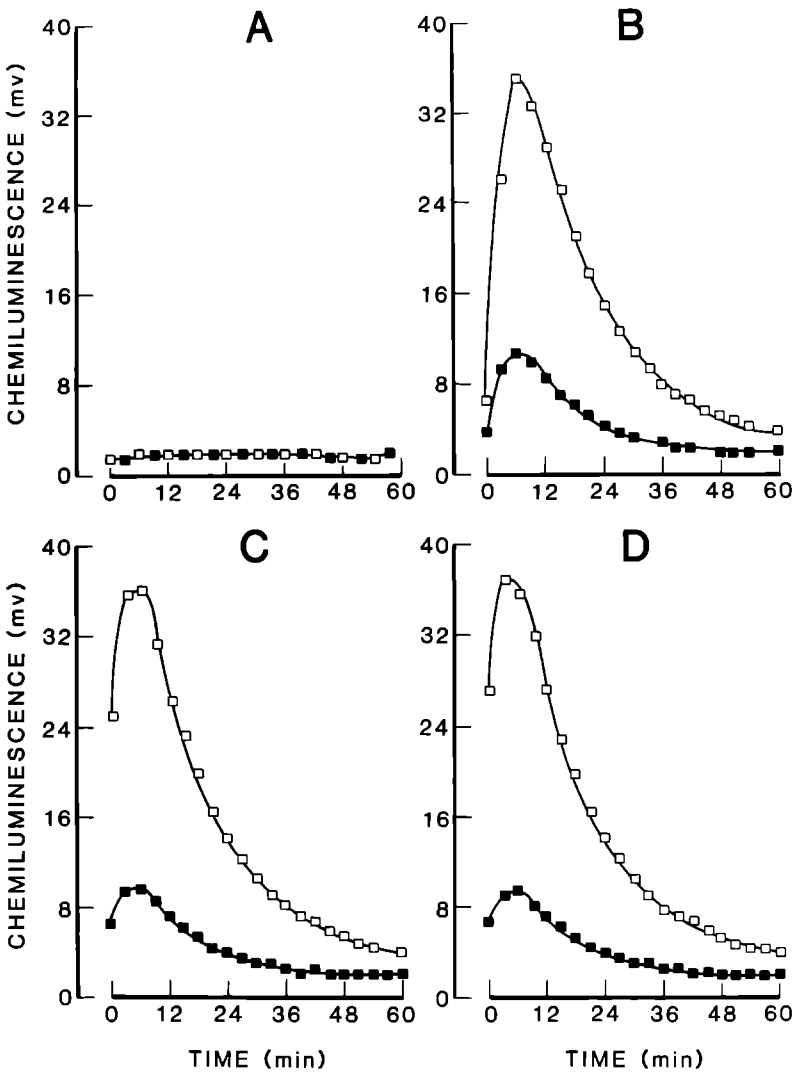

Fig. 9. Typical tracings of the resting chemiluminescence of *in vitro* stimulated PMN leukocytes of blood in the presence and absence of SOD. The chemiluminescence was determined before (A), and after 15 (B), 90 (C), and 120 (D) min of *in vitro* PMN leukocytes stimulation. □, resting chemiluminescence in the absence of SOD; ■, resting chemiluminescence in the presence of SOD.

chemiluminescence equivalent to the pre-stimulated level. If a decrease in the chemiluminescence activity of *in vivo* stimulated PMN leukocytes is due to the release of OFR from the PMN leukocytes, then the resting blood chemiluminescence would be higher when compared to that of the non-stimulated blood. Indeed the resting CL was higher in the beginning in zymosan treated dogs when compared to the pre-treatment values. The values were high only till 15 min and returned to pre-stimulated values at 30 min and after. These findings suggest that the oxygen free radicals metabolizing agents (SOD and catalase), which are normally present in the tissue, might have metabolized the superoxide anion and H_2O_2 [20, 21].

The resting chemiluminescence of blood was very high in those studies where the blood samples were collected from dogs and subsequently treated *in vitro* with zymosan. Also there was a marked decrease in the SOD inhibitable OFR and oxygen derived free radical chemiluminescent activity of in *in vitro* stimulated PMN leukocytes. However

there were practically no changes in the chemiluminescent activity of the non-stimulated PMN leukocytes. The decrease in the PMN chemiluminescent activity of *in vitro* stimulated PMN leukocytes in the early stage and the recovery thereof in the later stage might be due to reasons similar to those stated in the studies for *in vivo* stimulated PMN leukocytes. The decrease in the chemiluminescent activity of pre-stimulated PMN leukocytes may not be due to the loss of cell viability at room temperature for two reasons. Firstly there was a recovery of chemiluminescence of the pre-stimulated PMN leukocytes at the end of two hours. Secondly the chemiluminescent activity of non-stimulated PMN leukocytes *in vitro* practically remained unchanged for similar period of observations. There was, however, a decrease in the activity at 30 min in the non-stimulated PMN leukocytes. The reason for such finding is not known at present.

The resting chemiluminescence of the zymosan treated blood increased markedly at 5 min of zymosan treatment and remained increased throughout the period of observation. Opsonized zymosan releases oxygen free radicals from the PMN leukocytes. The high values of resting chemiluminescence, then may be because of a decrease in the metabolism of released oxygen free radicals. The decrease in the metabolism might be due to a) absence or low values of oxygen free radical metabolizing agents (SOD and catalase) in the blood and b) non-circulation of blood through the various organs in the body that contain large amounts of metabolizing agents (SOD, catalase, glutathione peroxidase) [20, 21]. Blood and extracellular fluid contain SOD and catalase [22, 23] hence the possibility that the absence of these enzymes may play a role can be ruled out. The second possibility might be more plausible because in the studies of the *in vivo* stimulated PMN leukocytes, the resting chemiluminescence was not as high as in the studies of the *in vitro* stimulated PMN leukocytes.

The finding that most of the resting chemiluminescence (approximately 70%) in blood of pre-stimulated PMN leukocytes *in vitro* was SOD inhibitable is interesting. This finding suggests that superoxide anion is present in high quantity in the blood of *in vitro* pre-stimulated PMN leukocytes. However it is known that the half life of superoxide anion is few milliseconds. This high persistant val-

Table 1. Changes in the total white blood cells (WBC) and polymorphonuclear (PMN) leukocytes counts in blood with and without zymosan treatment in *in vivo* and *in vitro*

Time (min)	PMN counts (Giga/L) (n = 7)			WBC counts (Giga/L) (n = 7)		
	Non stimulated *in vitro*	Zymosan stimulated *in vitro*	Zymosan stimulted *in vivo*	Non stimulated *in vitro*	Zymosan stimulated *in vitro*	Zymosan stimulated *in vivo*
0	4.70 ± 1.00	3.93 ± 0.98	4.25 ± 0.52	6.73 ± 1.30	5.77 ± 1.38	6.30 ± 0.44
5	4.70 ± 1.00	3.68 ± 0.85	1.14* ± 0.20	6.71 ± 1.30	5.59 ± 1.25	1.76* ± 0.44
15	4.73 ± 1.00	3.65 ± 0.85	1.35* ± 0.40	6.71 ± 1.30	5.54 ± 1.23	2.32* ± 0.92
30	4.74 ± 1.00	3.55 ± 0.83	3.03 ± 0.55	6.61 ± 1.30	5.57 ± 1.18	3.84 ± 0.97
60	3.90 ± 1.00	3.74 ± 0.85	2.58 ± 0.70	5.40 ± 0.90	5.59 ± 1.24	2.63* ± 0.40
90	4.90 ± 1.10	3.82 ± 0.86	2.78 ± 0.82	6.93 ± 1.40	5.84 ± 1.22	2.89 ± 0.74
120	5.00 ± 2.10	3.91 ± 0.90	2.30 ± 0.78	6.97 ± 1.30	6.04 ± 1.25	2.37* ± 0.81

Values are expressed as mean ± SEM.

* $P < 0.05$ comparison of values at different time intervals with respect to the values before zymosan treatment (0 min).

ues of superoxide anion can be explained if the dismutation of superoxide anion in the studies of *in vitro* stimulated PMN leukocytes is very slow. However such phenomenon have not been reported in the literature.

The circulating WBC and PMN leukocytes decreased in those studies where zymosan was administered in *in vivo* while there were no changes in WBC and PMN leukocyte counts in the studies where zymosan was added to the blood in *in vitro*. The decrease in the PMN leukocyte counts in *in vivo* studies might suggest their destruction after activation with zymosan. However the *in vitro* studies indicated that PMN leukocytes are not destroyed after activation with zymosan. Other possibility for a decrease in the PMN leukocytes in *in vivo* studies would be the trapping of neutrophils in the vasculature of various organ systems and therefore a decrease in the circulating PMN leukocytes. This possibility is supported by the fact that neutropenia occurs with complement activation [24]. Complement exposed PMN leukocytes are stimulated both to adhere to other surfaces [25] and to aggregate [26]. Pulmonary microvascular stasis of leukocytes have been shown to occur from complement activation [27, 28]. Several studies have indicated that PMN leukocytes are attracted to the myocardium by chemotactic factors released from ischemic heart [29–31]. These changes in the total WBC counts were similar to those of PMN leukocytes indicating that PMN leukocytes were the major component in decreasing the total WBC counts.

This study demonstrates that the pre-stimulated PMN leukocytes have decreased capacity to further produce and/or release oxygen derived free radicals with subsequent stimulation with zymosan. However, this activity recovers with time. The precise mechanism of decreased chemiluminescence is not known. It might be that zymosan administration had already released oxygen free radicals and hence additional zymosan (for stimulation of leukocytes for chemiluminescence study) would release less oxygen free radicals leading to a decrease in the chemiluminescence. The recovery of chemiluminescence with time may be related to the recovery of the activity of the PMN leukocytes. This study also indicates that the PMN leukocytes are not destroyed when stimulated to release oxygen-derived free radicals.

Acknowledgements

This investigation was supported in part by grants from the Saskatchewan Heart Foundation Inc. and the Saskatchewan Health Research Board. Dr. A.K. Chaudhary is the recipient of a Saskatchewan Health Research Board Fellowship. We wish to thank Mr. P.K. Chattopadhyay and Mr. D. Duncan for their technical assistance.

References

1. Del Maestro RF, Thaw HH, Bjork J, Planker M, Arfors KE: Free radicals as mediators of tissue injury. Acta Physiol Scand 492 (Suppl): 43–57, 1980
2. Jolly SR, Kane WJ, Bailie MB, Abrams GD, Lucchesi BR: Canine myocardial reperfusion injury: Its reduction by the combined administration of superoxide dismutase and catalase. Circ Res 54: 277–285, 1984
3. Perkowski SZ, Havill AN, Flynn JT, Gee MH: Role of intrapulmonary release of eicosanoids and superoxide anion as mediators of pulmonary dysfunction of endothelial injury in sheep with intermittent complement activation. Circ Res 53: 574–583, 1983
4. Prasad K, Kalra J: Oxygen free radicals and heart failure. Angiology 39: 417–420, 1988
5. Prasad K, Kalra J, Buchko G. Acute hemorrhage and oxygen free radicals. Angiology 39: 1005–1013, 1988
6. Prasad K, Kalra J & Bharradwaj B: Increased chemiluminescence of polymorphonuclear leukocytes in dogs with volume overload heart failure. Br J Exp Path 70: 463–468, 1989
7. Prasad K, Kalra J, Chan WP, Chaudhary AK: Effect of oxygen free radicals on cardiovascular function at organ and cellular level. Am Heart J 117: 1196–1202, 1989
8. Hess ML, Okabe E, Kontos HA: Proton and free oxygen radical interaction with the calcium transport system of cardiac sarcoplasmic reticulum. J Mol Cell Cardiol 13: 767–772, 1981
9. Fantone JC, Ward PA: Role of oxygen-derived free radicals and metabolites in leukocytes-dependent inflammatory reactions. Am J Pathol 107: 397–417, 1982
10. Dzau VJ, Packer M, Lilly LS, Swartz SL, Hollenberg NK, Williams GH: Prostaglandins in severe congestive heart failure. Relation to activation of the Renin-Angiotensin system and hyponatremia. N Engl J Med 310: 347–352, 1984
11. Babior BM: The respiratory burst of phagocytes. J Clin Invest 73: 599–601, 1984

12. Kato T, Wokalek H, Schöpf E, Eggert H, Ernst M, Rietschel ET, Fischer H: Measurement of chemiluminescence in freshly drawn human blood. Klin Wochenschr 59: 203–211, 1981
13. LKB-Wallac 1251 Luminometer Manual. The study of phagocytosis and opsonisation using luminol enhanced chemiluminescence. Application Note 513. Wallac of Finland. pp 1–5, 1985
14. Tono-oko T, Veno N, Matsumoto T: Chemiluminescence of whole blood. A simple and rapid method for the estimation to phagocytic function of granulocytes and opsonic activity in whole blood. Clin Immun Immunopath 26: 66–75, 1983
15. Hastings MJG, Petricevic I, Williams AJ, Cole PJ, Easmon CSF: The effect of culture media on the production and measurement of luminol-dependent chemiluminescence. Brit J Exp Path 63: 147–153, 1982
16. Holt ME, Ryall MET, Campbell AK: Albumin inhibits human polymorphonuclear leukocyte luminol-dependent chemiluminescence: evidence for oxygen free radical scavenging. Brit J Exp Path 65: 231–241, 1984
17. Nelson RD, Herron MJ, Schmidtke JR, Simmons RL: Chemiluminescence response of human leukocytes: Influence of medium components on light production. Infection Immun 17: 513–520, 1977
18. Dechatelet CR, Shirley PS: Evaluation of chronic granulomatous diseases by a chemiluminescent assay of microlitre quantities of whole blood. Clin Chem 27: 1739–1741, 1981
19. Dewald B, Baggioleni M, Curnette JT, Babior BM: Subcellular localization of the superoxide forming system in human neutrophils. J Clin Invest 63: 21–29, 1979
20. Godin DV, Wohaieb SA, Garnett ME, Goumeniouk AD: Antioxidant enzyme alterations in experimental and clinical diabetes. Mol Cell Biochem 84: 223–231, 1988
21. Guarnieri C, Flamigni F, Caldarera CM: Role of oxygen in the cellular damage induced by reoxygenation of hypoxic heart. J Mol Cell Cardiol 12: 797–808, 1980
22. Abdalla DSP, Monteiro HP, Oliveira JAC, Bechara EJH: Activities of superoxide dismutase and glutathione peroxidase in schizophrenic and manic-depressive patients. Clin Chem 32: 805–807, 1986
23. L'abbé MR, Fischer PWF: An automated method for the determination of Cu, Zn-superoxide dismutase in plasma and erythrocytes using an ABA-200™ discrete analyzer. Clin Biochem 19: 175–178, 1986
24. Hammerschmidt DE, Stroncek DF, Bowers TK, Lammi-Keefe CJ, Kurth DM, Andrew Ozalins BA, Nicoloff DM, Lillehei RC, Craddock PR, Jacob HS: Complement activation and neutropenia occuring during cardiopulmonary bypass. J Thorac Cardiovasc Surg 81: 370–377, 1981
25. Fehr J, Jacob HS: *In vitro* granulocyte adherence and *in vivo* margination. Two associated complement dependent functions. J Exp Med 146: 641–652, 1977
26. Craddock PR, Fehr J, Brigham KL, Kronenberg RS, Jacob HS: Complement and leukocyte mediated pulmonary dysfunction in hemodialysis. N Eng J Med 296: 769–774, 1977
27. Craddock PR, Hammerschmidt DE, White JG, Dalmasso AP, Jacob HS: Complement (C_{5a})-induced granulocyte aggregration in vitro. A possible mechanism of complement mediated leukostasis and leukopenia. J Clin Invest 60: 261–264, 1977
28. Hammerschmidt DE, Craddock PR, McCullough J, Kronenberg RS, Dalmasso AP, Jacob HS: Complement activation and pulmonary leukostasis during nylon fiber filtration leukopharesis. Blood 51: 721–730, 1972
29. Engler RL, Dahlgren MD, Peterson MA, Schmid-Schoenbein G: Role of leukocytes in the response to acute myocardial ischemia and reflow in dogs. Am J Physiol 251: H314–H323, 1986
30. Engler RL, Dahlgren MA, Dobbs A, Schmid-Schoenbein G: Accumulation of polymorphonuclear leukocytes during 3 hour experimental myocardial ischemia. Am J Physiol 251: H93–H100, 1986
31. Forman MB, Puett DW, Virmani R: Endothelial and myocardial injury during ischemia and reperfusion: Pathogenesis and therapeutic implications. J Am Coll Cardiol 13: 450–459, 1989

Address for offprints: K. Prasad, Department of Physiology, College of Medicine, University of Saskatchewan, Saskatoon, Saskatchewan S7N 0W0 Canada