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The Free Radical Forming System of Granulocytes and Macrophages: Further Studies

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ABSTRACT

The main features of the leukocyte oxidase system which is responsible for the production of oxygen-derived free radicals are briefly reviewed. One of the most important components of NADPH oxidase is the low potential cytochrome b. Here some additional evidence in favour of its proposed participation to superoxide formation by neutrophils and macrophages are reported: Cytochrome b co-purifies with enzymatic activity, in cells activated in anaerobiosis it is reduced in parallel with the expression of the oxidase on the membrane and a protein of 32 kDa belonging to cytochrome b is phosphorylated in PMA- and zymosan-stimulated neutrophils. Antibodies raised against the 32 kDa protein bind to a protein which is restricted in its expression to phagocytic cells.

KEYWORDS

NADPH oxidase; cytochrome b; superoxide; antibody; free radicals.

INTRODUCTION

The phagocytes have a peculiar oxidative metabolic pathway, called respiratory burst, which consists in a rapid increase of oxygen consumption, triggered when the cells are engaged in phagocytosis or activated by soluble agents. By this pathway the molecular oxygen is converted into superoxide anion (O_2^-) and then into hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^\cdot$) according to the following reactions:

$$NADPH + 2O_2 \longrightarrow NADP^+ + 2O_2^- + H^+ \qquad (1)$$

$$2O_2^- + 2H^+ \dashrightarrow O_2 + H_2O_2 \quad (2)$$

$$O_2^- + H_2O_2 \dashrightarrow O_2 + OH^- + OH^\cdot \quad (3)$$

The primary reaction is the one-electron reduction of oxygen to superoxide and is catalysed by an enzyme, or enzymatic system called NADPH oxidase. The two other reactions are the dismutation of superoxide (n. 2) which occurs spontaneously or is catalysed by superoxide dismutase, and the Haber-Weiss reaction (n. 3) which is catalysed by metals such as iron. The most powerful compound is the hydroxyl radical but the quantitative relevance of reaction 3 is not well established. Production of all these derivatives of oxygen reduction by phagocytes and by subcellular particles or partially purified systems from activated cells has been widely documented (Babior et al, 1973; Badwey and Karnowsky, 1980; Kakinuma and Kaneda, 1980; Rossi et al, 1982; Bannister et al, 1982). It is known that oxygen free radicals are involved in the antibacterial and antitumor function of granulocytes and macrophages, as well as in the tissue damage of the inflammatory reactions (Johnston et al, 1975; Klebanoff and Clark, 1978; Murray et al, 1979; Fantone and Ward, 1982; Nathan, 1982).

In the present paper the structure and properties of NADPH oxidase (reaction 1) will be considered on the basis of recent findings from our and other laboratories.

PROPERTIES OF THE O_2^--GENERATING SYSTEM OF THE MEMBRANES.

The role of NADPH oxidation in the phagocyte metabolism was firstly described by Rossi and Zatti (1964). The enzyme responsible for the NADPH oxidation and O_2^- formation has not jet fully characterized but a series of informations regarding its properties has been accumulated in the past years. The main features of NADPH oxidase are: 1) It uses NADPH as physiologic substrate and not NADH, and generates 2 mol of O_2^- for 1 mol of NADPH oxidized. 2) Its activity accounts for all oxygen metabolism during the respiratory burst, while mitochondrial oxygen consumption is not significantly affected by cell activation. 3) It is dormant in unstimulated cells and is turned on when the cells are engaged in phagocytosis or come in contact with soluble agents such as concanavalin A, formylated peptides, phorbol myristate acetate (PMA), complement fragments, immunocomplexes, cytochalasins, etc. 4) It works on the plasmamembrane as an electron transport system from NADPH (inside) to oxygen that is released as O_2^- outside the cell or in the phagocytic vacuole. During phagocytosis only the enzyme in contact with the ingested particle is selectively activated. 5) It is probably a multicomponent system in which a b-type cytochrome and a flavoprotein (FAD) appear to play a major role. It has been proposed that these components are functionally linked in a chain where the flavoprotein would act as NADPH-dehydrogenase-cytochrome

b reductase and the cytochrome b as a terminal oxidase (Cross et al, 1982a; Michell, 1983; Green et al, 1983; Borregaard and Tauber, 1984; Gabig and Lefker, 1985). 6) It is absent or inactive in chronic granulomatous disease (CGD), a syndrome with many variants that have in common the complete lack of phagocyte oxidative metabolism in the homozigous form. The gene that is abnormal in the X-linked form of the disease has been recently cloned (Royer-Pokora et al,1986).

STUDIES ON PARTIALLY PURIFIED ENZYME

In recent years we have been engaged in a series of attempts to extract and purify the NADPH oxidase from the membranes of neutrophils and macrophages. The enzyme has proven to be very labile on detergent extraction, in keeping with its supposed nature of multicomponent system. In order to solubilize and purify the enzyme in active form, strong detergents and high ionic strenghts couldn't be used. Therefore the enzyme was isolated not as a single protein but as a high-molecular-weight proteolipid complex. This physical state preserved the O_2^--forming activity but hampered a complete purification.

The best purification procedure was adopted with pig neutrophils, due to the large availability of blood cells (Bellavite et al, 1985). It consisted of extraction from NaCl-washed membranes with Lubrol-PX and deoxycholate, followed by gel filtration chromatography and glycerol density gradient centrifugation. Table 1 reports the composition and some properties of partially purified NADPH oxidase from pig neutrophils. The enzymes from guinea pig neutrophils (Serra et al, 1984, Bellavite et al, 1984), human neutrophils (Bellavite et al, 1986) and guinea pig peritoneal macrophages (Berton et al, 1985) were also partially purified and their content and properties were similar to those of the enzyme from pig neutrophils.

Since a 32 kDa peptide was the only band that in the electrophoresis copurified with cytochrome b_{-245} we suggested that it belongs to this cytochrome (Bellavite et al, 1985), but it should be noted that many different reports of the molecular weight of cytochrome b-245 have been published (for a review see Rossi et al, 1986). The reason for these discrepanciecies is still matter of discussion but probably they are due to the different procedures adopted by the various investigators or to the presence in the cytochrome of several subunits that may be in different aggregation states according to the experimental conditions.

THE ROLE OF CYTOCHROME b_{-245}

The involvement of a cytochrome b in the respiratory system of phagocytes was firstly suggested by Shinagawa et al (1966) and

Table 1. Composition and some properties of NADPH oxidase extracted and partially purified from pig neutrophils.

1. It contains high concentration of cytochrome b_{-245} (1,500--2,000 pmoles/mg protein).
2. It contains phospholipids (1,800 nmoles/mg protein), mostly P.E., P.C., Sph.
3. It contains very little FAD (40-50 pmoles/mg protein) and no quinones.
4. Its only electron acceptor is molecular oxygen. No diaphorase activity is present in the enzyme complex.
5. Turnover number with respect to cytochrome b_{-245}: 8-10/sec.
6. The proteolipid complex loses the activity when it is dissociated by high detergent and salt concentration.
7. The purification is associated with increase of concentration of a protein of 31.5-32 kDa (in SDS-electrophoresis).
8. The 32 kDa protein is markedly phosphorylated in the activated enzyme complex.

was extensively studied by Segal and Jones (Segal et al, 1978). A large series of evidence in favor of the participation of this cytochrome (called b_{-245} from its characteristic low mid point potential, or b_{558} from the α peak of the reduced-oxidized spectrum , here also referred simply as cytochrome b) to the electron transfer from NADPH to oxygen has been reported (for a review see Rossi et al, 1985). Particularly important in this respect have been the studies on CGD leukocytes, where it has been shown that cytochrome b-245 is absent or functionally inactive (Segal et al, 1983). The reduced-oxidized visible spectrum of this cytochrome is shown in fig. 1.

On the other hand, the participation of cytochrome b to O_2^- formation has been questioned on the basis of the following findings: First, the reduction of cytochrome b in anaerobiosis by NADPH is very slow and does not account for the enzymatic activity in aerobiosis (Babior, 1983), second, the cytochrome b according to Iizuka et al (1985) does not bind CO and therefore has not the properties of a terminal oxidase, third the NADPH oxidase purified by Markert et al (1985) and by Doussiere and Vignais (1985) did not contain visible spectrum of cytochrome b. However, in our opinion these findings do not exclude a role for the cytochrome b in the NADPH oxidase. In fact, as far as the first point is concerned, Cross et al (1985) showed that the reduction of cytochrome b in aerobiosis is fast enough to account for the overall transfer of electrons from NADPH to oxygen; second, some authors have reported that cytochrome b_{-245} actually binds CO, although at a very slow rate (Cross et al, 1982b; Bellavite et al, 1983, Pember et al, 1984) and in any case it cannot be excluded that the cytochrome reacts with oxygen not by way of the formation

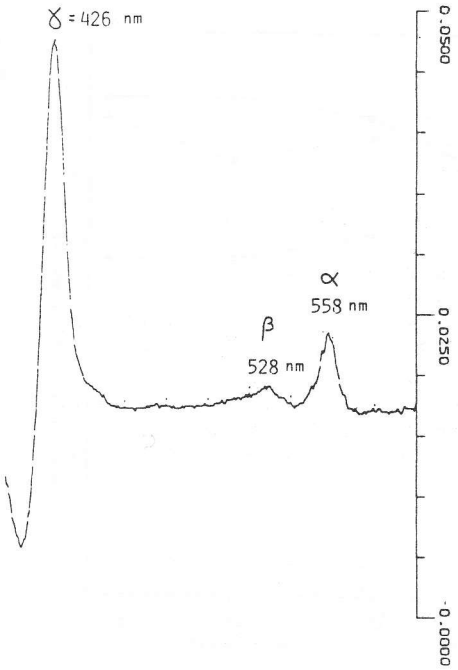


Fig. 1.

Reduced-minus-oxidized spectrum of cytochrome b_{-245} partially purified from guinea pig peritoneal macrophages (Berton et al, 1985). Protein concentration: 0.3 mg/ml.

of an oxygenated form but through a direct contact between molecular oxygen and the heme edge, as suggested by Iizuka et al (1985); third, as far as the purification of the NADPH oxidase is concerned, the lack of cytochrome b spectrum in some purified oxidase preparations does not exclude that it participates to the reaction in the whole membrane or in the enzyme complex isolated with different procedures.

In our experience the cytochrome b_{-245} was always associated with the NADPH dependent O_2^--forming activity, irrespective of the isolation method employed. We also repeated the work of Markert et al (1985) and we found that the NADPH oxidase isolated from human neutrophils by this procedure actually contains a substantial amount of cytochrome b-245 (Bellavite et al, 1986).

In order to better understanding the role played by cytochrome b in the neutrophil metabolism we have investigated the relationship between the cytochrome b reduction in intact cells activated in anaerobiosis and NADPH oxidase activation. Figure 2 shows that cytochrome b reduction occurs only when the cells are activated and in the same range of PMA concentration that causes also the activation of both NADPH oxidase and respiratory burst, measured as oxygen consumption. Figure 3 shows that in the time after the addition of a fixed dose of PMA there is a progressive activation of the oxidase and of cytochrome b reduction, with similar kinetics. The only difference is that cytochrome b reduction

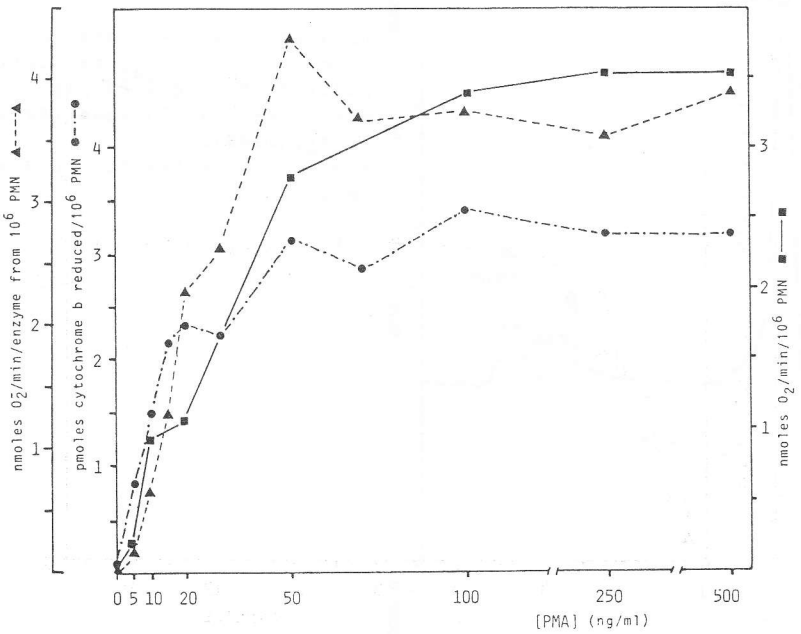


Fig. 2. Cytochrome b-245 reduction in neutrophils activated in anaerobiosis compared with the corresponding NADPH oxidase activity of cell-free particles and with oxygen consumption of intact cells. 4 x 10^7 pig neutrophils were incubated at 37 °C in a stoppered cuvette under a continuous flux of oxygen-free nitrogen and under continuous stirring, in 1 ml of Krebs-Ringer phosphate buffer supplemented with 10 mM glucose, 1 U/ml heparin, 200 U/ml catalase and 0.4 mg of glucose oxidase. The reference cuvette was not closed and contained the same mixture without glucose oxidase. The indicated doses of PMA were added to the sample cuvette and after 10 min the difference spectrum was recorded with a 576 Perkin Elmer spectrophotometer and the cytochrome b reduction was calculated at 426-405 nm using the extinction coefficient of 106 mM^{-1} cm^{-1}. The cells were immediately frozen in liquid nitrogen, thawed and sonicated. The cell-free particles were prepared and the NADPH oxidase activity was assayed as described (Bellavite *et al*, 1983). Oxygen consumption was measured with Clark oxygen electrode using cells (4 x 10^7) suspended in the above midium without glucose oxidase.

reaches the maximum a couple of minutes before the maximum of NADPH oxidase activity. The reason for this discrepancy could be due to the fact that cytochrome b reaches a plateau already when a maximum of 60% of total cell cytochrome b is reduced and therefore at this point a thermodynamic equilibrium between the

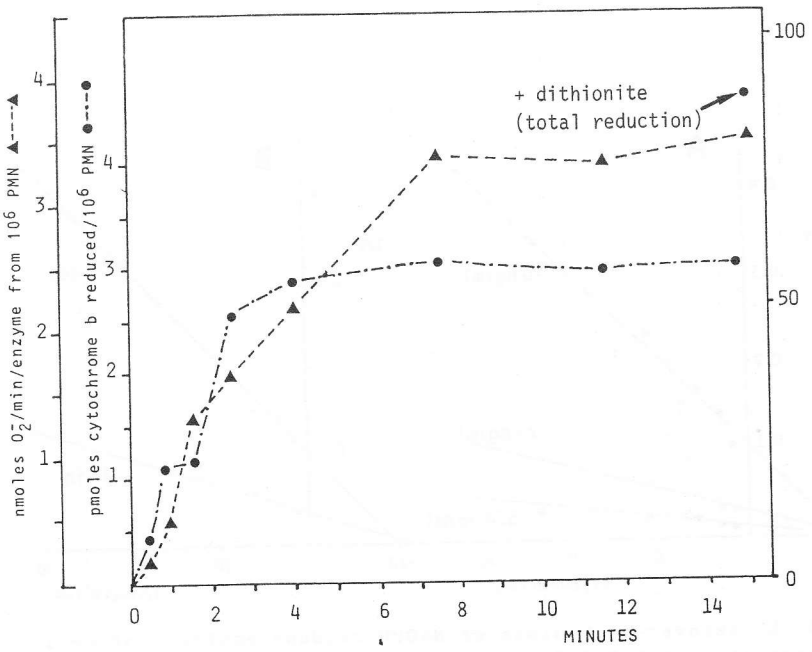


Fig. 3. Time-course of cytochrome b-245 reduction in neutrophils activated by PMA in anaerobiosis and NADPH oxidase activity of cell-free particles. The incubation and assay conditions were as in Fig. 2. Neutrophils were treated with 50 ng/ml PMA.

oxidized and the reduced forms could be reached. On the other hand the NADPH oxidase is assayed as O_2^- production in aerobic conditions and an increased activation would be reflected in increased superoxide production.

On samples at different activation state and cytochrome b reduction we measured the kinetic properties of NADPH oxidase (Fig. 4) Th enzyme from cells activated with different doses of PMA (panel A) and with the same dose for two different times showed the same Km for NADPH, while the maximum velocity of the reaction increased by increasing the activation. This indicates that in the course of the activation there is not a progressive increase of the affinity for the substrate of all the enzymes, but there is an higher efficiency in the transport of electrons from NADPH to oxygen. These data suggest that the superoxide forming activity depends on the number of oxidase-cytochrome b_{-245} complexes that enter into an active (capable of transporting electrons) state. The limiting step of the activation mechanism would be the recruitment of cytochrome b molecules in an active complex and not the affinity of the enzyme for NADPH.

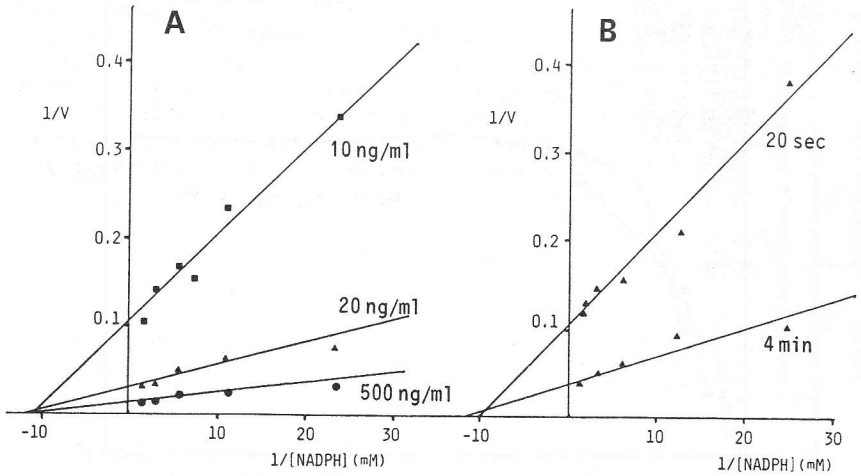
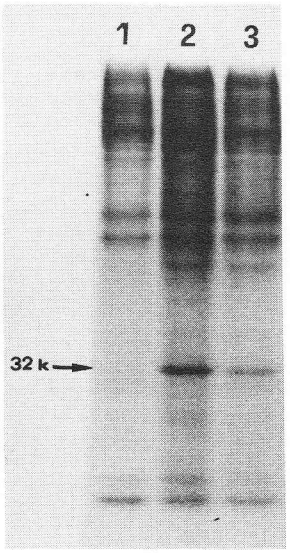


Fig. 4. Lineweaver-burk plots of NADPH oxidase activity of cell-free particles isolated from neutrophils at variable activation state. The cell-free particles were isolated from pig neutrophils activated in anaerobiosis as described in Figs. 2 and 3, with various doses of PMA for 10 min (panel A) and with 50 ng/ml PMA for different time (panel B).

These data are consistent with our recent findings that cytochrome b or a protein associated with it is phosphorylated in activated cells (Bellavite et al 1985, Papini et al 1985). As it can be seen in Fig. 5, the protein of 32 kDa, that we have found to be associated with cytochrome b throughout the purification (Table 1), markedly increased the incorporation of ^{32}Pi in the membranes from both PMA-activated and phagocytosing neutrophils. This finding suggests that the phosphorylation of cytochrome b or of one of its putative associated proteins is a fundamental step of the activation mechanism, possibly by facilitating the interaction with some proximal electron carrier (Bellavite et al, manuscript in preparation). Whether other concomitant modifications of the membrane are also necessary in order to trigger the respiratory burst remains to be investigated. The stimulatory effect of fatty acids on the NADPH oxidase in cell-free system (Bromberg and Pick, 1984) suggests that the modification of the properties of the lipid bilayer may facilitate the interaction between crucial membrane and cytosolic proteins.

Fig. 5.



Phosphoprotein patterns of resting and stimulated neutrophils. Pig neutrophils were labeled with ^{32}Pi and then incubated for 10 min without stimulant (lane 1), with 100 ng/ml PMA (lane 2) and with 4 mg/ml of opsonized zymosan (lane 3). The membranes were then isolated as described (Bellavite et al, 1985) and subjected to SDS-polyacrylamide electrophoresis according to Laemmli (1970). The gel was dried and exposed to X-ray film as described (Papini et al, 1985).

PRELIMINARY RESULTS WITH ANTIBODIES TO NADPH OXIDASE

After having identified the 32 kDa peptide of neutrophil's membranes as an important component of the NADPH oxidase and probably the cytochrome b_{-245} itself, we decided to raise antibodies against this protein in order to better investigate its role in the oxidase complex. The rationale of this approach was the attempt of obtaining antibodies that could interfere with the enzymatic function or that could be employed to purify the protein by affinity chromatography.

Polyclonal antibodies from rabbit serum and a monoclonal mouse antibody (designated 1G4-D9) were obtained by conventional procedures, using as antigen the 32 kDa protein excised and eluted from SDS-polyacrylamide gels stained with Coomassie blue R-250. The antigens recognized by polyclonal and monoclonal antibodies were demonstrated in partially purified samples of NADPH oxidase separated in SDS-polyacrylamide gels and blotted onto nitrocellulose. Fig. 6 shows a typical immunoblot of the NADPH oxidase sample. As it can be seen, polyclonal antibodies react with the 32 kDa protein and with two other proteins of approximately 28 and 15 kDa, while 1G4-D9 reacts with the 15 kDa protein. The reason why a monoclonal antibody obtained using as antigen the 32 kDa protein binds to a protein of 15 kDa of the NADPH oxidase sample deserves further investigation, although it is conceivable that the 15 kDa protein is a proteolytic fragment of the 32 kDa protein.

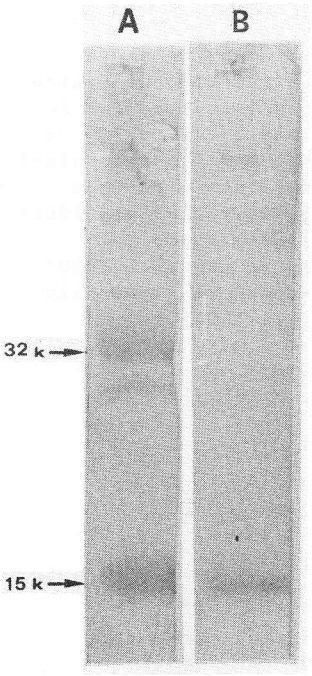


Fig. 6.

Immunoblot of partially purified NADPH oxidase from pig neutrophils. Polyclonal rabbit IgG and mouse monoclonal antibody IG4-D9 arised against the 32 kDa protein, were purified by ammonium sulphate precipitation and DEAE-cellulose chromatography. NADPH oxidase was partially purified according to Bellavite et al (1985), separated on SDS-polyacrylamide gel and blotted onto nitrocellulose. The antigens were visualized with an indirect enzyme-linked immunosorbent assay using as second antibody goat anti rabbit or goat anti mouse IgG conjugated with horseradish peroxidase. Lane A: immunoblot made with polyclonal rabbit IgG. Lane B: immunoblot made with monoclonal mouse antibody IG4-D9.

The antigen recognised by 1G4-D9 appears to be highly restricted in its expression, as assessed by indirect binding assays to cells and detergent extracts. There was no binding of 1G4-D9 to extracts of pig liver, brain, erythrocytes and lung tissues. The antibody bound, besides to pig neutrophils, to human normal and CGD neutrophils, either as intact cells or cell lysates. The specificity of 1G4-D9 antigen was also assessed by incubating pig spleen cryostatic sections with 1G4-D9 antibody. Fig. 7 shows that also in spleen sections the reactivity was confined to granulocytes.

Attempts to investigate the effect of IG4-D9 monoclonal antibody on neutrophil functions excluded any influence on O_2^- forming activity of intact cells or of partially purified NADPH oxidase. No effect could be demonstrated also on exocytosis. New experiments using as immunogen partially purified and active NADPH oxidase preparations have been undertaken in our laboratory with the aim to isolate monoclonal antibodies against the 32 kDa protein in its native and enzymatically active form.

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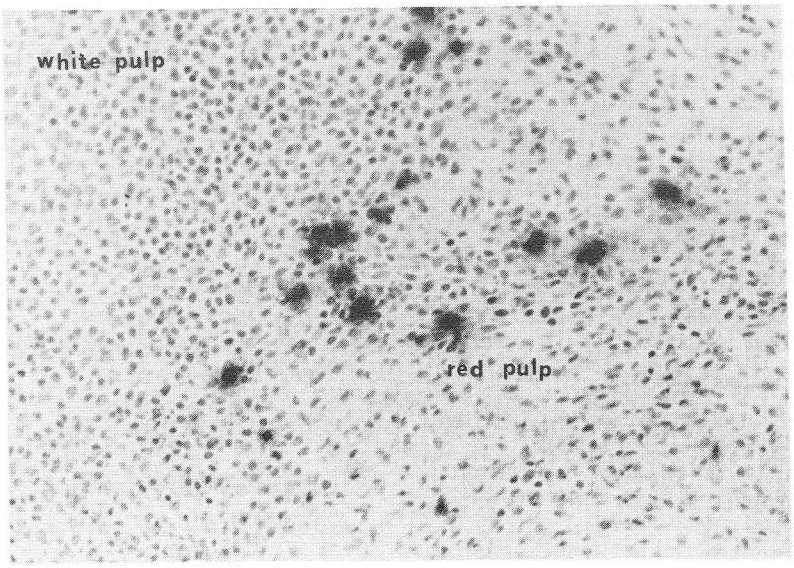

Fig. 7. Immunohistochemical characterization of monoclonal antibody IG4-D9. Spleen cryostat sections from swine were fixed in cold acetone, dried and processed with a standard alkaline phosphatase technique (APAAP) described by Cordell et al (1982). Levamisole was used for inhibition of endogenous alkaline phosphatase activity. Sections incubated with non-immune mouse serum or omitting first antibody using the same APAAP staining procedure were negative.

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