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The measurement of superoxide anion production by granulocytes in whole blood. A clinical test for the evaluation of phagocyte function and serum opsonic capacity

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Abstract. The paper reports a simple, sensitive and time-saving procedure for the assay of the function of the phagocytes on microsamples of whole blood. The method consists in the evaluation of the stimulation of superoxide anion (O_2^-) production (as superoxide dismutase-sensitive cytochrome c reduction) by leukocytes in whole blood challenged with (a) phagocytosable particles (opsonized zymosan); (b) particles that become phagocytosable by virtue of the opsonizing capacity of the plasma of blood samples (zymosan); and (c) a soluble agent such as phorbol myristate acetate. Preliminary studies indicate that this procedure can be used as a routine test because it enables information to be obtained about the respiratory responsiveness of phagocytes and about cellular and humoral defects of phagocytosis.

Key words. Phagocytosis, superoxide anion, granulocyte defects, opsonization.

Introduction

Peripheral blood phagocytes play a crucial role in the elimination of invading micro-organisms. These cells perform their task by migrating towards the infected area, recognizing and ingesting the invaders, and subsequently by killing and digesting them. The opsonization of the micro-organism by serum factors is necessary for efficient phagocytosis. The process of phagocytosis is accompanied by a dramatic increase of the oxidative metabolism of phagocytic cells which results in the producton of superoxide anion (O_2^-) and hydrogen peroxide. These products of the oxygen metabolism are mainly utilized for the bactericidal activity of the phagocytes [1–5]. Defects in the opsonic capacity of serum or in the function of phagocytes are often, if not always, associated with an increased susceptibility to infections. The identification of such defects is of obvious importance in order to link them to specific disease states. Most of the laboratory tests of phagocytic function so far available are often not useful for routine studies as they require special reagents (e.g., radioactive compounds) and instrumentation. They are also time consuming due to the procedures of isolation of granulocytes, and are difficult to carry out with small amounts of blood.

To overcome these inconveniences and limitations, we have developed a test for the quantitative evaluation of the phagocytic function and serum opsonic capacity based on the measurement of superoxide anion production by granulocytes in whole blood in the presence of zymosan. Defects in serum opsonic capacity or in cellular activation are seen by a lack or a decrease in O_2^- production. The humoral or cellular nature of the defect can be revealed by carrying out parallel assays with blood incubated with zymosan opsonized with normal serum and/or phorbol myristate acetate. The latter two stimulants do not require the opsonization step and reveal directly the O_2^- forming activity of the phagocytes.

Materials and methods

Human venous blood from healthy laboratory personnel or from blood donors was collected with disposable plastic syringes and anticoagulated with 10 units of heparin per ml. Blood was drawn from the patients and their parents after having obtained their consent. When required, granulocytes or mononuclear cells (monocytes + lymphocytes) were isolated from blood using dextran sedimentation followed by centrifugation of leukocytes on Ficoll-Hypaque [6].

Reagents

Zymosan (Zymosan A, Sigma Chemical Co., St. Louis, Missouri, U.S.A.) was suspended in 0·9% NaCl to a concentration of 20 mg/ml and boiled for 10 min. After washing, it was resuspended in Krebs-Ringer phosphate buffer, pH 7·4, containing 0·5 mmol/l $CaCl_2$

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and 5 mmol/l glucose (KRP) at a concentration of 10 mg/ml. Serum-treated zymosan was prepared by incubating the zymosan with a pool of fresh human serum (15% final concentration) at 37°C for 30 min under continuous shaking. After centrifugation and washing, the particles were resuspended in KRP at a concentration of 10 mg/ml.

Phorbol 12-myristate 13-acetate (PMA, Sigma Chem. Co.) was dissolved in dimethylsulphoxide at the concentration of 0·2 mg/ml.

Superoxide dismutase and cytochrome c, type VI, were purchased from Sigma Chem. Co. A molar absorption coefficient of 24500 was used for reduced cytochrome c at 550–468 nm. Other reagents were of the highest available purity.

Assay of superoxide anion (O_2^-) production

O_2^- production by whole blood was measured in the absence and in the presence of agents that stimulate the oxidative metabolism of granulocytes. In a complete experiment, four pairs of 10 ml plastic test-tubes, labelled 1A–1B, 2A–2B, 3A–3B, 4A–4B, were utilized. To each tube, 0·4 ml of KRP and 1·5 mg of cytochrome c were added. Tubes 'B' also contained 50 μg of superoxide dismutase. In addition, tubes 2 (A and B) contained 0·5 mg of zymosan; tubes 3 (A and B) 0·5 mg of serum treated zymosan, and tubes 4 (A and B) 0·5 μg of PMA. The tubes were preincubated in a water bath for 5 min at 37°C. Then, 0·1 ml of blood was added to each tube and the reaction was allowed to proceed for the time required, under continuous shaking (about 100 rpm). The incubation was stopped by the addition of 2 ml of ice-cold KRP containing 1 mmol/l N-ethylmaleimide and the tubes were centrifuged at 1500 *g* for 10 min. The absorbance of the cell-free supernatants was then measured at 550–468 nm, that is the absorbance at 550 nm minus that at 468 nm. The absorbance values (550–468) of the supernatants in tubes B were subtracted from those of the corresponding tubes A, in order to correct for the O_2^--independent reduction of cytochrome c. The resulting absorbance was multiplied by 2·5 (dilution factor) and divided by 0·0245 (extinction coefficient μmol/l of cytochrome c determined at 550–468), thus giving the nmoles of O_2^- produced by 0·1 ml of blood. The results were also expressed as nmol of $O_2^-/10^6$ granulocytes, on the basis of the total and differential counts of white blood cells.

Results

Optimal conditions for the opsonization of zymosan were determined by the experiments reported in Figs 1 and 2. The zymosan was opsonized either using various concentrations of fresh human plasma (Fig. 1) or by incubating it for various times with 15% plasma (Fig. 2). The stimulating power of these preparations was tested with blood washed with KRP in order to eliminate any trace of plasma. Fig. 1 shows that untreated zymosan is completely ineffective in stimulating O_2^- production and that optimal opsonization is reached using zymosan opsonized with 10–15% plasma. Microscopic examintion of smears made with samples from the reaction mixtures at the end of the incubation showed that zymosan, which is not opsonized, is not phagocytosed, whereas maximal uptake of zymosan (2–5 particles/granulocyte) was observed under conditions of optimal opsonization. The time course of zymosan opsonization is reported in Fig. 2. The opsonization takes place after a lag of 3–4 min and is completed after 10 min of incubation.

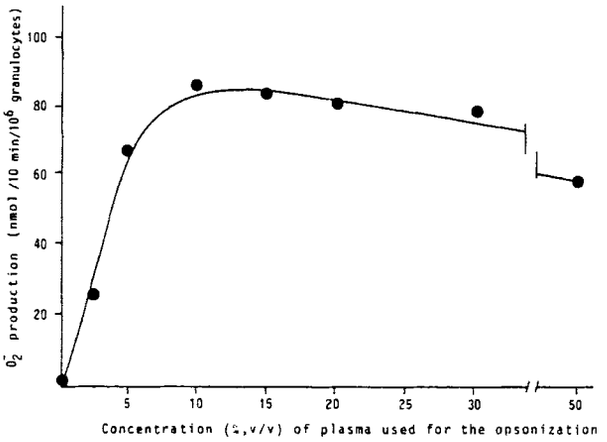

Figure 1. Production of O_2^- by blood incubated for 10 min with zymosan opsonized with various concentrations of plasma. Each point represents the mean value of duplicate readings.

The time course of O_2^- production by whole blood is shown in Fig. 3. In the absence of stimulants, extremely low levels of O_2^- are produced. With serum-treated zymosan and PMA, the production of O_2^- starts immediately and continues at a linear rate for 15–20 min. With zymosan, the initial rate of O_2^- production is slow due to the lag time required for opsonizaton. The reaction starts after about 5 min and then reaches a rate similar to that induced by the other

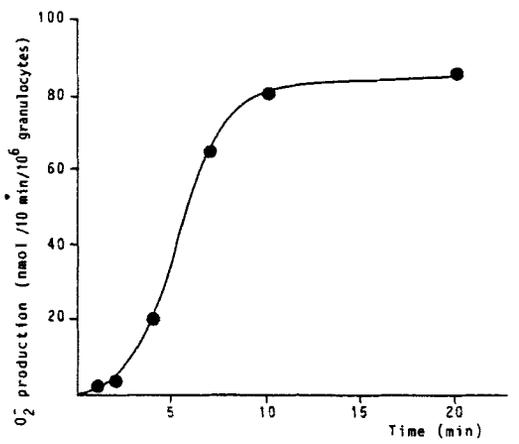

Figure 2. Production of O_2^- by blood incubated with zymosan treated for various times with 15% plasma. Each point represents the mean value of duplicate readings.

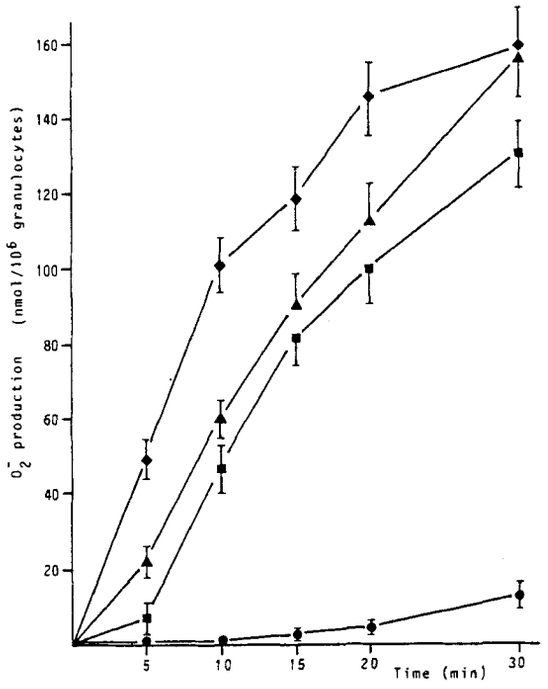

Figure 3. Time course of O_2^- production by whole blood incubated in the absence of stimulants (●) and in the presence of opsonized zymosan (♦), of PMA (▲) and of zymosan (■). Results are means ± SEM of eight experiments.

stimulants. On the basis of these results, an incubation time of 15 min was used in the subsequent experiments.

The influence of leukopenia and of leukocytosis was studied by testing samples of blood in which the concentration of granulocytes was either decreased (by filtration through nylon wool) or increased (by adding pure granulocytes). The O_2^- production was a function of the granulocyte concentration in the blood, over the range of 700–9000 granulocytes/mm^3 (data not shown).

Other blood cells do not interfere with the assay system. In fact, it is known that erythrocytes, platelets and lymphocytes do not exhibit an enhanced metabolic response to agents that stimulate the granulocytes [7–8]. As regards the monocytes, we observed that isolated monocytes incubated with serum treated zymosan produce about 40 nmol of O_2^- 15 min^{-1} 10^6 cells^{-1}. Therefore, the interference of monocytes is minimal and for practical purposes it may be neglected when the percentage of these cells in blood stays at below 5–6%.

Effect of haemolysis and of blood storage

The use of whole blood makes the method subject to interference by haemolysis. We have observed that the addition of increasing amounts of red cell haemolysate to the incubation mixture caused a decrease in the O_2^- measured. This could be due either to the presence of superoxide dismutase of the red cells or to the interference of haemoglobin with the spectrophotometric measurement of reduced cytochrome c. This indicates that it is necessary that the blood sample be free from haemolysis. In our experience, however, this problem has never arisen.

The effect of storing whole blood at room temperature and in an ice bucket is shown in Fig 4. Aliquots were taken at intervals and processed for superoxide production with the method described. The phagocytic function assayed on blood maintained at room temperature did not significantly change until after 4–6 h, whereas in the case of blood maintained in ice it decreased after only 2 h from blood sampling. These data indicate that blood must be stored at room temperature and assayed for O_2^- production within 4 h of its collection.

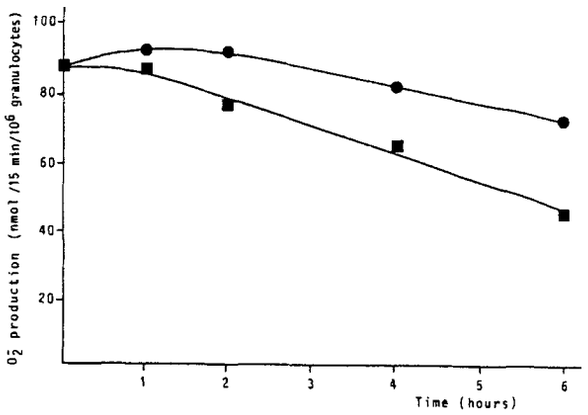

Figure 4. Effect of storage on the production of O_2^- by whole blood incubated with zymosan. Aliquots were taken at intervals from blood maintained at room temperature (●) and at 0–4°C (■). Each point represents the mean value of duplicate readings.

Normal values and preliminary clinical results

The results obtained during the standardization of the method with blood from normal individuals are reported in Table 1. In the absence of stimulants, the production of O_2^- is very low, but in the presence of zymosan, serum-treated zymosan, or PMA, it increases 30–40 times. This marked difference between basal and stimulated O_2^- production indicates that the assay is highly sensitive. No differences between values obtained respectively from males and females were found.

The distribution of O_2^- forming activity by whole blood incubated with zymosan in a group of normal subjects is reported in Fig. 5. It is evident that only a small number of individuals exhibited very low values. In all of these subjects an opsonizaton defect was found.

It is worth noting that repeated assays carried out on blood samples drawn from the same subject at different time intervals over a period of several months, gave similar results (data not shown). Moreover in assays carried out in triplicate or quadruplicate on the same blood sample, the standard deviation obtained did not

Table 1. Superoxide production by whole blood from normal subjects and from patients with cellular and with humoral defects

	Superoxide production (nmol O_2^- 15 min^{-1} 10^6 granulocytes^{-1})			
	No stimulants	+ Zymosan	+Serum-treated zymosan	+Phorbol myristate acetate
Normal males	2·3 ± 1·9 (48)	85·8 ± 20·0 (87)	117·4 ± 25·9 (12)	107·0 ± 39·5 (29)
Normal females	2·9 ± 1·9 (11)	82·6 ± 18·5 (27)	105·3 ± 30·5 (5)	101·5 ± 32·2 (9)
A.G. (CGD, male)	0·1	0·0	0·05	0·4
F.S. (CGD, male)	0·0	0·6	0·0	0·0
C.F. (CGD, male)	0·0	0·4	NT	0·5
V.M. (Mother of A.G.	2·7	25·1	NT	32·3
B.O. (Mother of C.F.)	3·8	16·8	NT	55·4
B.B. ('catarrhal' child)	3·1	6·4	123·5	NT
N.D. (newborn)	3·9	23·7	131·9	NT
S.R. (staphylococcal osteomyelitis)	1·7	14·2	NT	87·5
S.R. (the same patient after recovery)	3·5	69·6	NT	87·8

Results from normal subjects are mean values ± SD (number of subjects). CGD = chronic granulomatous disease. NT = not tested.

exceed 5% of the mean. This indicates that the method is highly reproducible and that the SD reported in Table 1 are mainly due to inter-individual variations in the control group.

Table 1 also reports preliminary results obtained during the first clinical applications of the test. The whole blood from the patients with chronic granulomatous disease (CGD) did not produce any O_2^- with any of the three stimulants employed. The mothers of the CGD patients gave intermediate values of O_2^- production. The O_2^- forming activity of CGD children's blood and of their mothers correlated well with the NBT test and with other metabolic parameters measured on isolated granulocytes, such as the oxygen consumption, the production of O_2^- and of H_2O_2 (data not shown). A humoral defect was found in a child with bronchitis and chronic hypersecretion from the upper respiratory tract (clinically classified as a 'catharral' boy), and in a newborn (two other newborn children had normal opsonizing and O_2^- forming activities). One child with low opsonizing capacity reached normal values after recovery from osteomyelitis.

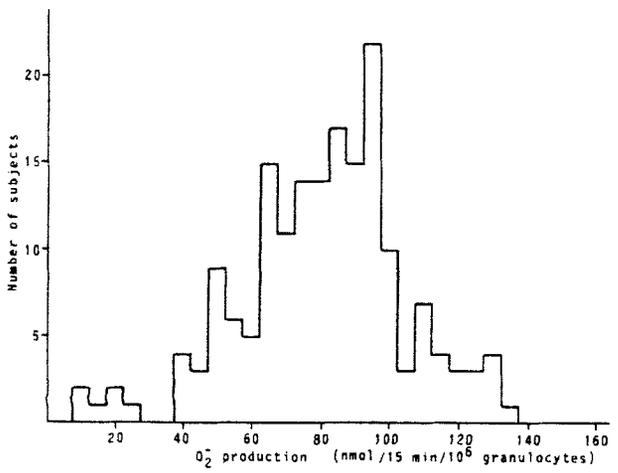

Figure 5. Distribution, in a group of healthy subjects, of the superoxide forming activity by whole blood incubated with zymosan.

Discussion

The present method is a quantitative, time-saving and simple test for the evaluation of the phagocytic function of granulocytes in whole blood. It enables the opsonizing capacity of plasma and the metabolic excitability of the granulocytes to be measured simultaneously under conditions similar to those occurring *in vivo*. A defect in O_2^--forming activity in the presence of zymosan, indicates that a defect is present either at the humoral or at the cellular level. In order to distinguish between these two possibilities, it is sufficient to incubate the blood with serum treated zymosan or with PMA. Under these conditions, O_2^- production will show normal results. In contrast, if a cellular mechanism is involved, O_2^- production will remain low. The use of PMA has the further function of aiding the diagnosis of rare diseases in which granulocytes fail to respond to opsonized zymosan but respond normally to soluble stimulants [9–11]. For a rapid screening, however, it is advisable to carry out an assay only with non-opsonized zymosan as stimulant, thus utilizing two test tubes only (2A and 2B, see methods).

During the standardization of the method, we observed a number of apparently healthy individuals with defective opsonization. These observations confirm those of Soothill and Harvey [12] who found a high incidence (4 in 72) of a similar defect in healthy adults. The nature of the defect is still unknown, but it probably lies in the alternative complement pathway, since zymosan is opsonized by fragments generated by direct activation of this pathway [13]. On these bases, it must be pointed out that the complement-linked opsonizing defects can be revealed by using zymosan in the assay.

Cellular defects, whose prototype is CGD [14], may be detected with our method. The intermediate values

of O_2^- production in the mothers of CGD patients, indicate that it is also possible to detect the carriers of this disease.

Several methods have been described for a quantitative evaluation of the phagocytic function of granulocytes [15–19]. The use of these methods during routine laboratory practice, however, is hampered by the fact that the isolation of granulocytes is necessary together with a large quantity of blood and is time consuming. Other methods which utilize whole blood, such as the method reported by Takeshige *et al.* [20] or the Nitroblue tetrazolium test [21–22], are limited to the diagnosis of cellular defects. The method of Nakamura *et al.* [23] can be extended to the evaluation of the opsonic capacity of plasma, but it requires the use of special equipment such as the Clark-type oxygen electrode which is not found in most laboratories.

In conclusion, the test described in this paper has the following main advantages: (a) it evaluates the opsonic capacity of the plasma and the metabolic response of phagocytes; (b) the technique is simple and only requires the use of a thermostated bath, a centrifuge and a spectrophotometer; and (c) the fact that the test requires a small amount of blood makes it useful in studies of paediatric patients or when repeated blood collections are required.

The main clinical application of the method is the screening for primary humoral or cellular disorders of phagocytosis. Other possible applications are for the follow-up of patients whose phagocytic defense could be reduced during the course of diseases such as leukaemia [24, 25], myeloma [26], rheumatoid arthritis [27, 28], burns [29, 30], liver diseases [31, 32], chronic diarrhoeal states [33], or by the effect of drugs such as corticosteroids [34, 35], sulphonamides [36], phenylbutazone [37] and myelotoxic agents.

Acknowledgements

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