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Erythrocyte Membranes Inhibit Respiratory Burst and Protein Nitration during Phagocytosis by Macrophages

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Summary

Phagocytosis is associated with respiratory burst producing reactive oxygen and nitrogen species. Several studies imply that erythrocytes can inhibit the respiratory burst during erythrophagocytosis. In this work we studied the mechanisms of this effect using control and *in vitro* peroxidized erythrocyte membranes. We demonstrated that autofluorescence of peroxidation products can be used for visualization of phagocytozed membranes by fluorescence microscopy. We also found that respiratory burst induced by a phorbol ester was inhibited by control membranes (5 mg/ml) to 63 % ($P<0.001$), and to 40 % by peroxidized membranes ($P<0.001$). We proved that this effect is not caused by the direct interaction of membranes with free radicals or by the interference with luminol chemiluminescence used for the detection of respiratory burst. There are indications of the inhibitory effects of iron ions and free radical products. Macrophages containing ingested erythrocyte membranes do not contain protein-bound nitrotyrosine. These observations imply a specific mechanism of erythrocyte phagocytosis.

Key words

Phagocytosis • Macrophages • Erythrocyte membranes • Respiratory burst • Nitrotyrosine

Introduction

The activation of macrophages associated with the generation of reactive oxygen and nitrogen species (ROS and RNS, respectively) is of critical importance in the process of a nonspecific immunological response. It leads to the killing of invading bacteria and inflammatory tissue injury. ROS, comprising mainly of superoxide, hydrogen peroxide and the hydroxyl radical originate from a metabolic pathway referred to as a respiratory burst, characterized by non-mitochondrial oxygen consumption. The enzyme NADPH oxidase plays the key role in this process. It is activated during phagocytosis and generates superoxide as the primary product (Maly and Schurer-Maly 1995). Superoxide is the precursor of other ROS, such as hydrogen peroxide, which can be formed during superoxide dismutation, both spontaneous and enzymatic. We have documented production of hydrogen peroxide by activated macrophages in several studies (Wilhelm *et al.* 1996, 1997, 2003).

Macrophage activation is also accompanied by the induction of inducible nitric oxide synthase and

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sustained release of NO (MacMicking *et al.* 1997). An important compound among RNS is represented by peroxynitrite ($ONOO^-$), which produces a wide range of biological effects, including protein tyrosine nitration (Beckman and Koppenol 1996). Protein nitrotyrosine is thus a widely used indicator of RNS generation.

Erythrocytes have a limited life span of about 110 days and after this period are cleared from the circulation by the reticuloendothelial system. Free radical damage to erythrocytes leads to the loss of deformability of their membranes and increased their scavenging by spleen macrophages (Jain 1988). In this way, erythrocytes damaged by free radicals in various pathologies (Ramachandran and Iyer 1984, Laszlo *et al.* 1991, Peuchant *et al.* 1994, Wilhelm and Herget 1999, Skoumalová *et al*. 2003) could enhance the generation of ROS and RNS during their clearance by macrophages. On the other hand, it was found that phagocytosis of immunoglobulin G-coated erythrocytes was followed by a depression of macrophage functions including respiratory burst, whilst phagocytosis of erythrocyte ghosts did not depress macrophage function (Loegering *et al.* 1996). Inhibition of phagocytosis and oxidative burst were also observed after ingestion of *Plasmodium falciparum*-infected erythrocytes (Schwarzer *et al.* 1992). This inhibition is probably mediated by the stable end products of lipid peroxidation (Schwarzer *et al.* 2003).

Thus the mechanisms of inhibition of macrophage respiratory burst by erythrocytes are of great medical importance and therefore we addressed this question in this study. We investigated the effects of isolated human erythrocyte membranes, either control or peroxidized *in vitro*, on the respiratory burst of RAW macrophages and on the generation of nitrated proteins during phagocytosis. The results indicate inhibition of macrophage activities by erythrocyte membranes.

Methods

The remnants of blood samples left after blood donor testing were obtained from the Department of Hematology (Second Medical Faculty, Charles University, Prague). The blood, anticoagulated by K-EDTA, was pooled, 100 ml of blood was lyzed in ACK lyzing buffer (0.15 M NH_4Cl, 0.01 M $KHCO_3$, 0.0001 M Na_2 EDTA, pH 7.2) and left for one hour. The lyzed blood was centrifuged (40 000g, 40 min). The sediment was resuspended in 300 ml of PBS and centrifuged again under the same conditions. This procedure was repeated once more, then the sediment was resuspended in a small volume of PBS and dialyzed overnight against the large volume of PBS. The membranes were then sedimented by centrifugation, resuspended in a small volume of PBS and the protein concentration was assayed according to Lowry *et al.* (1951). One ml aliquots were stored at –70 °C until used.

Membranes were diluted to a concentration of 2 mg protein/ml in 10 mM Tris.HCl buffer containing 600 μM EDTA, pH 7.4. Lipid peroxidation was initiated by the addition of $FeSO_4$ (final concentration 300 μM) and ascorbate (final concentration 600 μM). The mixture was incubated at laboratory temperature in sealed conic flasks for 24 h. During this period, samples were taken at specific time intervals and oxidized and control membranes were frozen at –70 °C until used.

Mouse monocyte macrophages RAW 264.7 were obtained from ECACC, through Sigma-Aldrich (Prague). The cells were cultivated in Dulbecco´s modified Eagle´s medium (Sigma) supplemented with 10 % fetal calf serum (BioClot, Germany). Cells were routinely grown at 37 °C in humid atmosphere equilibrated with 5 % CO_2. Experiments were performed with cultures near the confluency.

Macrophages after overnight cultivation with peroxidized or control erythrocyte membranes were studied using fluorescence microscope Nikon Eclipse 400 (excitation filter 330-380 nm, barrier filter 420 nm, dichroic mirror 400 nm).

The concentration of lipofuscin-like pigments (LFP) was assayed according to Goldstein and McDonagh (1976). The chloroform-isopropanol mixture (6 ml, 3:2, v/v) was added to 250 μl of control or peroxidized membranes. The mixture was extracted for 1 h on a motor-driven shaker. After extraction, 2 ml of water were added to achieve phase separation, the samples were centrifuged (100 g, 10 min) and the lower chloroform layer was used for the assay. The same procedure was adopted for the LFP assay of macrophages cultivated in the presence of erythrocyte membranes.

Fluorescence spectra of chloroform extracts were measured on Aminco-Bowman 2 spectrofluorometer. Tridimensional excitation spectral arrays were measured in the range of 250-400 nm for the emissions set between 300-500 nm with a step of 10 nm. For the quantitative assay of LFP the excitation and emission maxima found in the 3D spectra were used, instrument was calibrated with standard No. 5 from the instrument manufacturer. LFP concentration was expressed in

relative fluorescence units (RFU/mg protein).

Chemiluminescence measurements were carried out similarly as in our previous study (Wilhelm *et al.* 2003). We measured the luminol-dependent chemiluminescence originating from the reaction between hydrogen peroxide and luminol catalyzed by horse-radish peroxidase. For one assay $1x10^5$ cells was used. They were stimulated with protein kinase C activator phorbol-myristate-acetate (PMA), final concentration 1.6 μM. The measurements were carried out in plastic cuvette at 37 °C. The basic chemiluminescence (without PMA) was measured at the beginning of each experiment. Data are expressed as means ± SD of three separate experiments.

The measurement of the effects of erythrocyte membranes on the ROS production in xanthine/xanthine oxidase system was based on our previous study (Wilhelm and Vilim 1986). The reaction mixture contained in 1 ml of phosphate buffer (50 mM KH_2PO_4-KOH, 10 mM EDTA, pH 7.5) 0.25 mM luminol and 0.5 mM xanthine. The reaction was started by addition of 0.02 U of xanthine oxidase. The erythrocyte membranes (either control or peroxidized, 5 mg/ml) were added before xanthine oxidase.

The concentration of nitrotyrosine was measured in the fraction of soluble macrophage proteins. Cells were homogenized by freezing and thawing twice and extracted by TBS pH 8.4. The content of 3-nitrotyrosine was determined by previously described an enzyme immunoassay (EIA) method (Herget *et al.* 2000). Briefly, wells of polystyrene plates (Nunc) were coated with nitrated bovine serum albumin (BSA). Standard solutions of nitrated BSA (the content of 3-nitrotyrosine of standard was estimated from absorption at 430 nm) or extracts of RAW cells were mixed with monoclonal antibody NO-60-E3, that was developed in our laboratory, and incubated in coated wells of plate for 90 min under gentle shaking. After washing the wells were incubated with anti-mouse IgG antibody conjugated with peroxidase (P206, Dako) for next 90 min and developed with o-phenylenediamine. Standard curves and concentration of 3-nitrotyrosine in samples were calculated according to Rodbard's four parameter equation (Rodbard and McClean 1977).

Results

Firstly, the isolated erythrocyte membranes were subjected to lipid peroxidation *in vitro*. The free radical damage was detected by means of intrinsic fluorescence of the end products of lipid peroxidation. Figure 1 shows the 3D fluorescence spectra of control (part A), and peroxidized membranes (part B). The free radical damage results in the accumulation of fluorophore with maximum emission at 430 nm after excitation at 350 nm. The concentration of this fluorophore in peroxidized membranes increased almost fivefold, from 0.25±0.06 to 1.21±0.12 RFU/mg protein ($P<0.001$).

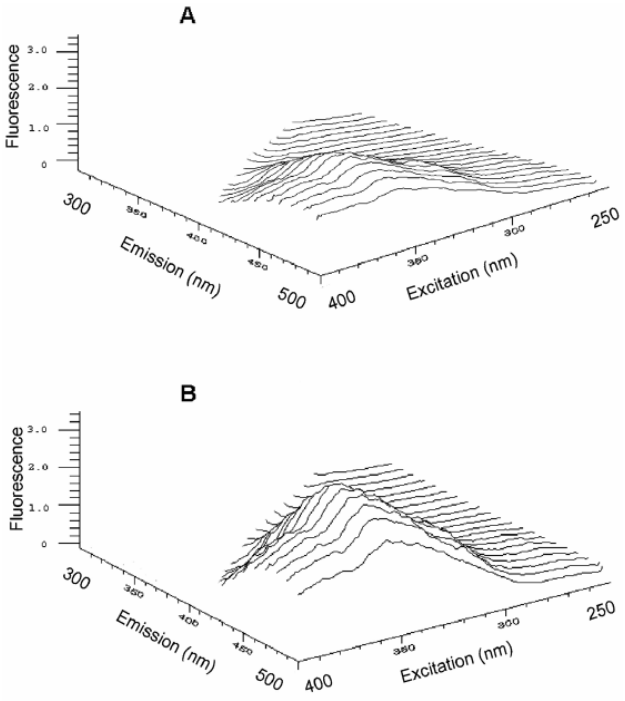

Fig. 1. Tridimensional spectral arrays of fluorescent products of lipid peroxidation. A - control erythrocyte membranes, B - membranes after 24 h of in vitro lipid peroxidation. Fluorescence intensity given in arbitrary units.

Both peroxidized and control membranes were incubated with RAW macrophages for 12 h. Figure 2 represents typical examples of fluorescence microscopy pictures of macrophages with ingested peroxidized membranes (upper part) and non-peroxidized control membranes (lower part). As this technique uses the autofluorescence of peroxidative products, the photo of the macrophage with ingested control membranes is much dimmer. Nevertheless, this picture documents that highly peroxidized erythrocyte membranes are eagerly phagocytozed by macrophages.

Secondly, we tested the effect of membrane phagocytosis on the respiratory burst. As the respiratory burst induced by membranes occurs in RAW cells in 1-5 h (Pfeifer *et al.* 2001) and it would be difficult to maintain the cell fully viable in the cuvette of

luminometer for this time period, we choose another approach. We triggered the respiratory burst by the addition of PMA either in the absence or in the presence of membranes. Figure 3 shows that the peak chemiluminescence was reached in 18 min in macrophages without membranes (curve 1). The addition of 1 mg/ml of membrane protein shortened the time to peak chemiluminescence to about 11 min. The peak intensity was reduced by 26 % with control membranes (curve 2) or by 37 % with peroxidized membranes (curve 3). The quantitative expression of three separate experiments gave 25.5±0.7 mV for the control macrophages, 19.1±0.5 mV for the non-peroxidized membranes ($P<0.05$), and 16.2±0.5 mV for the peroxidized membranes ($P<0.01$).

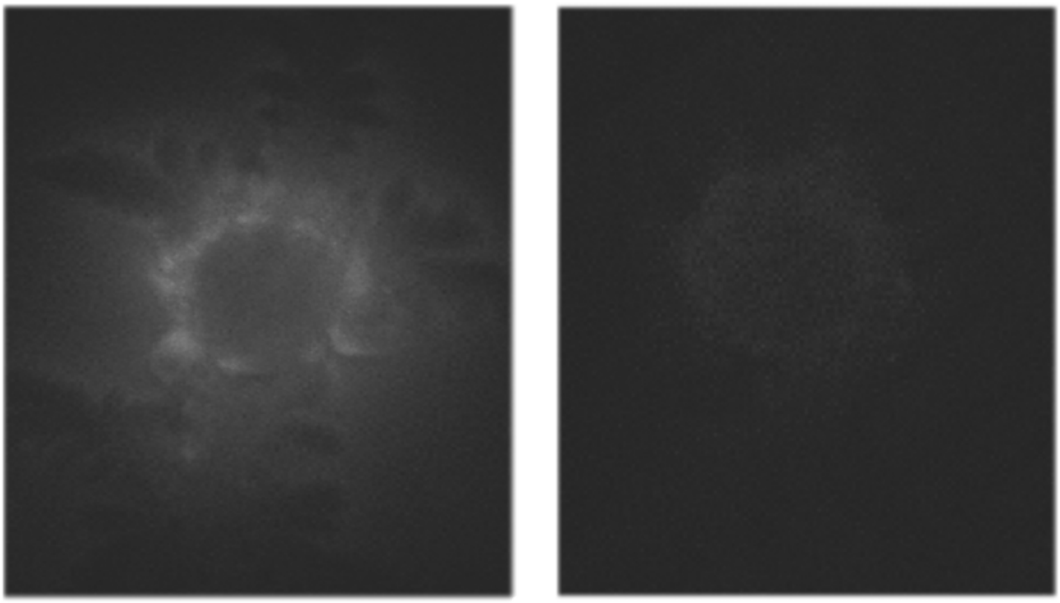

Fig. 2. Autofluorescence of phagocytes filled with erythrocyte membranes. Photos from fluorescence microscope showing a phagocytic cell with ingested peroxidized erythrocyte membranes (left part), or control membranes (right part).

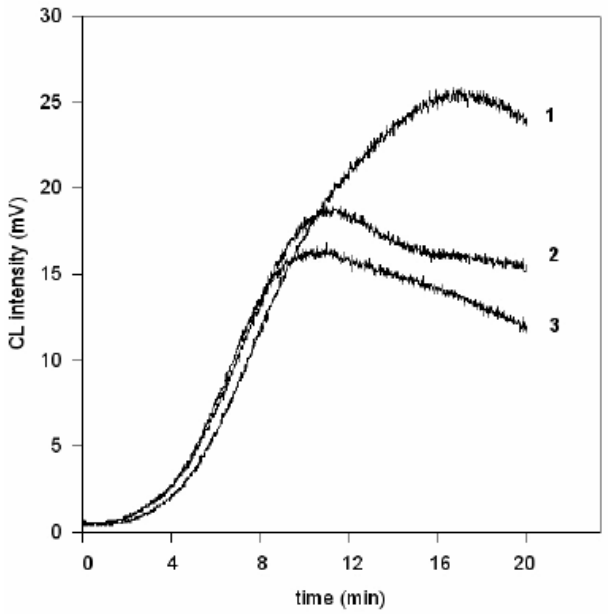

Fig. 3. Chemiluminescence tracing of respiratory burst induced by PMA. Curve 1 - without erythrocyte membranes, curve 2 - control erythrocyte membranes (1 mg/ml), curve 3 - peroxidized erythrocyte membranes (1 mg/ml).

Furthermore, we studied the effect of membrane concentration on chemiluminescence inhibition. When the membrane concentration was increased fivefold, the peak intensity was reduced by 10 % with control membranes (Fig. 4A), i.e. the inhibition of the total chemiluminescence was 37 % in relation to the control. In quantitative terms, the chemiluminescence intensity decreased from 19.1±0.5 to 17.6±0.7 mV and the decrease was not significant. The same increase in concentration of peroxidized membranes reduced the peak chemiluminescence by 40 % (Fig. 4B); in this case the inhibition of the total chemiluminescence was 60 % as compared to the control. Quantitatively expressed, the chemiluminescence intensity decreased from 16.2±0.5 to 10.1±0.7 mV ($P<0.01$).

One possible explanation for the observed inhibition of chemiluminescence by erythrocyte membranes might be the direct interaction of the membranes with the ROS generated by activated macrophages. We tested this possibility by measuring the effects of membranes on chemiluminescence produced in

xanthine/xanthine oxidase system, that generates similar mixture of ROS as activated macrophages. Figure 5 (curve 1) shows that the addition of xanthine oxidase results in a short burst of chemiluminescence. In the presence of erythrocyte membranes (curve 2), the initial peak is followed by sustained chemiluminescence. There was practically no difference between the control and the peroxidized membranes, both preparations increased the chemiluminescence intensity. Thus the inhibitory effect of membranes on chemiluminescence generated by activated macrophages cannot be explained by direct interaction of membranes with ROS.

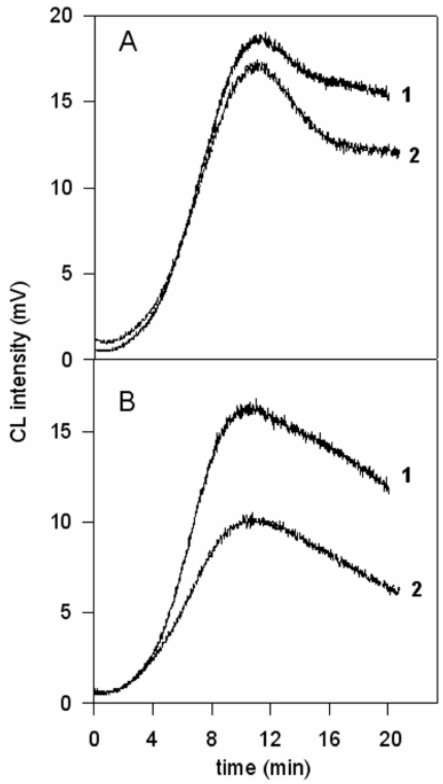

Fig. 4. The effect of membrane concentration on respiratory burst. Part A - control membranes, part B - peroxidized membranes. Curve 1 - protein concentration 1 mg/ml, curve 2 - protein concentration 5 mg/ml.

In order to evaluate the production of RNS during erythrocyte membrane phagocytosis we measured the concentration of protein nitrotyrosine. In RAW cells iNOS induction occurred with a lag phase of 4 h, and the fall in the rate of NO release was observed between 6 and 15 h (Pfeifer *et al.* 2001). Therefore, we measured protein nitrotyrosine after 12 h, when most membranes had already been ingested, as documented by Figure 2. The nitrotyrosine content in non-phagocytosing macrophages was 2.007±0.017 μmol/g of protein, in macrophages with phagocytozed control membranes 1.977±0.035 μmol/g of protein, and in macrophages with phagocytozed peroxidized membranes 2.095±0.059 μmol/g of protein. The differences were not statistically significant. Apparently, RNS production was not activated during erythrocyte membrane phagocytosis.

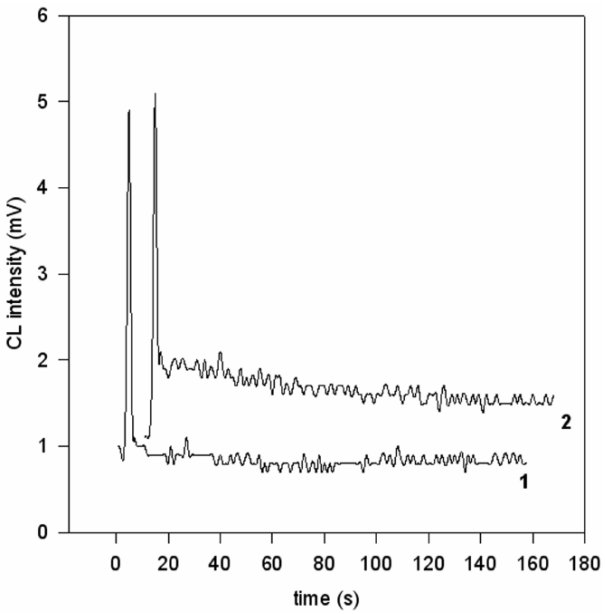

Fig. 5. Luminol chemiluminescence in xanthine oxidase system. Curve 1 - without erythrocyte membranes, curve 2 - in the presence of peroxidized erythrocyte membranes, protein concentration 5 mg/ml.

Discussion

Erythrophagocytosis in the reticuloendothelial system is the usual fate of erythrocytes at the end of its life span. From the teleological point of view it would be advantageous for the organism if erythrophagocytosis under physiological conditions would not comprise respiratory burst and tissue damage. However, this phenomenon might have its dark side. Several clinical studies have shown that increased erythrophagocytosis induces phagocyte dysfunction which may contribute to increased susceptibility to infection under situations such as burn injury (Pruitt and McManus 1984), salmonellosis (Hook, 1961), or malaria (Schwarzer *et al.* 1992).

It was deduced that the erythrocyte content is responsible for the macrophage dysfunction, as the ingestion of erythrocyte ghosts had no effect on macrophage function (Commins *et al.* 1990). However, our study shows that even the washed erythrocyte membranes inhibit respiratory burst induced by PMA. This could have been caused by oxidative inactivation of

protein kinase C, the effector of PMA signal. Currently, we do not know the specific site of the pathway leading from activated protein kinase C to superoxide production, which has been affected. However, our results correspond to the study which showed that phagocytosis of *Plasmodium falciparum* malarial pigment hemozoin, which induces free radical production inside the phagocyte, inactivates protein kinase C (Schwarzer *et al.* 1993). In our experiments even the non-peroxidized membranes contained some residual iron and it was suggested in another study that hemoglobin-derived iron interacts with ROS to cause oxidant damage to phagocytes (Loegering *et al.* 1996).

Inhibition of PMA-elicited respiratory burst was higher in the presence of peroxidized membranes. This preparation contained some extra iron from the *in vitro* lipid peroxidation experiment and also the products of membrane lipid peroxidation as revealed by fluorescence measurements. In the recent study (Schwarzer *et al.* 2003) it has been shown that some stable products of lipid peroxidation, specifically monohydroxy derivatives of fatty acids, are toxic to phagocytes. This could explain the increased inhibition by peroxidized membranes. The highest mebrane concentration used in our study (5 mg protein/ml) produced the same inhibition (60 %) as the optimum concentration of monohydroxy fatty acid in the afore mentioned study. We could not study higher membrane concentrations, because the opacity of the solution interfered with the chemiluminescence assay.

Inhibition of respiratory burst did not supress phagocytosis of the peroxidized membranes as revealed by fluorescence microscopy. At the time of completed phagocytosis we did not observe any change in protein nitration. This observation is different from the experiments which showed the increase of nitrotyrosine concentration in murine peritoneal macrophages activated *in vitro* with interferon-γ/lipopolysaccharide (Pfeifer *et al.* 2001). Thus it appears that phagocytosis of erythrocyte membranes does not activate iNOS and this effect is not dependent on membrane peroxidation.

In summary, we observed that erythrocyte membranes inhibit PMA-elicited respiratory burst by a mechanism that might involve oxidant dependent inhibition of protein kinase C, and/or the related pathway of superoxide production. The present study was not designed to localize the specific site. This question should be addressed in the future studies. Our experiments proved that this effect is not caused by interference of the membranes with the detection system which uses luminol chemiluminescence. We demonstrated that autofluorescence of peroxidation products can be used for visualization of phagocytozed membranes by fluorescence microscopy. Macrophages containing ingested erythrocyte membranes do not contain protein-bound nitrotyrosine. These observations imply a specific mechanism of erythrocyte phagocytosis.

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