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Studies on the NADPH Oxidase of Phagocytes

PRODUCTION OF A MONOCLONAL ANTIBODY WHICH BLOCKS THE ENZYMATIC ACTIVITY OF PIG NEUTROPHIL NADPH OXIDASE*

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We describe in this paper a monoclonal antibody to pig NADPH oxidase which inhibits enzymatic activity. This antibody, designated 1H8.2, was selected from a group of monoclonal antibodies produced against active preparations of purified NADPH oxidase and which showed selectivity of binding. 1H8.2 is an IgM restricted in binding to pig NADPH oxidase and showing higher binding to NADPH oxidase purified from phorbol myristate acetate-stimulated than from resting neutrophils. The antibody inhibits by about 90% the oxidase activity at 20–50 μg/ml. Inhibition is due to a decrease of the V_{max} of the oxidase, and the K_m is not affected. Incubation of the NADPH oxidase with 1H8.2 in the presence of concentrations of NADPH up to 25-fold the K_m does not prevent the inhibition. Together with the evidence that the antibody does not inhibit the neutrophil superoxide dismutase-insensitive NADPH cytochrome *c* reductase and the liver NADPH-cytochrome *c* reductase this observation indicates that the 1H8.2 does not bind to an epitope belonging to the NADPH-binding site. Experiments of immunoprecipitation of iodinated membrane proteins and of immunoaffinity purification showed that 1H8.2 recognizes a heterodimer of apparent molecular mass of 16/18 and 14 kDa. These polypeptides can be involved in the NADPH oxidase activity or represent still unrecognized molecules able to modulate its function.

Production of toxic oxygen molecules by phagocytes is due to the activation of an electron transport system which is universally known as NADPH oxidase. Starting from its recognition as the enzymatic activity which is responsible for the respiratory burst triggered during internalization of bacteria (1), NADPH oxidase has been the object of intensive investigation during the past 20 years (see Refs. 2–4 for reviews).

There is a general agreement that the NADPH oxidase which transfers electrons from NADPH to O_2 is indeed composed of more than one component, and strong evidence has been presented for the existence of a flavoprotein which would represent the NADPH-binding site, and a low potential cytochrome *b* which would represent the terminal component able to reduce O_2 to O_2^- (see Refs. 2–4).

Recent studies provided unequivocal evidence about the molecular nature of the low potential cytochrome *b* in human neutrophils (5, 6) which was shown to be a heterodimer consisting of a 91-kDa glycosylated heavy chain and a 22-kDa light chain. The genes for both chains have been cloned (7, 8), and mutations in the heavy chain genes are responsible for the *X*-linked forms of chronic granulomatous disease, a disease characterized by the inability of phagocytes to undergo a respiratory burst upon challenge.

Uncertainty still exists about the molecular nature of other components involved in the NADPH oxidase activity. Active preparations of NADPH oxidase practically devoid of cytochrome *b* were shown to consist of only three major components of 67, 48, and 32 kDa (9). The use of NADPH analogues allowed to identify in phagocytes membranes NADPH-binding proteins of 45 (10) and 66 (11) kDa. A flavoprotein of 51 kDa has been recently claimed to be a component of the oxido-reductase complex (12).

In order to characterize molecules involved in, or able to modulate, the NADPH oxidase activity we have started to isolate monoclonal antibodies against active preparations of NADPH oxidase. A monoclonal antibody raised against a partially purified preparation of guinea pig NADPH oxidase has permitted the identification of a species-restricted proteolipid Ag whose cross-linking on the cell surface triggers guinea pig neutrophils functions (13, 14). For the first time, we describe in this paper a monoclonal antibody able to inhibit the activity of NADPH oxidase. The monoclonal antibody is directed against pig NADPH oxidase and recognizes a heterodimer consisting of polypeptides of 16/18 and 14 kDa. This finding opened the way to the characterization of new polypeptides involved in the activity of the NADPH oxidase or regulating its function.

MATERIALS AND METHODS

Immunogen and Immunization—NADPH oxidase was purified from detergent-solubilized plasma membranes of diisopropyl fluorophosphate (DFP)[1]-treated, phorbol myristate acetate (PMA)-activated pig neutrophils as described in Ref. 15. The preparation used as immunogen displayed an O_2^--producing activity of 294 nmol/min/mg of proteins and contained 1700 pmol of cytochrome b_{558}/mg proteins. BALB/c mice were immunized intramuscularly in the hindleg with 100 μg/ml of the oxidase preparation diluted 1:1 with complete Freund adjuvant. After 20 days, booster injections with the same dose of immunogen diluted 1:1 with incomplete Freund adjuvant were made at identical sites. Mice serum was tested after 10 days for binding to NADPH oxidase immobilized to plastic (see below), and

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[1] The abbreviations used are: DFP, diisopropyl fluorophosphate; PMA, phorbol 12,13-myristate acetate; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

two mice received an intraperitoneal injection of 100 μg of the immunogen 4 days before the fusion.

Production of Hybridoma—Spleen cell suspensions were fused with the nonsecreting mouse myeloma line NSO/U originally isolated by M. R. Clark, B. W. Wright, and C. Milstein (see Ref. 16) and kindly supplied by C. Milstein (Cambridge). The fusion protocol was essentially as described in (16) with a ratio of 1 parental myeloma line/7 spleen cells. Selected hybridoma were cloned by limiting dilution.

Binding Assays—The culture medium from wells where growth of hybridoma occurred was first tested for binding to the NADPH oxidase preparation used as immunogen. NADPH oxidase was immobilized to 96-wells plates (Flow Laboratories, Irvine, Scotland) with a procedure adopted from Michl *et al.* (17). Purified preparation of NADPH oxidase were diluted to 0.1–0.2 mg/ml with 50 mM sodium phosphate buffer, pH 8.0, supplemented with 2 mM NaN_3, 1 mM phenylmethylsulfonyl fluoride, 1 mM EGTA, 0.4% sodium deoxycholate, and 0.4% Lubrol-PX. After 30 min of incubation in ice, 100 μl were distributed in 96-well plates which had been previously derivatized with poly-L-lysine and glutaraldehyde (17). After incubation at 4 °C for 4 h, the plates were washed five times with phosphate-buffered saline (PBS), and the wells filled with PBS containing 5 mg/ml of casein and incubated overnight at 4 °C to block unreacted aldehyde groups. After five washings with PBS the wells were filled with 100 μl of hybridoma culture medium or purified preparation of antibodies diluted in PBS and incubated at 4 °C for 1–2 h. Binding of the first antibody was revealed by an enzyme-linked immunoadsorbent assay with a horseradish peroxidase-labeled rabbit anti-mouse IgG + IgM (H + L chains) (KLP, Gaithersburg, MD). Controls included binding to wells coated only with the quenching solution (PBS-casein) and blanks done using control medium or purified preparations of mouse immunoglobulins. For binding to proteins solubilized for erythrocytes or hepatocytes membranes, ghosts of pig erythrocytes were obtained as described in Ref. 18, and hepatocytes membranes were obtained from postmitochondrial supernatants of pig liver homogenates pelleted at 100,000 × *g* for 30 min. Membrane pellets were suspended in the solubilization buffer used for the NADPH oxidase (see above) at 1 mg/ml and immobilized to plastic as described above.

Assays of NADPH Oxidase and Studies on the Ability of the Antibodies to Alter the Oxidase Activity—NADPH oxidase obtained from PMA-stimulated neutrophils was assayed as superoxide dismutase-sensitive cytochrome *c* reductase activity in the conditions described in Ref. 19. To study the effects of antibodies on the NADPH oxidase activity, these were incubated for 1–2 h in ice with purified preparations of NADPH oxidase, and then aliquots of the incubation mixture were assayed. Different preparations of antibodies were used to study their effects on the NADPH oxidase activity. i) An initial screening was done by incubating 100 μl of hybridoma culture supernatants, diluted 1:1 with PBS, with 10–20 μg of NADPH oxidase preparations. Experiments with culture media gave erratic results and did not allow to observe unequivocal alterations of the oxidase activity. ii) As a second step of screening, antibodies were concentrated from culture media or ascites by ammonium sulfate (45%) precipitation and extensively dialyzed first against 5 mM phosphate buffer, pH 7.4, and then against PBS. Preparations of antibodies and NADPH oxidase were incubated in PBS at a final concentration of 1 mg/ml and 0.2–0.3 mg/ml, respectively. These experiments allowed identifying the antibody designated 1H8.2 as capable of inhibiting the NADPH oxidase, while no inhibitory activity was observed with the other five antibodies, control medium treated as the hybridoma cultures supernatants, and a preparation of control mouse immunoglobulins. iii) Studies with purified preparations (see below) of antibodies secreted by two clones of the 1H8.2 hybridoma were done by incubating the antibody and the NADPH oxidase in PBS. The concentration of the antibody was routinely 20–50 μg/ml and that of the NADPH oxidase 200–300 μg/ml.

Antibody Purification—Antibodies were precipitated from mouse serum and ascites with 30% and from conditioned medium with 45% ammonium sulfate. The precipitates were dissolved in water and dialyzed first against 5 mM sodium phosphate buffer and then against PBS. Immunoglobulin fractions purified from mouse serum by anion exchange chromatography were used to raise anti-mouse immunoglobulins in rabbits. IgG purified from rabbit serum were coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) at 5–10 mg/ml resin and used for affinity purification of ammonium sulfate precipitates of control mouse serum and medium conditioned by a 1H8.2 hybridoma clone. Fractions eluted from the affinity column with 0.1 M propionic acid, pH 4.0, were immediately neutralized with solid Tris and dialyzed against PBS. To characterize monoclonal antibodies, preparations purified from conditioned media were immobilized to plastic and binding of rat monoclonal anti-mouse IgM and anti-IgG subclass (Jansen, Beerse, Belgium) detected with horseradish peroxidase-labeled sheep anti-rat immunoglobulins (Amersham International plc, Amersham, United Kingdom).

Immunoprecipitation of Antigens—Plasma membranes isolated from DFP-treated, PMA-stimulated neutrophils were purified by sucrose density gradient centrifugation (15) and suspended at 50 μg/ml in 100 mM sodium phosphate buffer, pH 7.5, 5 mM EDTA, 2 mM NaN_3, 1.5 μM leupeptine, 2 μM pepstatin, 1 mM phenylmethylsulfonyl fluoride supplemented with 0.1% SDS. After 1 h in ice, membrane proteins were iodinated by the chloramine-T method and chromatographed through a G-25 column (Pharmacia) equilibrated with the above buffer containing 1% Nonidet P-40 instead of SDS. The material eluted in the void volume (3.0 ml) was centrifuged in a microcentrifuge (13,000 × *g*) for 5 min and the supernatant used for the immunoprecipitation experiments. These were done in two ways: i) 200–400 μl of iodinated membrane proteins were diluted 2.5–5 times with the same buffer and incubated with 200 μl of Sepharose 4B coupled with antibodies (see below). Incubations were at 4 °C for 2 h in a rotating wheel. ii) 200–400 μl of iodinated membrane proteins were incubated in ice for 1 h with 10 μg of antibodies and the immunocomplex precipitated with rabbit anti-mouse immunoglobulins bound to Protein A-Sepharose (Pharmacia). Immunoprecipitates were washed three times with 1 ml of each of the following buffers: i) 10 mM Tris, pH 8.0, 500 mM NaCl, 0.5% Nonidet P-40, 0.05% SDS; ii) 10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.05% SDS; iii) 10 mM Tris, pH 8.0, 0.05% SDS.

SDS-PAGE and Autoradiography—Samples for PAGE were incubated at 60 °C for 30 min in 60 mM Tris, pH 6.8, 8% glycerol, 4% SDS and with or without 2% 2-mercaptoethanol prior to separation in 12% polyacrylamide gels (20). After fixation, staining with Coomassie Blue and destaining, gels were dried and exposed to x-ray films (Kodak X-Omat MA, France) as described in Ref. 20.

Immunoaffinity Purification of the 1H8.2 Antigen—Mouse serum immunoglobulins and ascites from mice injected with a clone of 1H8.2 hybridoma were concentrated by ammonium sulfate precipitation and coupled to CNBr-activated Sepharose 4B (Pharmacia) at a concentration of 5–10 mg/ml of resin. Plasma membranes isolated from DFP-treated, PMA-stimulated neutrophils were suspended in the same buffer used for the immunoprecipitation experiments (see above) containing 2% Nonidet P-40 and 20% glycerol, and after stirring for 10 min at 4 °C, centrifuged for 1 h at 100,000 × *g*. 7 ml of the supernatant (1.2 mg protein/ml) were first applied on a mouse immunoglobulin-Sepharose 4B column to adsorb proteins binding nonspecifically to the resin, and the eluate was then applied on a 1H8.2-Sepharose column. Columns containing 4 ml of resin and with heights of 2.3 and diameters of 1.5 cm were used for both the control- and the 1H8.2-Sepharose and were previously equilibrated in the same buffer used for the solubilization of membranes containing 0.5% Nonidet P-40. After washing the affinity column with 20 ml of this buffer supplemented with 0.5 M NaCl, the antigen was eluted with 0.1 M acetic acid, pH 4.0, containing 0.1% Nonidet P-40 and 20% glycerol. Fractions of 2.5 ml were collected into tubes containing 0.6 ml of 1 M Tris-Cl, pH 8.5. Proteins were precipitated with acetone at −20 °C for 1 h, centrifuged for 10 min at 10,000 × *g*, and analyzed by SDS-PAGE.

RESULTS

Selection of Antibodies Raised against NADPH Oxidase—The first step in our strategy of screening to select antibodies against the NADPH oxidase was based on the selectivity of binding to NADPH oxidase. The supernatants of 40 hybridomas (from a total of 193) which showed binding to NADPH oxidase were tested against cytosolic fractions of neutrophils and proteins extracted from membranes of erythrocytes ghosts and hepatocytes.

Table I shows the binding properties of some of the antibodies which are representative of the results obtained with all those tested. Some of the antibodies (represented in Table I by 1E7), showed, as the serum of mice whose spleens were used for the fusion, no restriction of binding to NADPH oxidase. 20 of 40 (1G3, 1H8.2, 2B10, and 3B10 in Table I)

TABLE I

Selectivity of binding of different antibodies to NADPH oxidase

Ag were prepared and immobilized to 96-well plates derivatized with poly-L-lysine and glutaraldehyde as described under "Materials and Methods." The results of binding of 1:100 dilution of immune serum and plain hybridoma culture media are reported. After 1 h of incubation at 4 °C with the first antibody, binding was revealed with a horseradish-peroxidase-labeled anti-mouse IgG + IgM using *ortho*-phenylenediamine as substrate. Aspecific binding of the second antibody gave values of optical density of 0.02–0.04 which were not subtracted from the values reported. ND = not determined.

Antibody	Optical density at 492 nm				
	PMA-activated NADPH oxidase	Resting NADPH oxidase	Cytosol	Erythrocytes ghosts extracts	Hepatocytes membranes extracts
Immune serum	1.586	1.380	0.644	1.197	1.109
1G3	0.938	ND	0.048	0.016	0.042
1H8.2	1.390	0.783	0.044	0.030	0.039
2B10	0.714	ND	0.046	0.056	0.038
3B10	0.696	ND	0.081	0.044	0.087
3C1	0.248	0.141	0.043	0.053	0.248
1E7	0.913	0.900	1.353	1.222	ND

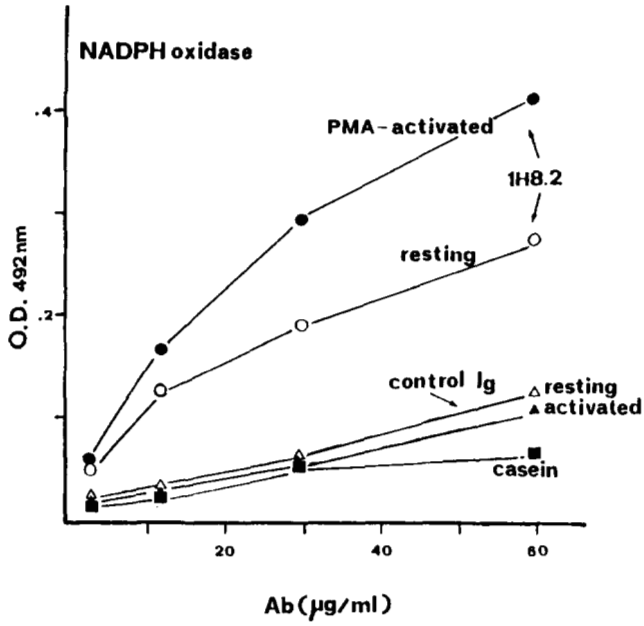

FIG. 1. **Binding of 1H8.2 to NADPH oxidase.** NADPH oxidase was purified from resting and PMA-activated neutrophils as described in Ref. 15. Both preparations were diluted to the same protein concentration and bound to plastic as described under "Materials and Methods." Control mouse immunoglobulins and 1H8.2 were purified by affinity chromatography and binding was revealed as described under "Materials and Methods" and the Table I legend.

showed, however, apparent selectivity of binding to NADPH oxidase. These were concentrated from hybridoma cultures (see "Materials and Methods") and tested for the ability to alter the NADPH oxidase activity. One antibody, designated 1H8.2, inhibited the NADPH oxidase activity.

Binding Properties of 1H8.2, an Antibody Which Inhibits the NADPH Oxidase—As shown in Fig. 1, 1H8.2 binds to NADPH oxidase, and binding is dependent on antibody concentration. The binding is higher to the NADPH oxidase extracted from neutrophils stimulated with PMA when compared with similar preparations extracted from resting neutrophils. We did not detect any binding to NADPH oxidase preparations of rat, guinea pig, or human neutrophils (not shown). 1H8.2 is an IgM.

Inhibition of the NADPH Oxidase Activity by 1H8.2—As shown in Fig. 2, 1H8.2 inhibits the activated NADPH oxidase activity in a dose-dependent fashion. Inhibition is almost complete, and in 10 independent experiments performed with different oxidase preparations we observed an inhibition of 88.9 ± 11.9% with 25–50 μg/ml antibody. Inhibition of the NADPH oxidase activity required more than 30 min of incubation with the antibody and this had no effect if added in the assay. Controls with purified preparations of mouse or rabbit immunoglobulins or with five other antibodies derived from the same fusion gave invariably negative results. 1H8.2 did not inhibit the activity of other NADPH-dependent cytochrome *c* reductases (see below).

Different observations indicated that the inhibitory activity of 1H8.2 is not due to binding to an epitope belonging to the NADPH-binding site. As shown in Fig. 3, 1H8.2 inhibited the V_{max} of NADPH oxidase and did not affect the K_m of the enzyme. Incubation of NADPH oxidase with 1H8.2 in the presence of up to 2 mM NADPH did not prevent inhibition (not shown). Finally, the antibody had no effect on the activity of pig neutrophil superoxide dismutase-insensitive NADPH cytochrome *c* reductase nor on NADPH cytochrome *c* reductase extracted from pig liver hepatocytes (not shown).

Immunoprecipitation and Affinity Purification of 1H8.2 Ag—The first attempts to identify the 1H8.2 Ag were done with Western blots of SDS-PAGE of purified preparations of NADPH oxidase or membrane extracts of PMA-stimulated neutrophils. 1H8.2 did not, however, recognize any polypeptide in Western blots, and we, therefore, tried to immunoprecipitate extracts of iodinated membranes.

As shown in Fig. 4*A*, immunoprecipitation experiments indicate that 1H8.2 recognizes a heterodimer of apparent molecular mass of 16/18 and 14 kDa. The same molecule is recognized by another antibody derived from the same fusion and designated 2B10, which however does not inhibit NADPH oxidase.

Purification of the 1H8.2 Ag with an affinity column allowed us to obtain enough material to stain with Coomassie Blue after SDS-PAGE. As shown in *panel B* of Fig. 4, the two components purified by affinity chromatography showed comparable staining with Coomassie Blue, suggesting that the heterogeneity observed in the experiments of immunoprecipitation of iodinated proteins (see *panel A*) is likely due to a different extent of iodination of the 16/18- and the 14-kDa polypeptides.

Purification of the 1H8.2 Ag by affinity chromatography was done at concentrations of detergents which inactivated the NADPH oxidase. In the conditions used, it was, however, possible to detect the spectrum of cytochrome b_{558}, the most

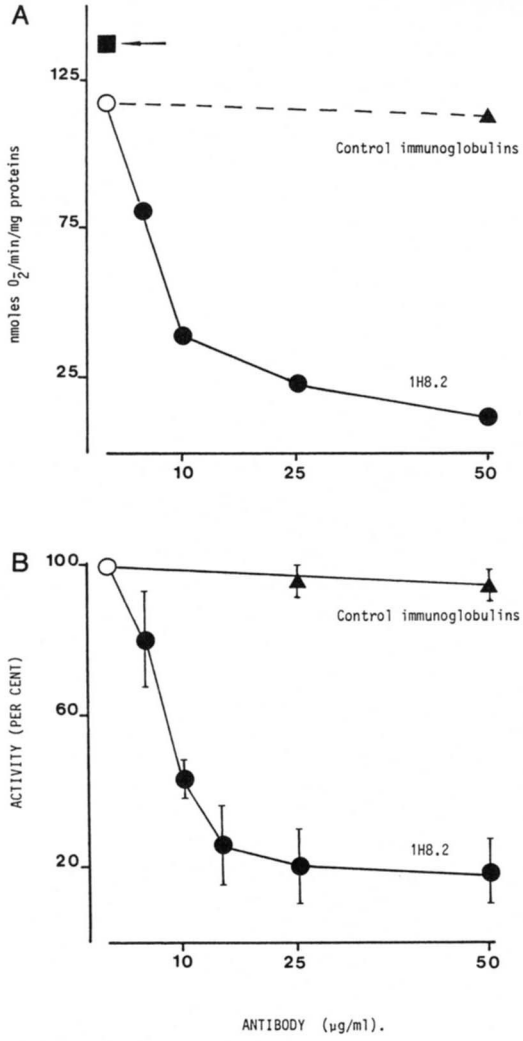

FIG. 2. **Inhibition of NADPH oxidase activity by 1H8.2.** *A*, NADPH oxidase extracted from plasma membranes of PMA-stimulated neutrophils and purified as described in Ref. 15 was diluted to 200 µg/ml in PBS and incubated with different concentrations of 1H8.2 purified by affinity chromatography. Incubation was for 1 h in ice. Duplicate aliquots of 50 µg were then assayed with concentrations of NADPH of 0.15 mM as described in Ref. 19. Mean results of duplicate assays of one experiment are reported. The symbol indicated by an *arrow* shows activity of the oxidase preparation before starting the incubation with control immunoglobulins or 1H8.2. *B*, the mean results ± S.D. of four independent experiments expressed as percent of control activity are reported. Incubations of NADPH oxidase with the antibody and assay were as described above. O_2^- generation by the oxidase preparations used and assayed after 1 h of incubation in ice in the absence of antibody (100% in the figure) were 120, 22, 40, and 62 nmol/min/mg proteins.

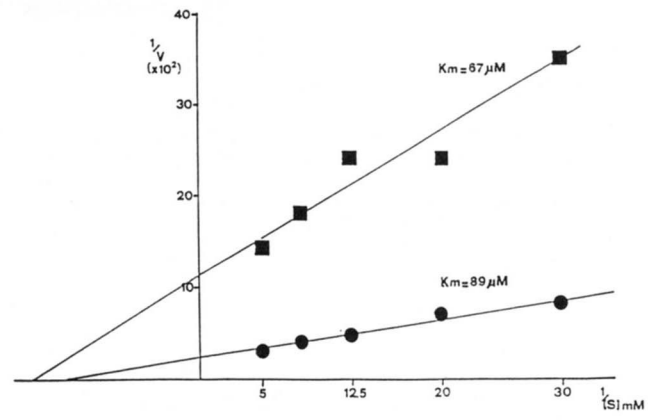

FIG. 3. **Effect of 1H8.2 on the K_m of NADPH oxidase.** NADPH oxidase was incubated as described in Fig. 2 with 10 µg/ml antibody and the activity assayed at different concentrations of NADPH as indicated. *Abscissa:* 1/NADPH, mM; *ordinate:* 1/V, nmol O_2^-/min/mg proteins.

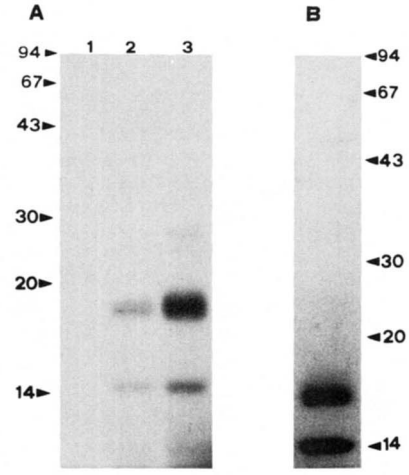

FIG. 4. **Immunoprecipitation and affinity purification of 1H8.2 antigen.** *A*, NADPH oxidase was iodinated as described under "Materials and Methods" and immunoprecipitated with 1) control mouse immunoglobulins-Sepharose; 2) 2B10-Sepharose; and 3) 1H8.2-Sepharose. The immunoadsorbent was washed free of unbound radioactivity and the bound material eluted in a SDS-containing buffer and then analyzed by SDS-PAGE and autoradiography. *Numbers on the left* indicate migration of marker proteins (from top to bottom: phosphorylase *b*, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, α-lactalbumin). The same result was obtained in four independent experiments, in one of which the Ag was precipitated with rabbit anti-mouse immunoglobulins and Protein A-Sepharose. The samples were analyzed in reducing conditions, and the same result was obtained in nonreducing conditions. *B*, NADPH oxidase solubilized from plasma membrane of DFP-treated, PMA-stimulated neutrophils was solubilized with 2% Nonidet P-40, passed through a mouse immunoglobulins-Sepharose column and then on a 1H8.2-Sepharose column. After washing of the affinity column with 0.5 M NaCl, the Ag was eluted with 0.1 M acetic acid, pH 4.0, concentrated by precipitation with acetone and analyzed by SDS-PAGE. A gel stained with Coomassie Blue is shown. *Numbers on the right* indicate migration of marker proteins.

characterized component of the NADPH oxidase.

At least 90% of the cytochrome *b* was not retained by the affinity column and another small, imprecisely quantifiable aliquot eluted during the washings of the column with high salts buffers (see "Materials and Methods"). No cytochrome *b* spectrum was detected in the antigen eluted with acetic acid and used for the electrophoretic characterization reported in Fig. 4*B*.

DISCUSSION

We have isolated a monoclonal antibody to pig NADPH oxidase which is the first so far characterized as able to block the enzyme activity. This antibody, that we have designated 1H8.2, is one of several which showed apparent selectivity of binding to partially purified preparations of NADPH oxidase but is the only one we found to inhibit oxidase activity. 1H8.2 is restricted to pig and does not react with rat, guinea pig, and human NADPH oxidase. The antibody shows higher binding to NADPH oxidase extracted from membranes of PMA-activated neutrophils than to NADPH oxidase extracted from resting cells. This could be an indication that there is also an intracellular pool of 1H8.2 Ag which is translocated to the plasma membrane after stimulation with

PMA. Alternatively, activation of NADPH oxidase with PMA could cause conformational changes in 1H8.2 Ag which facilitate interaction of antibody with appropriate epitopes.

1H8.2 blocks NADPH oxidase activity almost completely at about 50 μg/ml. Inhibition derives from a decrease in the V_{max} of the enzyme while its K_m is not affected. Conceivably, incubation of 1H8.2 with NADPH oxidase in the presence of concentration of NADPH up to 25-fold higher than the calculated K_m (see Fig. 3) did not prevent the inhibitory effect. These data indicate that 1H8.2 does not bind to an epitope belonging to the NADPH binding region. This conclusion is also supported by the observation that the antibody has no effect on the superoxide dismutase-insensitive NADPH-dependent cytochrome *c* reductase and on liver NADPH-cytochrome *c* reductase. Polyclonal antibodies to an enzyme containing a nucleotide-binding site were shown to inhibit unrelated enzymes also possessing a structural domain for the binding of NADPH, and the inhibition was prevented by NADPH (21). Antibodies to cytochrome P-450 reductase have been shown to inhibit, although by at most 30%, also NADPH oxidase (22).

1H8.2 immunoprecipitates a heterodimer consisting of two components of apparent molecular weight of 16/18 and 14 kDa (Fig. 4). The 1H8.2 Ag was characterized both by immunoprecipitation of iodinated membrane proteins and immunoaffinity purification. The affinity purified Ag appeared to consist of polypeptides staining with the same intensity with Coomassie Blue thus indicating that, assuming a similar binding of the dye, the two proteins are associated in equimolar amounts. The apparent predominance of the 16/18-kDa protein in the experiments of immunoprecipitation of iodinated material is therefore likely due to a different extent of iodination.

We do not know the nature of 1H8.2 Ag. From studies of immunoprecipitation, we believe the molecule recognized by 1H8.2 is not cytochrome *b*. Cytochrome *b* was not retained by the affinity column, and the eluted Ag did not show any cytochrome *b* spectrum. Moreover, the molecular weights reported for the purified cytochrome *b* of both human and pig neutrophils are different from that of 1H8.2 Ag. Cytochrome *b* purified from human neutrophils consists of two components of 91 and 22 kDa (5, 6). We have shown that with pig neutrophils, cytochrome *b* copurifies with a major band of 32 kDa with weakly staining components of 90–120 kDa (23).

The 1H8.2 Ag could well be a component of the NADPH oxidase or a still unrecognized molecule able to regulate the oxidase activity. Uncertainty still exists about the other components that, besides cytochrome *b*, are involved in the NADPH oxidase activity. Polypeptides of 67, 48, and 32 kDa have been found to copurify with highly enriched NADPH oxidase preparations which contain only trace amounts of cytochrome *b* (9). NADPH-binding proteins of 45 (10) and 66 (11) kDa or a 51-kDa polypeptide (12) have been claimed to represent the putative flavoprotein which transfers electrons from NADPH to cytochrome *b*.

The Ag recognized by 1H8.2 does not apparently correspond to any of these molecules. It shows, however, analogy with one of the polypeptides identified by eluting from isoelectric focusing gels a pI 5.0 band displaying NBT-reducing activity. Polyclonal antibodies to the pI 5.0 band inhibit the O_2^--generating activity of NADPH oxidase solubilized from pig neutrophil membranes by about 50% and recognize, in Western blots, proteins of 70/72, 28/32, and 16/18 kDa (24).

Studies initiated by Bromberg and Pick (25) have established that the NADPH oxidase can be activated in a cell-free system by detergents and that cytosolic factor(s) are involved in the activation. 1H8.2 does not recognize cytosolic proteins, and we therefore believe that the heterodimer of 16/18 and 14 kDa is not the cytosolic factor identified in studies of NADPH oxidase activation in cell free-systems.

1H8.2 is the first monoclonal antibody so far characterized that is able to inhibit the NADPH oxidase. The results presented in this paper establish that monoclonal antibodies can be used to identify molecules involved in the NADPH oxidase activity. Investigations are underway to define the nature of 1H8.2 Ag and its role in the function of the NADPH oxidase.

Note Added in Proof—After the submission of this paper we became aware that J. Doussiere and P. V. Vignais ((1988) *FEBS Lett.* **234,** 362–366) reported that an antiserum that inhibits the respiratory burst of bovine neutrophils binds to polypeptides of 18 and 16 kDa.

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