Source of Hydrogen Peroxide and of Chemiluminescence Observed in Activated Human Platelet Preparations

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Human platelet suspensions can be observed to produce small amounts of H$_2$O$_2$ (0.04 nmoles H$_2$O$_2$/min/2.5 x 10$^5$ cells/cu mm) and measurable chemiluminescence when exposed to target particles for phagocytosis, such as latex spherules. Both H$_2$O$_2$ production and chemiluminescence are characteristic of phagocytosing polymorphonuclear leukocytes (PMN) and analysis of the purified platelets indicates contamination by PMN at the level of 0.2%. The amount of H$_2$O$_2$ produced and the chemiluminescence observed can be duplicated by adding latex spheres to a preparation of PMN at a concentration equivalent to the contaminant in the platelet preparations. We conclude that the H$_2$O$_2$ produced and chemiluminescence observed from activated platelets is due to the presence of small amounts of contaminating PMN. These studies emphasize the importance of controlling for PMN contamination in studies of platelet biochemistry and physiology.

The human polymorphonuclear leukocyte (PMN) and the human platelet both possess granules which are composed of a variety of potent enzyme systems. Phagocytosis is well established for the PMN and has recently been well documented as a property of the platelet. These considerations have led to the hypothesis that the blood platelet might play a role in inflammation or the host's defense against infection.

There are several characteristic changes which accompany phagocytosis by PMN, including reduction of nitroblue tetrazolium, a reaction also observed in phagocytosing platelets, production and release of H$_2$O$_2$, production of the superoxide radical, and the appearance of chemiluminescence (CL). The present study is a comparison of the production of H$_2$O$_2$ and CL by both phagocytosing PMN and phagocytosing platelets.

MATERIALS AND METHODS

Venous blood from normal individuals who had abstained from all medications, including aspirin, for at least 10 days was collected in plastic syringes and mixed immediately (5:1) with acid citrate anticoagulant in plastic tubes. Platelets were separated by centrifugation for 10 min at 200 g at 22°C, yielding platelet-rich plasma. Gel-filtered platelets were prepared from this material by a modification of the agarose gel filtration technique of Tangen et al. recently described by us. The coarse nylon mesh at the column base was composed of 500-µm diameter monofilament threads with a mesh count of 24 threads/in. The upper circle of fine mesh was composed of 50-µm diameter threads with a mesh count of 210 threads/in, which yielded a calculated pore size of 71 µm. It is possible that the use of a finer nylon mesh with a smaller
pore size would favor the exclusion of contaminating PMN. The eluting buffer was a modification of Tyrode’s albumin solution and contained 120 mM NaCl, 9 mM NaHCO3, 6 mM dextrose, 11 mM sodium citrate, 11 mM Tris, 1 mM KH2PO4, 3 mM KCl, 1 mM MgCl2, 2 mM CaCl2, and 5.0 g/100 ml salt-poor albumin, which had previously been dialyzed against the same buffer. The final pH was 7.35.

Granulocyte suspensions were prepared from acid-citrate anticoagulated blood simultaneously collected from the donors, and then were transferred into 50-ml plastic conical centrifuge tubes. Dextran (Sigma Chemical Co., St. Louis, Mo., Lot 124C-0415; average molecular weight 234,000) was added at a ratio of 1 ml of dextran solution (5 g/100 ml of 0.154 M NaCl) for each 2 ml of whole blood, as described by Babior et al. The blood was allowed to sediment for 45 min; then the supernatant was transferred to plastic tubes and centrifuged for 12 min at 500 g. The resulting leukocyte button was exposed to three parts iced distilled water for 30 sec to lyse erythrocytes, and then one part 0.6 M NaCl was added. The suspension was spun at 350 g for 4 min, and the leukocyte button was again exposed to distilled water, 0.6 M NaCl was added, and the suspension was centrifuged and finally washed with 0.154 M NaCl. A leukocyte suspension containing 4-5 x 10^6 leukocytes/cu mm was made with the eluting buffer described above, minus albumin. Differential counts showed 90%–95% of these leukocytes were PMN.

Direct measurement of H2O2 was done by assaying the decrease in fluorescence of scopoletin catalyzed by horseradish peroxidase. This reaction, originally described by Andreae, has been used by Root et al. and by us to determine the rate of release of H2O2 from activated granulocytes. Fluorescence was determined in an Aminco-Bowman spectrofluorometer (American Instrument Co., Silver Spring, Md.) at an excitation wavelength of 350 nm and an emission wavelength of 460 nm.

CL of PMN and platelets was determined by the method of Johnston et al. 2.5 ml of the cell suspension was placed in plastic scintillation vials which had been exposed only to red light and was counted out of coincidence in a liquid scintillation detector, model LS-100C (Beckman Instruments Inc., Fullerton, Calif.). Platelet and leukocyte counts were obtained on all cell suspensions in order to assess the degree of contamination of one cell type by the other. The PMN suspensions were essentially free of platelets, but all platelet suspensions contained small numbers of PMN. For this reason, platelet studies were performed with simultaneous studies of a pure suspension of isologous granulocytes diluted to the level found to be contaminating the platelet suspension as a control. In each group of experiments statistical significance was determined by Student’s t test for paired data.

H2O2 (30% solution, J. T. Baker Chemical Co., Phillipsburg, N.J.) was prepared fresh each day by dilution in distilled water and stored at 4°C until used. Scopoletin (Sigma) 1 mM in distilled water was stored at 4°C. Peroxidase, type II from horseradish (Sigma), 100 150 purpurogal- lin U/ml was made up in distilled water at 1.0 mg/ml and stored at 4°C. Superoxide dismutase (Sigma) was dissolved in distilled water at 500 μg/ml.

Dow latex particles (Pitman-Moore Division, Dow Chemical Co., Indianapolis, Ind.) with uniform diameters of 1.09 μm in a 10% solid suspension were used for all experiments involving PMN. For platelet studies, 0.109-μm-diameter latex particles were also used. *Staphylococcus aureus*, Newman D2C strain (Sigma), was suspended in 0.9% NaCl and added to cell suspensions at a final concentration of 90 μg/ml. Bovine thrombin (Parke-Davis & Co., Detroit, Mich.) was diluted in Tris buffer at pH 7.6 to a concentration of 8 U/ml and stored at -20°C until used. Disodium adenosine diphosphate (ADP, Sigma) was dissolved in barbital buffer (pH 7.35) at 10 mg/100 ml, adjusted to pH 6.8, and stored at -20°C.

RESULTS

H2O2 production was measured at 2-min intervals for 20 min at 37°C after the addition of excess latex particles (10 particles/cell) to the PMN suspension. As shown in Fig. 1, PMN at 7-10 x 10^1/cu mm released an average of 0.47 nmol H2O2/min. The amount of H2O2 produced decreased as a function of the number of PMN present. Suspensions of 2-3 x 10^3 platelets/cu mm were found to be contaminated by 4-6 x 10^2 PMN/cu mm. The addition of 0.109-μm latex particles to these platelet suspensions yielded a mean production
H$_2$O$_2$ AND CHEMILUMINESCENCE IN PLATELETS

Granulocytes + Latex Particles

Platelets + ADP Thrombin Latex Staphylococci

Fig. 1. H$_2$O$_2$ production by suspensions of human granulocytes stimulated by the addition of 1.09-$\mu$m latex particles (10 particles/cell) and by suspensions of human gel-filtered platelets stimulated by the addition of either ADP (10$^{-5}$ M), thrombin (0.32 U/ml), 0.109-$\mu$m latex particles (10 particles/cell), or Staphylococcus aureus (90 $\mu$g/ml). Incubations were carried out for 20 min at 37$^\circ$C.

of 0.04 nmoles H$_2$O$_2$/min, an amount not significantly different from the H$_2$O$_2$ production attributable to the contaminating PMN (Fig. 1). Thrombin at 0.32 U/ml and staphylococci at 90 $\mu$g/ml platelet suspensions yielded no measurable H$_2$O$_2$.

ADP added to the platelet suspensions at 10$^{-5}$ M yielded a result similar to that seen following exposure to latex. In order to evaluate whether this effect was dependent on the presence of platelets, ADP at 10$^{-5}$ M was added directly to the PMN suspension, at both 10$^3$/cu mm and 10$^4$/cu mm. In a series of five experiments, the H$_2$O$_2$ produced by resting PMN was not significantly different from that observed in PMN treated with ADP.

PMN were resuspended in autologous cell-free plasma at 2.5 $\times$ 10$^4$/cu mm and 2.5 ml of this suspension was used for CL measurements. The addition of latex particles (1.09 $\mu$m) at 10 particles/cell resulted in a rapid burst of CL, as shown in Fig. 2. Superoxide dismutase in excess (50 $\mu$g/ml) produced a 65% mean inhibition in CL (data not shown here) when added to the cell suspension prior to the latex particles.

Platelet-rich plasma at 2–3 $\times$ 10$^5$/cu mm was similarly studied. The small amount of CL observed following exposure to latex particles (0.109 $\mu$m) was
not different from that attributable to a suspension of $6 \times 10^2$ PMN/cu mm in plasma exposed to the same particles. A mean of 600 PMN/cu mm contaminated the platelet-rich plasma in these studies. Platelets, in either plasma or buffer, did not exhibit CL after exposure to ADP, thrombin, or staphylococci, beyond that amount of CL which was attributable to contaminating PMN.

DISCUSSION

Platelets have the capacity to phagocytose several particles, including latex, ferritin, silicon dioxide, and thorotrast.\textsuperscript{1} This process is accompanied by metabolic changes,\textsuperscript{18} although it is still not clear whether the changes are due to phagocytosis or the aggregation which can follow exposure of platelets to particulate materials.\textsuperscript{19} The phagocytic process in PMN also results in significant metabolic changes, which include increases in $O_2$ consumption,\textsuperscript{7} $H_2O_2$ production,\textsuperscript{8} hexose monophosphate shunt pathway activity,\textsuperscript{20} superoxide production,\textsuperscript{9} and nitroblue tetrazolium reduction.\textsuperscript{6} Although the phagocytic mechanism may be similar, platelets increase anaerobic and aerobic glucose metabolism and the activity of the tricarboxylic acid cycle\textsuperscript{18} rather than increasing hexose monophosphate shunt pathway activity, as observed with phagocytosing PMN.\textsuperscript{20}

We have taken advantage of the sensitivity of the scopoletin assay for $H_2O_2$\textsuperscript{13} to measure $H_2O_2$ production in platelet preparations. As seen in Fig. 1, the
addition of latex spherules to gel-filtered platelets results in a small but measurable production of H$_2$O$_2$, which is not seen following addition of thrombin or bacteria to this preparation. Inasmuch as PMN contamination of the platelet preparations used here amounted to 0.2%, we studied the relationship between H$_2$O$_2$ production and the number of PMN. Fig. 1 also indicates that amounts of H$_2$O$_2$ comparable to that produced in our platelet preparations were produced by PMN at the level of contamination. The latter observation strongly suggests that the H$_2$O$_2$ measured in our gel-filtered platelet preparations was produced by contaminating PMN. Inasmuch as our assay measures only extracellular H$_2$O$_2$, we are not in a position to evaluate whether phagocytosing platelets increase intracellular H$_2$O$_2$ production, which could be metabolized by H$_2$O$_2$-consuming reactions and enzymes at a rate which would prevent leakage to the extracellular space.

The ADP-induced stimulation of H$_2$O$_2$ formation observed in Fig. 1 could conceivably have been due to a direct action of ADP on the PMN, or an indirect effect due to the ADP-induced aggregation of platelets in the presence of PMN. Our observation that the addition of ADP to PMN suspensions did not result in any additional H$_2$O$_2$ production suggests that the effect is mediated by the presence of platelets. The formation of small platelet aggregates following the addition of ADP might initiate phagocytosis and subsequent H$_2$O$_2$ production in the contaminating PMN.

Even more compelling are the CL data presented in Fig. 2. As first reported by Allen et al. and confirmed frequently since then, phagocytosing PMN produce CL which can be readily detected in a liquid scintillation counter. Although the exact source of this CL is still not known, it has proven to be a useful tool for following the activation of PMN. With respect to platelets, we can observe an increase in CL above baseline when platelet-rich plasma is exposed to latex. This increase, however, is the same as that observed when platelet-poor plasma, supplemented with PMN at the level of the contamination of platelet-rich plasma, is treated with latex. We therefore conclude that the CL observed from latex-treated platelet-rich plasma arises from the PMN contaminant. In our experience, many methods of platelet preparation result in contamination of 200-2500 leukocytes/cu mm. These observations emphasize the importance of controlling for contamination by other cell types when studying platelet reactions. In particular, the production of H$_2$O$_2$ and free radicals by the PMN which contaminate platelet suspensions may contribute to the loss of platelet function during storage.


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