into consideration when one attempts to explain the historical visual observations is the visibility or contrast of the features themselves and, consequently, the reliability of visual observations in general. McGovern, Gross, and Rasool (*7*), who studied several sets of visual observations, have concluded that Mercury's rotation period is 58.4 ± 0.4 days. While we fully agree with this conclusion, we strongly question the means by which they arrived at this result, since their rotation period was strongly influenced by four pairs of Antoniadi's observations (*2*). In examining the published drawings of Antoniadi, we find other observations which are in serious conflict with three of the pairs used by McGovern *et al.* Thus, the 59-day rotation period could be obtained by selecting only those observations which tend to support it, and by ignoring those which do not. We therefore conclude that at least some of the accepted visual observations are completely inaccurate, probably because of the marginal visibility of surface features on Mercury.

Photographs of Mercury taken in red light on 25 April 1968 show a well-defined feature of nontypical conspicuousness at 240° longitude located near the center of the disk. Photometric calibration applied to the photographic plates permits an evaluation of both the apparent and intrinsic contrasts of this marking. The value for the instrinsic contrast is about 0.20, somewhat less than the dark areas on the moon (0.4) and Mars (0.3 in yellow light). However, the apparent contrast of this feature is only 0.08, because Mercury was necessarily observed through the illuminated terrestrial daytime sky. Less conspicuous markings had contrasts less than half as great, and this certainly represents marginal visibility. Typical surface features on Mercury, therefore, are quite difficult to detect by direct visual observation. It remains for high-contrast photography to provide the most reliable means for mapping the surface of Mercury.

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8. The prime meridian is defined as passing through the subsolar point at the instant of perihelion passage on 1 May 1968. The assumed 2:3 resonance between rotation period and orbital revolution would assure redefinition of the prime meridian at every other perihelion passage. We further assume that the rotation axis is perpendicular to the orbital plane.
9. We thank C. F. Knuckles, T. P. Pope, and A. S. Murrell who obtained the Mercury photographs, T. C. Bruce for programming, and the Computer Center of New Mexico State University for making CDC 3300 and IBM 1130 time available. Supported in part by NASA grants NsG-142-61 and NGR-32-003-027.

17 September 1968

Deficiency of Reduced Nicotinamide-Adenine Dinucleotide Oxidase in Chronic Granulomatous Disease

Abstract. *Reduced nicotinamide-adenine dinucleotide oxidase of normal human polymorphonuclear leukocytes has properties that would qualify it as the enzyme responsible for the respiratory burst during phagocytosis. The enzyme was deficient in leukocytes of five patients with chronic granulomatous disease. This lack of adequate reduced nicotinamide-adenine dinucleotide oxidase could be the basis for the metabolic abnormalities characteristic of these leukocytes and for their diminished bactericidal activity.*

When polymorphonuclear leukocytes phagocytize, they exhibit increased respiration, increased flow of glucose via the hexose monophosphate shunt (HMP) compared with that through the Embden-Meyerhof-Parnas pathway (*1*), and formation of hydrogen peroxide (*2*). The ingestion process itself and these metabolic concomitants are insensitive to cyanide (*1*), and the metabolic energy needed for the process has been considered to come from glycolysis (*1*).

In the phagocytic leukocytes of peripheral blood of children with chronic granulomatous disease (a genetic defect expressed as impaired intracellular killing of certain bacteria), the respiratory burst, the stimulation of glucose-6-phosphate oxidation, and formation of peroxide during phagocytosis are lacking (*3*), whereas the increase in glycolysis is normal. The leukocytes from these patients do ingest bacteria normally, but since they fail to kill many types of organisms, the patients suffer from various chronic infections (*4*). Attempts have been made to establish the reason (*4*) for the functional metabolic deficiencies in these leukocytes (*5*) and to define possible connections between these metabolic lacks and the inability of the cells to kill bacteria normally.

In any search for a key enzyme responsible for the respiratory burst of normal phagocytizing cells, the following must be kept in mind: (i) the enzyme should be insensitive to cyanide; (ii) peroxide would probably be one of its products; (iii) the enzyme should be present in amounts adequate for the needs of the intact phagocytizing cell; and (iv) a relation between the respiratory enzyme and the hexose monophosphate pathway should be evident. There is evidence that the hexose monophosphate shunt itself is unimpaired in the patients' cells (*3*). Depression or lack of a terminal respiratory enzyme, which could be linked to the lack of bursts of respiration and HMP activity during phagocytosis, would not only indicate a key biochemical lesion in chronic granulomatous disease but would also help to establish that the enzyme is indeed responsible for the oxidative stimulations when normal cells phagocytize.

Recently, a flavoprotein enzyme, reduced nicotinamide-adenine dinucleotide (NADH) oxidase, was isolated and characterized from the soluble fraction of homogenates (prepared in isotonic KCl) from guinea pig peritoneal granulocytes (*6*). The enzyme is specific for NADH, and the products formed are hydrogen peroxide and nicotinamide-adenine dinucleotide (NAD^+) in equimolar amounts (*6*). The enzyme is insensitive to cyanide and is present, at least in guinea pig polymorphonuclear leukocytes, in adequate amounts to cover the respiratory burst during phagocytosis. This enzyme thus would satisfy points (i), (ii), and (iii) above. Evidence is available which links this enzymatic activity (that is, NAD^+ pro-

duction) to the hexose monophosphate pathway, control of which is determined by availability of nicotinamide-adenine dinucleotide phosphate ($NADP^+$) (*7*). The linkage has been shown to be via transhydrogenation by lactate dehydrogenase (*7*), and also via glutathione reductase (*8*), to satisfy point (iv).

A reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase insensitive to cyanide and confined to the granule fraction of 0.34*M* sucrose homogenates obtained from guinea pig peritoneal leukocytes has also been reported (*9*). The linkage of this enzyme to the hexose monophosphate pathway would be more direct than that of the NADH oxidase mentioned above, but no data have been provided as to the quantitative importance of this enzyme.

We report here quantitative determinations of both oxidase enzymes in human peripheral blood leukocytes and compare these activities to the respiratory burst in intact cells. The data bear on point (iii) above. Finally, we offer our results on NADH oxidase activity in the peripheral blood leukocytes from children with chronic granulomatous disease. This enzyme appears to be deficient in the patients' cells, and this lack could well be responsible for the metabolic abnormalities. We also suggest that it is this same enzymatic lack that leads to the depression of bactericidal activity in these cells.

Leukocyte respiration was measured manometrically in Warburg flasks (micro size) containing a final volume of 1 ml of 0.154*M* Krebs-Ringer phosphate salt solution (*p*H 7.4) and 3.0 to 6.0 $\times$ 10^7 leukocytes (3.0 to 5.5 mg of cellular protein). The respiratory burst, measured after addition of 0.1 ml of well-dialyzed latex particles (*10*), was 1.4 μl (63 nmole) of oxygen per hour for each milligram of cellular protein at 37°C.

Because Cagan (*6*) had found that the Michaelis constant (K_m) for NADH oxidase was 1.0 m*M* with respect to NADH, this enzyme was measured at 37°C in cuvettes (1-mm light path) containing a final volume of 0.4 ml of 0.05*M* K_2HPO_4–KH_2PO_4 buffer (*p*H 7.0), 1m*M* KCN, and 2.5 m*M* NADH, final concentration. There was almost enough enzyme activity recovered to explain the respiratory burst, when one considers the optimum *p*H for this type of enzyme (*p*H 5.0), which may obtain intracellularly during phagocytosis. Because of lack of sufficient material, we do not yet have data on the kinetic properties of the enzyme from human cells. Initial experiments suggest that the enzyme from human cells has similar properties to those of the guinea pig enzyme on which exhaustive studies have been made. If one considers that some losses must have occurred in handling the small amounts of material during homogenization and centrifugation, coupled with the point made in the previous sentence, we come within sight of satisfying point (iii) previously outlined.

Table 1. Reduced nicotinamide-adenine dinucleotide oxidase (nanomoles of oxygen per milligram of total cell protein per hour) of human polymorphonuclear leukocytes. Five patients were studied. All exhibited the clinical manifestations of the disease, and their polymorphonuclear leukocytes were deficient in the quantitative dye test which is diagnostic (*5*). The six controls were patients with other ailments. There were no differences between the two groups with respect to the proportions of cell types in the blood.

Control	Disease
33.6	11.1
20.4	5.2
57.9	10.6
21.0	8.2
18.0	1.7*
25.5	
29.5 $\pm$ 6.1	7.4 $\pm$ 1.7
$P < .01$	

* Female patient.

The cyanide-insensitive NADPH oxidase was assayed in the granule and supernatant fractions of human leukocyte homogenates in 0.34*M* sucrose after a 15-minute centrifugation at 30,000*g*. Assays performed spectrophotometrically (*11*), as well as with the oxygen electrode (*12*) indicate that enzyme activity was confined to the granule fraction. This activity is far less than that necessary for the respiratory burst, when measured either at 0.1 or 7 m*M* NADPH. It was certainly much lower than the NADH oxidase activity.

The data that pertain to these points, obtained for polymorphonuclear leukocytes from four human subjects, are summarized as follows: Q_{O_2} (microliters of oxygen per milligram of total cell protein per hour) of resting cells is 1.1; that of phagocytizing cells is 2.5. The difference, that is, respiratory stimulation during phagocytosis, may be expressed as 63 nanomoles of oxygen per milligram of total cell protein per hour. The total NADPH oxidase activity, measured at *p*H 7.0, is 4.9 nanomoles of oxygen per milligram of total cell protein per hour. At the same *p*H, NADH oxidase activity is 30. If the enzyme from human cells has a *p*H optimum profile similar to that of guinea pig cells, its activity at *p*H 5.0 (which may be closer to that of the intracellular milieu, especially during phagocytosis) would be double that at *p*H 7.0. Thus, the true available NADH oxidase activity may be in the vicinity of 60 nanomoles of oxygen per milligram of total cell protein per hour.

Since NADH oxidase rather than NADPH oxidase is more likely to be the primary enzyme involved in the cyanide-insensitive respiratory burst during phagocytosis, the lack of this enzyme or the inhibition of its activity could be the basis for the oxidative defect in the leukocytes of patients with chronic granulomatous disease. Accordingly, we determined the NADH oxidase activity in supernatants (30,000*g*) of homogenates of leukocytes in alkaline KCl (*6*). The cells were obtained from four male children and one female child with chronic granulomatous disease. We concluded that these leukocytes have a significant deficiency of NADH oxidase activity (Table 1) (*13*).

In the guinea pig polymorphonuclear leukocytes, the NADH oxidase produces H_2O_2 (*6*). If the enzyme of human cells is analogous to the guinea pig enzyme, depression of this oxidase in patients with chronic granulomatous disease would result in depression of H_2O_2 formation; indeed, a depression of H_2O_2 was noted in such phagocytizing cells (*3*). This situation could cause the depressed ability of chronic granulomatous disease cells to kill bacteria, a possibility suggested by the findings of Klebanoff *et al.* (*14*) regarding the role of H_2O_2 in bactericidal activity of leukocytes. Klebanoff *et al.* (*14*) have linked H_2O_2, myeloperoxidase, and a halide in this function of polymorphonuclear leukocytes. We therefore suggest that the depressed NADH oxidase activity we have demonstrated in chronic granulomatous disease cells is an important, and perhaps the cardinal, deficiency in the disease. These findings help to establish a connection between the metabolic events of phagocytosis and the important functional role of polymorphonuclear leukocytes, that is, their bactericidal activity.

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11. Spectrophotometric assays of NADPH oxidase were carried out at 37°C in cuvettes with a 1-cm light path, in a final volume of 1 ml; 0.1*M* phosphate buffer (*p*H 7.0) and a final concentration of 1 m*M* KCN; 0.1 m*M* NADPH; granule enzyme, 1 mg of protein.
12. Oxygen electrode assays of NADPH oxidase were carried out with a Clark membrane electrode (Instrumentation Laboratory, Inc., Boston) and a circulating water bath electrode chamber. The final volume was 1.3 ml of 0.1*M* phosphate buffer, *p*H 7.0 at 37°C. Final concentrations of KCN were 1 m*M*; 7 m*M* NADPH; granule enzyme, 2 mg of protein.
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15. Supported by PHS grants AI-03260, AI-08173, AM-37-26302, and by the John A. Hartford Foundation.

6 September 1968

Human Osteosarcomas: Immunologic Evidence Suggesting an Associated Infectious Agent

Abstract. *Immunofluorescent studies have revealed a high incidence of antibodies to osteosarcomas in the serums of patients with this disease and their close associates which react with a common antigen (or antigens) in osteosarcomas. The distribution of these antibodies suggests the association of an infectious agent with this neoplasm which is capable of producing unrecognized infections in healthy contacts of these patients.*

Although viruses have been established as oncogenic agents capable of inducing a wide variety of animal neoplasms, their role in the etiology of human neoplasia remains undetermined. This report will summarize studies on the distribution of antibodies to osteosarcoma in patients with osteosarcoma, their immediate families, and their close associates which suggests the association of an infectious agent with this neoplasm.

Serums randomly selected from four patients with osteogenic sarcoma, 14 members of their immediate families, four close associates of one patient, and 25 normal blood-bank donors have been tested by the indirect immunofluorescence technique for antibodies against four different osteosarcomas. Imprints of osteogenic sarcoma tissue were made on glass slides and stored in a liquid-nitrogen refrigerator until used in the immunofluorescence tests with techniques that have been reported (*1*).

When the previously frozen tumor imprints were used in this test they were fixed in acetone for 10 minutes and allowed to dry; then a drop of test serum was added. The slide was incubated at room temperature for 20 minutes in a moist chamber. Smears were washed twice in saline and a drop of properly diluted horse antiserum to human globulin, conjugated to fluorescein isothiocyanate and previously absorbed with porcine liver powder, was added for another 20 minutes. The smears were again washed twice, mounted in 50 percent glycerin in saline, and examined with a fluorescent microscope. Serums were routinely tested at dilutions of 1:10, 1:100, and 1:300 and only at the higher dilutions if they were positive at 1:300.

The direct immunofluorescent technique was used to study the reactivity of globulins from two patients against their own osteosarcomas (patients B and C). Globulins were precipitated from serum with ammonium sulfate and conjugated to fluorescein isothiocyanate. The labeled globulins were then tested for their reactivity against osteosarcomas by allowing a drop of these conjugated antiserums to incubate on the tumor imprint slide for 30 minutes, after which the slide was washed twice and examined. Blocking studies were performed by incubating the smears with unlabeled serum, washing, and then adding the labeled globulin. This was allowed to incubate for 30 minutes, after which the slide was washed twice and examined.

Results of this study are summarized in Tables 1 and 2. Each patient's serum was found to react not only with his own osteosarcoma but also with the tumors of other patients (Table 1). The titer of reactivity varied between 1:100 and 1:600 (Table 2). Some serums (particularly those of high titer) showed prominent prozones that were negative when tested undiluted or at a 1:10 dilution, but that became strongly positive at dilutions of 1:100 or 1:300. The immunofluorescence staining was principally limited to the cytoplasm and cell membrane, but faint nuclear staining was also observed (Fig. 1). The specificity of these immunofluorescence reactions was studied by testing high-titer serum from the four osteosarcoma patients at dilutions of 1:10, 1:100, and 1:300 against human neoplasms of other types (a melanoma and adenocarcinomas of the breast and colon). No reactions were observed between serums from osteosarcoma patients and these three neoplasms.

A high incidence of immunofluorescence antibodies was also demonstrated in serums from members of the immediate family (85 percent) and the serums from close associates of patient B (91 percent). The tests of serums from normal blood-bank donors revealed a significantly lower

Table 1. Incidence of antibodies against four different osteosarcomas in the serums of patients, their relatives, close associates, and normal blood-bank donors. Results are expressed as No. positive/No. tested.

Type of serums	Osteosarcoma from patient A	Osteosarcoma from patient B	Osteosarcoma from patient C	Osteosarcoma from patient D	Total	Percent positive
Osteosarcoma patients	2/2	4/4	3/3	4/4	13/13*	100
Patients' family members	5/11	7/7	8/8	14/14	34/40*	85
Associates of patients	1/1	4/4	3/3	3/4	11/12*	91
Blood-bank donors	1/10	4/19	8/25	7/16	20/70	29

* Incidence of reactivity is significantly different from blood-bank donors ($P < .01$).