Demonstration by the Nadi Reaction of Cytochrome Oxidase Activity in Cells of the Lymphoid and Myeloid Series Obtained from Normal Individuals and Patients Suffering from Hodgkin's and Other Diseases

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DURING THE PAST TEN YEARS numerous investigators have studied the cytochrome oxidase activity of a wide variety of tissues, principally by manometric methods. While the manometric method is specific and quantitative, it has one disadvantage. If the tissue under investigation is not homogeneous it is impossible to relate the quantitative data to the oxidase activity of the various cellular elements present. This difficulty presented itself during a collaborative investigation of the cytochrome system in lymphoid tissue[13, 21] and prompted us to explore the possiblities of a histochemical method utilizing the Nadi reaction.

The Nadi reaction consists of the simultaneous oxidation of a mixture of dimethyl-p-phenylene diamine and alpha naphthol and subsequent condensation of the initial oxidation products, insoluble indophenol blue being the end product. While the reaction appears to proceed spontaneously in the presence of molecular oxygen (auto-oxidation) its rate is greatly increased by the presence of indophenol oxidase, which is presumably identical with Keilin's cytochrome oxidase.[12, 22] Since cytochrome c is considered to be the exclusive substrate of cytochrome oxidase, the accelerating effect of the oxidase on the Nadi reagent must be mediated by cytochrome c. The real oxidant, then, would be ferricytochrome c rather than molecular oxygen. Since the Nadi reagents are present in excess, the limiting factors in the production of indophenol blue are the amounts of cytochrome oxidase and cytochrome c present in the cells. The staining reaction therefore demonstrates the sites of, and roughly quantitates the amount of, cytochrome activity.

Although the indophenol blue reaction has been known since 1885,[6] when Ehrlich used the Nadi mixture in the demonstration of oxidative cell functions, it has been used but infrequently as a histochemical procedure.[7, 15, 16]

In tissue sections the reaction has been found to be capricious due to the fact that much oxidase activity is lost in preparing frozen or paraffin sections and localization is made impossible because of rapid diffusion of the dye. Both of these difficulties were circumvented by applying the reaction to free cells in wet smears and thus obtaining a cytochemical preparation suitable for observation with oil immersion objectives. The sites of cytochrome oxidase activity in each of the various cell types could thus be visualized separately.

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Supported in part by grants from the National Cancer Institute of the U.S. Public Health Service, the American Cancer Society, and the Damon Runyon Memorial Fund.

Submitted March 2, 1951; accepted for publication April 18, 1951.

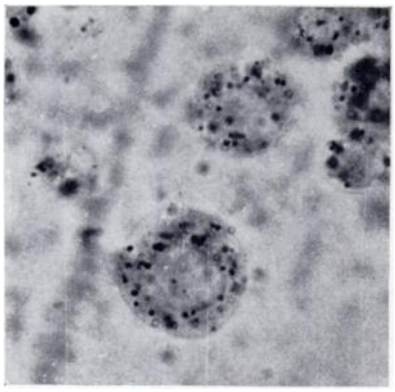
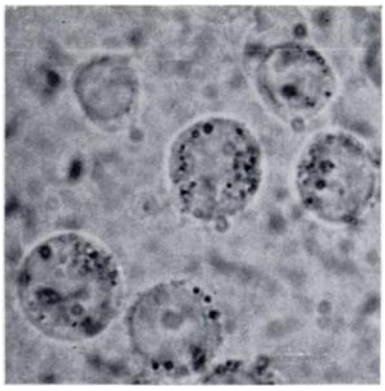


FIG. 1



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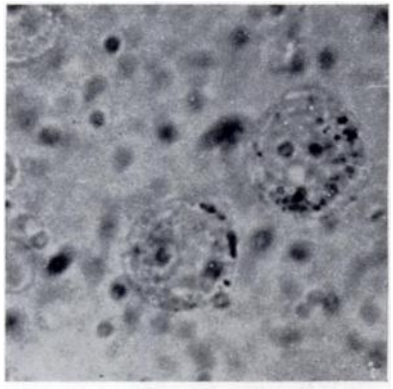

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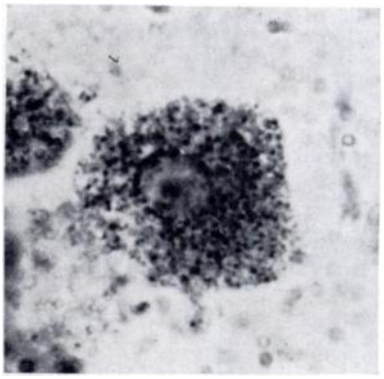

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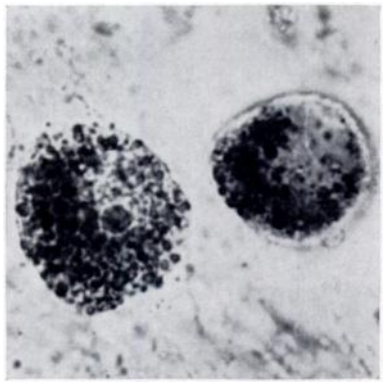

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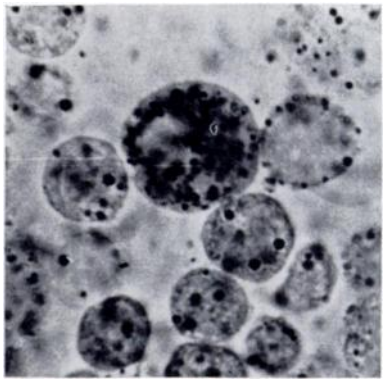

FIG. 6

FIG. 1.—Nadi-positive granules in cells from lymph nodes and blood. Photographed by bright field light (×900.) Large lymphocytes from lymph node showing simple lymphoid hyperplasia.

FIG. 2.—Lymphocytes from a node showing lymphosarcoma.

FIG. 3.—Cells from buffy coat of blood of patient with lymphatic leukemia.

FIG. 4.—Tubular epithelial cell from normal human kidney.

FIG. 5.—Macrophages from a lymph node.

FIG. 6.—Cells from a node showing Hodgkin's disease. Large cell is a monocyte or free reticulum cell with a lymphocyte to the right of it. Other cells are polymorphonuclear leukocytes.

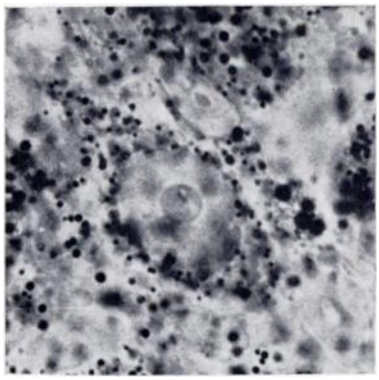

FIG. 7

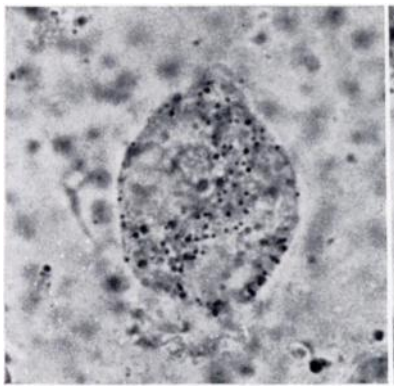

FIG. 8

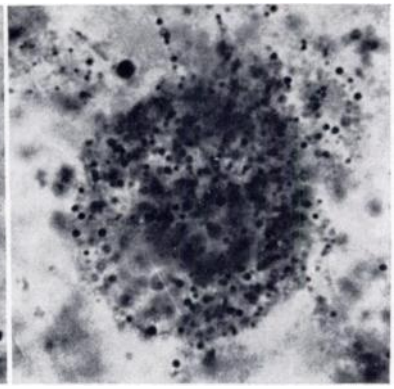

FIG. 9

FIG. 7.—Large reticulum cell from node showing Hodgkin's disease; note large nucleolus. Granules outside cell come from ruptured cells in vicinity.

FIGS 8 and 9.—Sternberg-Reed cells from node showing Hodgkin's disease.

MATERIALS AND METHODS

The material consisted of cells from normal and diseased lymph nodes removed by surgical biopsy and included those from cases of Hodgkin's disease, lymphosarcoma, leukemia and tuberculous lymphadenitis; cells from normal spleens and spleens of patients with Hodgkin's disease, lymphsarcoma and myeloid metaplasia; cells of normal blood and bone marrow and the blood and marrow of patients with myelogenous and lymphatic leukemia. Cells from pleural and peritoneal transudates were also examined.

Comparisons were made with tissues known to have a high content of cytochrome oxidase such as myocardium, liver and kidney from fresh autopsy material. All the cells studied were of human origin.

In tissues such as lymph nodes and spleen, free cells can be obtained from the freshly cut surface by squeezing the tissue and expressing a cellular fluid. Cells from other tissues such as muscle, liver and kidney may be obtained by macerating a small piece of tissue with scissors and dissecting needles. In normal and leukemic bloods and bone marrow aspirations the buffy coat was used and cells from transudates were also concentrated by centrifugation.

The Nadi reagent was prepared by dissolving 2 mg. of dimethyl and paraphenylenediamine in 1 ml. of distilled water and adding equal parts of freshly prepared, saturated, aqueous solution of alpha naphthol and 0.1 M. phosphate buffer at pH 7.4. The p-phenylene diamine was purified by sublimation in vacuo. This sublimate is faintly yellow and when

dissolved in buffer gives a faint pink color. In testing for azide inhibition phosphate buffer at pH 5.4 was used with .005 M. sodium azide. The acid buffer alone did not appreciably affect the intensity of staining reaction but slowed it down so that maximum intensity was not reached for 15 to 20 minutes.

One drop of each of the solutions was placed on a slide and mixed by gentle stirring with the cellular material to be examined. Stirring for 2 to 3 minutes helps to oxygenate the material and spread the cells evenly throughout the solution. A coverslip is then applied and gently pressed down to give a thin layer of cells floating in the fluid.

The identification of cell types in such a preparation is simplified by the use of a microscope which allows rapid shifting from phase contrast to bright field light without moving the specimen or objective. Our own[9] experience and that of others[1-3, 14] in phase contrast examination of unstained wet smears of many tissues has shown that a large number of cell types can be identified with as much accuracy as a stained smear would afford. One can identify lymphocytes, eosinophiles (by the size and refractility of the granules), polymorphonuclear neutrophiles, plasma cells, monocytes, macrophages, free reticulum cells, myelocytes and mesothelial cells in the type of material under discussion.

Results

In all the cell types examined, round, bright blue granules varying from 0.5 to 2.0 μ in diameter could be seen after treatment with the Nadi reagent. These were always round and always located in the cytoplasm. The maximum effect was usually reached within 10 minutes of the time of mixing cells and stain. The granules remained distinct and there was never any diffusion within the cells. No nuclear staining was ever observed. Granules often lay over the nucleus but fine focusing showed that they were in the cytoplasm, superimposed over the nucleus. In the presence of azide there was no observable staining within the first hour. After that time blue coloration developed in the solution. Cyanide inhibited the reaction completely for 90 minutes or more.

The number of stained granules varied considerably in the different cell types, the variation being roughly parallel to that found by other workers doing quantitative manometric experiments. Cells of cardiac muscle, liver and kidney were loaded with granules, while the various cells of blood and lymphoid tissue contained comparatively few.

Lymphocytes from normal lymph nodes and blood and from the nodes and blood of patients with Hodgkin's disease, leukemia, lymphosarcoma and tuberculous lymphadenitis contained some 5 to 20 round, deep blue, cytoplasmic granules. There was no appreciable quantitative difference in the staining reaction of the lymphocytes in the various diseases mentioned.

Polymorphonuclear leukocytes usually showed a smaller number of stained granules than lymphocytes but the difference was not marked. Myelocytes from normal bone marrow showed slightly more activity than mature leukocytes and myelocytes from a case of myelogenous leukemia had about the same activity as normal myelocytes.

Monocytes from blood, lymph nodes and spleen contained numerous granules, the activity being much more intense than that in lymphocytes.

Free reticulum cells from normal lymph nodes and spleen and lymph nodes from patients with Hodgkin's disease showed marked variation in the number of stained granules. In some the cytoplasm was loaded, while in others only a few

could be seen. Under observation by phase contrast no other cytologic differences between the cells could be established. In one case of Hodgkin's sarcoma the abnormal reticulum cells stained rather evenly, each containing 2 to 4 granules. Epitheloid cells from a node showing tuberculous adenitis did not contain any granules, while lymphocytes in the same preparation showed the usual activity.

In macrophages from lymph nodes and spleen there were both the small 0.5 to 2.0 μ granules and the larger ones measuring up to 4 μ in diameter. These latter were comparable in size and distribution to the fat vacuoles found in these cells.

Megakarocytes from a case of myeloid metaplasia of the spleen did not show any activity nor did those from normal bone marrow aspirations. Blood platelets did not show any activity.

Mesothelial cells from pleural and ascitic transudates showed very little activity considering the large amount of cytoplasm these cells contain.

The cytochrome oxidase activity in Sternberg-Reed cells from lymph nodes varied considerably. In some there was no staining at all, while in others the cytoplasm was loaded with blue granules.

In view of the fact that recent studies of the cytochrome oxidase content of various fractions of disrupted cells separated by ultracentrifugation indicate that this enzyme system is concentrated in the large granule (mitochondrial) fraction,[4, 10, 11, 19] we attempted to relate the granules visualized by the Nadi reaction to mitochondria. Since mitochondria are visible by phase contrast in unstained cells, there should be a correlation between the phase contrast appearance of granules in unstained cells and the blue granules seen by bright field light in stained material.

In none of the material examined did the number of blue granules in stained cells exceed the number of granules which were viable by phase contrast in unstained cells when the phase preparation was considered adequate. This cannot be appreciated, however, in any but the thinnest smears in which the cells are still viable, widely separated and surrounded by a fluid medium of the proper optical density. Smears from lymph nodes illustrate this point. In a wet smear made from fluid expressed from a lymph node it is very difficult to see any cytoplasm in the lymphocytes because of crowding of the cells. In cells which float freely in the moving channels of fluid between clumps of closely packed cells one can see a good rim of cytoplasm. In a thin smear of peritoneal or pleural fluid all the lymphocytes will show a cytoplasmic rim containing round granules and slightly elongated rods which correspond in number and location to those stained by the Nadi reaction. The stained granules are, however, always round. This may be due to variations in the normal osmotic relationships induced by the staining fluid. The granules seen by phase contrast in unstained cells also correspond in number and location to those bodies (mitochondria) which can be stained supravitally by janus green. Our observations indicate that the cytoplasmic granules which are seen in cells stained by Nadi reagent exist in a visible form in unstained cells, correspond in size to the "large granules" in ultracentrifuge fractions, and correspond in number and location to the particles which can be stained supravitally by janus green.

Discussion

All the cells of the lympho-reticulo-endothelial and myeloid systems studied by this method showed some degree of cytochrome oxidase activity. The activity of these cells is very slight, however, when compared with that of cardiac muscle, liver and kidney cells. This is in essential agreement with the results of manometric experiments in which lymphoid tissue has been shown to be relatively deficient in this enzyme system.[8, 13, 20, 21]

Within the limits of the rough quantitative measure afforded by this cytochemical method, the cells in blood and lymphoid tissue may be listed in the order of decreasing activity as follows: monocytes and free reticulum cells, macrophages, myelocytes, lymphocytes and polymorphonuclear leukocytes. This order is based on the average number of stained granules observed in the cytoplasm of the various cells and presupposes that the amount of activity is roughly proportional to the number of granules which take the stain. There were no visible differences in the intensity of the coloration of individual granules.

No quantitative comparison is possible, however, between cells of a higher order of activity because the cytoplasm of these cells appears to be loaded with blue granules. Thus, there is no observable difference between the staining reaction of human kidney and liver cells and comparable cells from the organs of rats and mice. It has been shown by manometric methods that, organ for organ, the amount of cytochrome c–cytochrome oxidase activity is much greater in the smaller animals than it is in man.

The observation that leukemic cells showed the same amount of activity as their normal counterparts is supported by the findings of Greenstein et al., in manometric experiments on spleen and lymph nodes from human beings afflicted with leukemia.

The demonstration, in intact cells, that granules which can be stained by the Nadi reaction correspond in size, number and location to the cytoplasmic bodies which can be stained supravitally by janus green tends to confirm the opinion that cytochrome oxidase activity takes place in, or has some connection with, mitochondria.

According to the present views cytochrome oxidase (indophenol oxidase), in conjunction with cytochrome c and possibly other cytochrome components (a, b), represents the universal, terminal oxidation product in aerobic cells which enables them to utilize molecular oxygen for the oxidation of intermediary metabolites to carbon dioxide and water. These intermediary metabolites, e.g., lactate, pyruvate, etc. are produced by the fermentation or glycolysis of carbohydrate which may be regarded as the anaerobic, preparatory phase of cellular metabolism.[17] It is known that certain cells, e.g., anaerobic bacteria, are able to exist on fermentation alone. However, from an energetic point of view, fermentation is considerably less efficient than respiration. Hence cytochrome oxidase is considered essential for the effective utilization of carbohydrates. Its low concentration in certain cells, as demonstrated in the present experiments, may either be interpreted to mean that their energy requirements are small or that sources of energy other than that derived from the oxidation of carbohydrates are available to these cells, e.g., energy-rich phosphate bonds in the form of

adenosine triphosphate. No experimental evidence seems to be available concerning this question.

Summary

Using the Nadi reaction, cytochemical studies of the cytochrome oxidase activity of cells of lymphoid and myeloid tissue were carried out. Normal cells and those from patients with Hodgkin's disease and leukemia were examined.

With the exception of monocytes and macrophages cells from lymph nodes, spleen, bone marrow and peripheral blood show a low level of cytochrome activity when compared with myocardial, liver and renal tubular epithelium.

Leukemic cells and those from lymph nodes affected by Hodgkin's disease contain about the same degree of cytochrome activity as their normal counterparts, under the conditions of this study.

The cytoplasmic particles stained by the Nadi reaction correspond in size, distribution and number to particles which can be stained supravitally by janus green.

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