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EVIDENCE THAT PHAGOCYTOSING CHICKEN POLYMORPHONUCLEAR LEUKOCYTES GENERATE HYDROGEN PEROXIDE AND SUPEROXIDE ANION

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Polymorphonuclear leukocytes (PMN) are known to produce hydrogen peroxide during the respiratory stimulation associated with the ingestion of particles (9,10,19,24). The oxygen taken up by the cell is univalently reduced to superoxide anion (O_2^-) which, in turn, dismutates with formation of hydrogen peroxide. The fate of hydrogen peroxide in these cells is dual, a) it may be destroyed within the cell by enzymes such as myeloperoxidase, catalase or glutathione peroxidase, and/or b) it may leak out of the cell, the amount of peroxide released depending at least on the rate of formation and the efficiency of the intracellular H_2O_2 destroying systems.

It has been reported that production of H_2O_2 concomitant with the increment of respiration occurring during phagocytosis is not demonstrable in chicken PMN (16). On this basis it has been suggested that these cells also fail to produce or release O_2^- (16). This would set chicken PMN apart from all other types of phagocytes as far as the mechanism of metabolic activation during phagocytosis is concerned.

In the present paper the oxidative metabolism of chicken PMN leukocytes has been reinvestigated as compared to guinea pig PMN, thus representing a classical model for the study of the metabolic concomitants of phagocytosis. We conclude that the oxidative metabolism of PMN from both species is qualitatively similar including O_2^- and H_2O_2 generation.

MATERIALS AND METHODS

Cell preparation. Male adult chickens (Ubard strain) were injected intraperitoneally with 70 ml of 1% sterile sodium caseinate solution. The exudate was collected 5 hr later. The exudates contaminated with red cells were discarded. The cells were collected by centrifugation at 150 g for 7 min and were then resuspended in calcium-free Krebs-Ringer-Phosphate buffer (KRP) pH 7.4. The average differential count of the preparations used was: neutrophils 80-85%, mononuclear cells 11-20%, eosinophils 0-4%.

Guinea pig leukocytes were obtained as previously described (13).

Bacteria. Bacillus mycoides was used throughout the experiments. The bacteria were grown on nutrient agar, autoclaved and opsonized for 20 min at 37 C with fresh homologous serum.

Oxygen uptake. The rate of oxygen uptake by resting and phagocytosing leukocytes was measured at 37 C with a Clark oxygen electrode attached to a plastic chamber, as previously described (24).

Hydrogen peroxide production. The formation of H_2O_2 was assayed with three different methods, a) polarographic, b) colorimetric and c) fluorimetric.

In the polarographic assay (24) the oxygen consumption by phagocytosing cells was recorded for 3-4 min in the presence or absence of 1 mM cyanide. Excess catalase (from beef liver, Boehringer, Mannheim, GmbH, Germany) was then added into the electrode chamber, and a backward deflection of the recording trace was observed indicating that oxygen was released in the medium from H_2O_2.

The colorimetric method was that described by Thurman et al. (23). After recording the oxygen uptake of phagocytosing PMN leukocytes with a Clark oxygen electrode for various time intervals, 1 ml of the mixture was rapidly drawn from the electrode chamber and trichloroacetic acid was added to it at a final concentration of 10% (w/v). After centrifugation, the clear supernatant was used for assays.

Two different procedures were employed for the fluorimetric assay of H_2O_2. In one of these, the decay of scopoletin fluorescence following its oxidation by horseradish peroxidase in the presence of H_2O_2 was measured. The basic principle of this technique has been described by Root et al.(19). Briefly, while oxygen uptake by phagocytosing cells was being recorded with a Clark oxygen electrode, aliquots of the mixture were drawn at various

time intervals from the electrode chamber and were immediately centrifuged in a 3,200 Eppendorf microcentrifuge. The clear supernatant was used for assays. The spectrophotofluorimeter cuvette contained 3 ml of KRP, 2.5 µM scopoletin (Sigma Chemical Co., St. Louis, Mo., USA) and 0.166 µM horseradish peroxidase (Sigma Chemical Co.). The volume of the samples that were added to the cuvette varied from 10 to 100 µl. The second procedure was based on the conversion of the nonfluorescent compound homovanillic acid to the highly fluorescent 2,2'-dihydroxy-3,3'-dimethoxybipheny1-5,5'-diacetic acid by horseradish peroxidase in the presence of H_2O_2 (7,8). Concentrations of H_2O_2 as low as 0.03 µM could be determined by this method. This high sensitivity made it possible to perform kinetic measurements of H_2O_2 production by phagocytosing cells as follows. The spectrophotofluorimetric cuvette was filled with 2.5 ml of KRP containing 5 mM glucose, 0.5 mM $CaCl_2$, 20 µM homovanillic acid (Fluka AG, Buchs, Switzerland), 20 µg of horseradish peroxidase and 2 x 10^6 cells. The recorder was turned on. Usually resting cells did not cause any appreciable increase in fluorescence. After a baseline was established for resting cells, phagocytosis was started by adding 2.0 x 10^8 heat killed B. mycoides. The cuvette content was continuously stirred throughout the experiments.

Superoxide anion production. The assay was based on the superoxide dismutase inhibitable reduction of cytochrome c (1). The assay medium was the same as for oxygen uptake measurements plus 200 µM cytochrome c (from horse heart, grade VI, Sigma Chemical Co.) and, when required, 35 µg/ml of superoxide dismutase. The experimental details have been described elsewhere (6).

Cell homogenization and preparation of the granules. The leukocytes were incubated with or without bacteria in KRP containing 0.5 mM glucose for 5 min at 37 C. After dilution with ice-cold KRP, the leukocytes were sedimented at 150 g for 10 min so that most of the bacteria remained in the supernatant. The packed cells were suspended in ice-cold 0.34 M sucrose (buffered at pH 7.0 with Na-bicarbonate) to a concentration of 3-4 x 10^8 leukocytes/ml and homogenization was performed in a Potter type homogenizer equipped with a teflon pestle driven by a motor. The homogenization was stopped when 90% of the cells were broken as judged by light microscopy examination. Usually this process did not take more than 3-4 min. The homogenate was diluted with 0.34 M sucrose and centrifuged at 250 g for 7 min to remove nuclei, cell debris and unbroken cells. The supernatant fraction was centrifuged at 20,000 g for 20 min and sedimented granules were suspended in 0.34 M sucrose.

Determination of NADPH oxidase activity. The rate of NADPH oxidation was measured by determining the rate of O_2 consumption with a Clark type oxygen electrode as previously described (15,

13). Enzymatically reduced NADPH was purchased from Boehringer (Mannheim, GmbH, Germany).

RESULTS

Oxygen uptake, O_2^- and H_2O_2 Generation. The results of simultaneous determinations are shown in Table I. Chicken leukocytes respond to phagocytosis with an increased oxygen consumption as the PMN of all the other species so far tested do. The respiratory increment in chicken leukocytes was considerably lower than that observed in guinea pig cells. The oxygen uptake by phagocytosing chicken leukocytes was not significantly affected by azide, whereas guinea pig leukocytes showed a higher oxygen consumption in the presence of this inhibitor than in its absence.

Appreciable amounts of H_2O_2 could be measured in the incubation medium of phagocytosing chicken leukocytes, with or without azide, both by the scopoletin and thiocyanate method. The amount of H_2O_2 measured with phagocytosing chicken cells accounted for 37% of the oxygen consumed in the presence of azide. With phagocytosing guinea pig cells the amount of H_2O_2 that accumulated in the medium in the absence of azide was comparable to that observed with chicken cells under the same experimental conditions. However, if the guinea pig cells were let to phagocytose in the presence of azide the amount of H_2O_2 was several times higher than in absence of the medium of phagocytosing chicken leukocytes. The H_2O_2 measured with phagocytosing guinea pig cells accounted for about 99% of the oxygen taken up in the presence of azide.

A small but definite amount of superoxide anion was measured in the incubation medium of chicken phagocytosing leukocytes. The amount of superoxide anion released by phagocytosing chicken leukocytes was considerably lower than that released by phagocytosing guinea pig leukocytes.

Generation of hydrogen peroxide by phagocytosing chicken polymorphs could be detected also by polarographic techniques, as demonstrated by Fig. 1. This figure shows the recording traces of oxygen consumption by the cells and of oxygen liberation from H_2O_2 after addition of catalase. The backward deflection of the recording trace is therefore an index of H_2O_2 accumulation. Hydrogen peroxide could be detected in the medium of phagocytosing chicken leukocytes both in the presence and in the absence of cyanide, whereas no H_2O_2 was detectable with guinea pig cells in absence of the inhibitor.

Fig. 2 shows the generation of H_2O_2 by phagocytosing chicken PMN as determined by the homovanillic acid method. In this experiment the production of peroxide was followed kinetically

TABLE I

Oxygen Uptake and Generation of O_2^- and H_2O_2 by Chicken and Guinea Pig Polymorphonuclear Leukocytes During Phagocytosis[a]

	Chicken PMN		Guinea Pig PMN	
	---	+ NaN_3	---	+ NaN_3
O_2	19.8 ± 1.4(4)	14.3 ± 1.3(6)	73.2 ± 3.7(5)	103.3 ± 5.1(5)
O_2^-	9.0 ± 0.7(6)	not tested	71.7 ± 7.8(5)	not tested
H_2O_2	3.33 (2)[a] 3.12 (1)[b] 3.26± 0.2(3)[c]	5.03 ± 0.5(4)[a] 5.87 (2)[b] 5.31 ± 0.3(6)[c]	2.8 (2)	102.6 ± 2.3(5)

[a]The basic assay medium contained 2 ml KRP pH 7.4, 0.5 mM glucose and 0.5 mM $CaCl_2$. For O_2^- assay, 200 µM ferricytochrome c and, when required, 35 µg/ml of superoxide dismutase was added. Opsonized B. mycoides was the phagocytosable particle (ratio cell/bacteria, 1/100). Temperature 37 C NaN_3, 2 mM.

The results, given as differences between phagocytosing and resting cells, are expressed as nmoles/2min/2 x 10^7 cells ± S.E.M. Number of experiments in parentheses.

a, fluorimetric method. b, colorimetric method. c, mean of a and b.

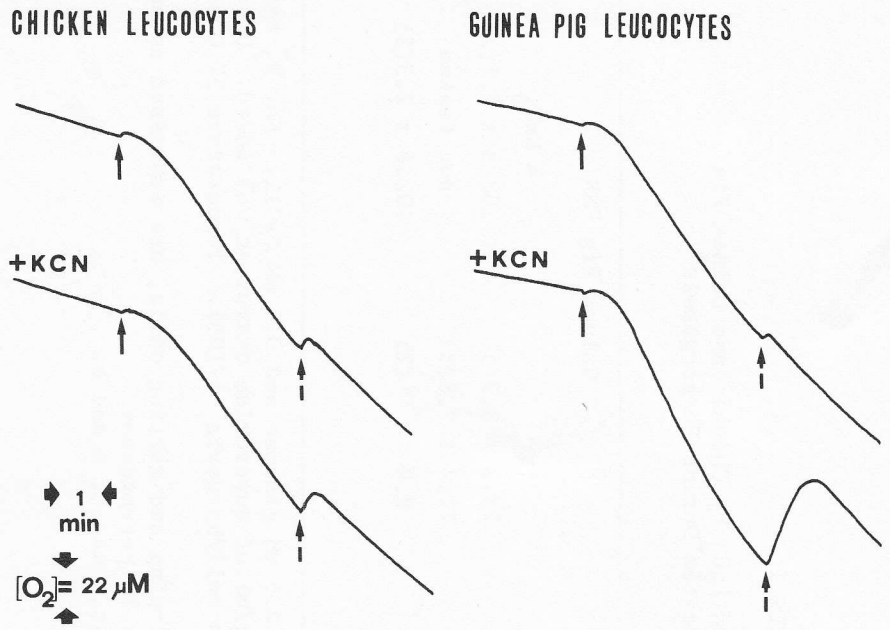

Fig. 1. Polarographic assay of oxygen uptake and H_2O_2 generation by chicken and guinea pig PMN. The basic assay medium was KRP pH 7.4 containing 0.5 mM glucose and 0.5 mM $CaCl_2$. Volume 2 ml. Temperature 37 C KCN, 1 mM. 9.5 x 10^7 chicken cells and 2.5 x 10^7 guinea pig cells were used in each assay. Opsonized B. mycoides was added at the solid arrows (ratio cell/bacteria, 1/100). Excess catalase was added at the broken arrows.

during phagocytosis. The initial part of the recording traces is flat indicating that no appreciable amount of H_2O_2 was produced. This initial part of the recording trace was given by cells at rest. Phagocytosis was started by adding bacteria at the arrows. After a lag time of about 90 sec the recording traces begin to go upwards, in dictating that the non-fluorescent compound 2,2'-dihydroxy-3,3'-dimethoxybiphenyl-5,5'-diacetic acid. The appearance of fluorescence is specifically due to H_2O_2 as indicated by the addition of catalase which prevents further increase in fluorescence. Omission of either horseradish peroxidase or homovanillic acid prevented any appearance of fluorescence. The addition

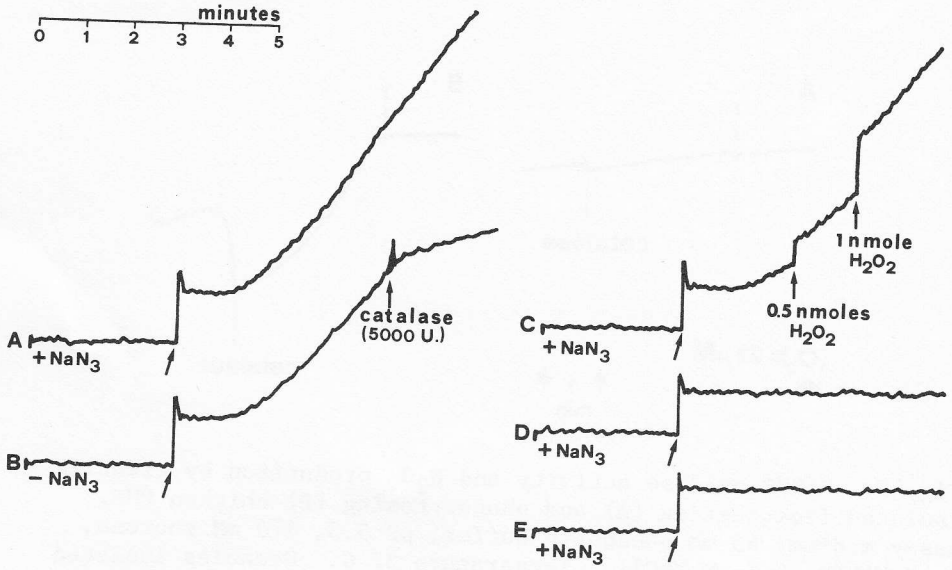

Fig. 2. Spectrophotofluorometric assay of H_2O_2 production by chicken PMN during phagocytosis. The basic assay medium was as follows: 2.5 ml KRP pH 7.4, 5 mM glucose, 0.5 mM $CaCl_2$, 20 µM homovanillic acid, 20 µg horseradish peroxidase, 2.0 x 10^6 cells and 2 mM NaN_3 where indicated. Opsonized B. mycoides was added at the arrows (ratio cell/bacteria, 1/100). In D and E homovanillic acid and horseradish peroxidase, respectively, were omitted from the assay medium.

of external standards of H_2O_2 during the recording of phagocytosis associated H_2O_2 production (curve C) shows that the fluorescence signal is a linear function of the amount of H_2O_2 added. Finally, it must be noted that the amount of H_2O_2 produced by phagocytosing chicken leukocytes is indentical in the presence and absence of sodium azide. In other animal species such as guinea pig, the amount of H_2O_2 measured in the presence of azide is much higher than in its absence. This difference is very likely due to the absence of the two H_2O_2-degrading enzymes catalase and myeloperoxidase from chicken polymorphs.

Oxidation of NADPH by chicken PMN granules. Fig. 3 shows that chicken leukocyte granules can oxidize NADPH and that this oxidation is higher in granules isolated from phagocytosing cells than from resting cells. The oxidation of NADPH was coupled with H_2O_2 production, the peroxide generated being stoichiometric to the oxygen consumed.

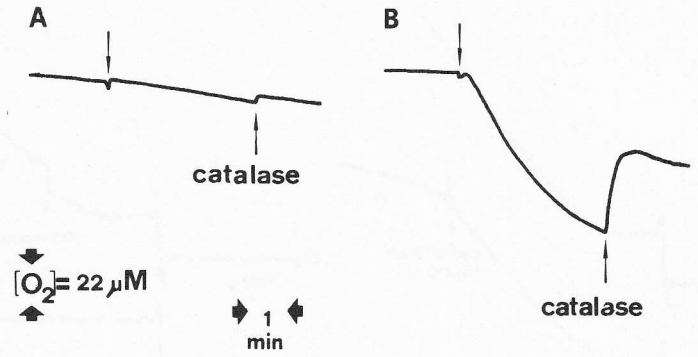

Fig. 3. NADPH oxidase activity and H_2O_2 production by granules isolated from resting (A) and phagocytosing (B) chicken PMN. Assay medium: 65 mM phosphate buffer, pH 5.5, 170 mM sucrose, 1 mM NADPH, 0.5 mM $MnCl_2$. Temperature 37 C. Granules isolated from 4 x 10^7 leukocytes were added at the arrows.

DISCUSSION

This paper shows that the metabolic burst of phagocytosing chicken leukocytes shares several features with that of phagocytosing guinea pig leukocytes. These include an increased oxygen consumption, an increased generation of superoxide anion and hydrogen peroxide and an activation of the NADPH oxidase of the granules which is coupled with an increased hydrogen peroxide production.

Some differences have been also observed between the two types of cell. A major one is that all the biochemical activities that have been recorded, are definitely lower in chicken cells than in guinea pig cells.

An increased respiration and hexose monophosphate shunt activity in phagocytosing chicken leukocytes has been already reported by Pennial and Spitznagel (16). These authors, however, failed to demonstrate generation of H_2O_2, and concluded that the inability of chicken leukocytes to produce H_2O_2 "sets these cells apart from the PMN of all other species". The original contribution of

the present paper is that we were able to demonstrate generation and extracellular release of both superoxide anion and hydrogen peroxide in phagocytosing chicken leukocytes. Four different techniques were used to measure H_2O_2 production, including the sensitive scopoletin and homovanillic acid methods. The failure of Pennial and Spitznagel to demonstrate H_2O_2 using the technique of formate oxidation may be explained by the absence of catalase in chicken PMN (3,4). This technique, in fact, relies on the peroxidatic activity of catalase which converts formate into CO_2 concomitantly with the phagocytic production of H_2O_2 (2,10). Formate oxidation has been reported to be stimulated by addition of exogenous catalase at least in phagocytosing human leukocytes (11,12). Pennial and Spitznagel could not demonstrate formate oxidation by phagocytosing chicken leukocytes, even in the presence of exogenous catalase. This result cannot be obviously accounted for by catalase deficiency in chicken cells. Since the formate oxidation technique reveals only a small fraction of the peroxide produced (9,12), it might be that the failure of Pennial and Spitznagel to show formate oxidation by phagocytosing chicken leukocytes in the presence of exogenous catalase is attributable to the low sensitivity of the method employed. The authors failed to show H_2O_2 production even with the scopoletin method which, instead, we have employed with positive results. In our experiments a modification of the method as originally described by Root and Chance (19) has been used. In the original method the oxidation of scopoletin in the presence of horseradish peroxidase is measured concomitantly with phagocytosis. Operatively, the cuvette of the spectrophotofluorimeter is filled with both the reagents for the scopoletin reaction and leukocytes, and then the reaction is started by addition of the phagocytic particles. The rate of decay of scopoletin fluorescence is recorded and the linear part of the recording trace is used for calculation of the initial rate of H_2O_2 production. The number of human cells used for each assay in the method described by Root *et al.* (19) is 2.5×10^6/ml. In preliminary experiments we have shown that this cell concentration was too low for an evaluation of H_2O_2 produced by chicken leukocytes. On the basis of this fact a concentration of 5×10^7 leukocytes/ml was used and the cells were let to phagocytose for variable periods of time. The medium was then quickly separated from the cells and aliquots of variable size were used for the scopoletin assay of H_2O_2. This procedure offers two advantages, a) a large number of cells can be used, and b) the peroxide accumulated for the desired period of time can be measured. With this technique, accumulation of H_2O_2 in the medium of phagocytosing chicken leukocytes was detected both in the presence and in the absence of azide. With phagocytosing guinea pig leukocytes, only a small amount of H_2O_2 accumulated in the medium without the inhibitor, but it was about 40 times higher when the inhibitor was present. This difference might be explained by the presence in guinea pig

leukocytes of two hydrogen peroxide destroying enzymes such as catalase and myeloperoxidase, which are both sensitive to azide, and by the lack of these enzymes from chicken leukocytes (3-5,17). In fact, we have recently shown that in phagocytosing guinea pig cells, about 90% of the H_2O_2 produced is degraded by catalase and myeloperoxidase (6).

A further demonstration that chicken leukocytes do produce hydrogen peroxide during phagocytosis was obtained by using the fluorimetric assay which employs homovanillic acid. With this technique the formation of a fluorescent compound is measured, at variance with the scopoletin method which measures the decrease of fluorescence. Furthermore, the fluorescent compound which is formed upon peroxidation of the homovanillic acid has a high fluorescence intensity which makes the method very sensitive. In fact, we could follow by this method the kinetics of H_2O_2 generation concomitant to phagocytosis with as low as 10^6 chicken cells/ml.

The enzymatic basis of the stimulated respiration in phagocytosing leukocytes, either polymorphs or macrophages, is still a debated issue (14,20,21). We have proposed in previous papers that the activation during phagocytosis of a NADPH oxidizing activity, which is bound to granules and is relatively insensitive to inhibition by cyanide, accounts for the increased respiration and H_2O_2 generation in either type of phagocyte (15,18,20-22). This paper shows that chicken leukocytes were able to oxidize NADPH. The 20,000 g fraction isolated from the homogenate of phagocytosing chicken cells had a higher NADPH oxidizing activity than the corresponding fraction isolated from resting cells. The final product of the granule catalized oxidation of NADPH was found to be hydrogen peroxide.

In conclusion, although the oxidative metabolism of chicken leukocytes is less active than that of guinea pig leukocytes, it seems to follow a pattern of oxidative response to phagocytosis similar to that of leukocytes from other species.

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