HISTOCHEMICAL DEMONSTRATION OF DEHYDROGENASE ACTIVITY IN THE CELLS OF NORMAL HUMAN BLOOD AND BONE MARROW

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ABSTRACT

Endogenous and succinic dehydrogenase activity was demonstrated in the living cells of normal human blood and bone marrow using a buffered nitro BT–succinate incubating solution. With this technique dehydrogenase activity was localized primarily in the granular leukocytes and the sites of enzymatic activity appeared to be non-mitochondrial. The addition of a non-ionic surface active agent to the incubating solution resulted in marked differences in the cellular and intracellular localization of dehydrogenase activity. With this method it was possible to demonstrate dehydrogenase activity in the mitochondria of most of the formed elements of the blood and bone marrow, including developing granulocytes and erythroid cells, agranulocytes, and blood platelets. Mature erythrocytes also exhibited a minimal dehydrogenase reaction with this procedure. This investigation indicated that in order adequately to demonstrate and evaluate dehydrogenase activity in the cells of the blood and bone marrow it was necessary to have increased cellular and mitochondrial permeability, as well as partially viable cells with an intact dehydrogenase system.

Few histochemical studies have been reported concerning the localization of dehydrogenase activity in the cells of the blood and bone marrow (1, 8, 14, 15, 17). Wachstein's studies (14, 15) indicated the presence of dehydrogenase activity in many of the cellular elements of normal and abnormal blood, bone marrow, and lymphatic tissue, as well as in exudates, by the use of a buffered neotetrazolium (2,2′-(*p*-diphenylene)-bis(3,5-diphenyl) tetrazolium chloride) incubating medium. These studies indicated further that the reaction was improved by the addition of sodium succinate or various activators to the incubating solution. Wachstein reported (14) the presence of dehydrogenase activity in 62 to 84 per cent of the granulocytes of the blood while only a small number of the circulating lymphocytes and monocytes exhibited enzyme activity. In contrast with the lymphocytes of the blood nearly half of the lymphocytic cells present in lymphatic tissue exhibited dehydrogenase activity (14). Other workers (3, 4, 12) have successfully demonstrated succinic dehydrogenase activity in the lymphocytic cells of lymphatic tissue employing various tetrazolium salts. The degree of reactivity of the lymphocytes of both blood (1, 14) and lymphatic tissue (3, 4) has been reported to vary in certain hematological diseases.

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Wachstein (14, 15) observed dehydrogenase activity in developing granulocytes (myelocytes) in human bone marrow but was unable to demonstrate activity in the more immature myeloblasts. He noted the apparent inverse relationship between the presence of mitochondria and histochemically demonstrable dehydrogenase activity in developing granulocytes and suggested (14) that this observation might be related to differences in aerobic and anaerobic metabolism reported in leukocytes (2).

Recently, De Souza and Kothare (8) localized succinic dehydrogenase activity in the cytoplasm of human granulocytes using the nitro BT (2,2′-di - *p* - nitrophenyl - 5,5′ - diphenyl - 3,3′ - (3,3′-dimethoxy-4,4′-biphenylene) ditetrazolium chloride) salt. These investigators noted the presence of endogenous dehydrogenase activity in the granulocytes but obtained a more intense reaction following the addition of succinate to the medium. Yakaitis (17, 18) using an incubating solution consisting of INT (2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride) dissolved in *N*,*N*-dimethyl formamide and phosphate buffer, pH 7.5, and in the absence of succinate or other substrates noted that the leukocytes of infant and old mice exhibited less dehydrogenase activity than those of young adult mice. In addition leukocytes of healthy adult mice of the AKR-susceptible strain exhibited a greater dehydrogenase reaction than the leukocytes from two resistant strains.

It was the purpose of this investigation to define the localization of dehydrogenase activity in the cells of normal human blood and bone marrow employing nitro BT as the tetrazolium salt (12). Two methods will be described and differences obtained in enzymatic localization will be discussed.

MATERIALS AND METHODS

Method A

Vital preparations were prepared in the following manner. A small drop of fresh blood was mixed on a coverslip with a drop of incubating solution consisting of 2.6 mg./ml. of sodium succinate and 1 mg./ml. of nitro BT[1] in 0.1 M Sorensen's phosphate buffer, pH 7.4. The coverslip was then inverted on a slide and the preparation examined with the bright-field and/or phase contrast microscope. Blue-purple deposits of formazan, indicative of sites of dehydrogenase activity, began to appear after 10 minutes and a maximal reaction was obtained between 30 and 60 minutes. Optimal incubation time was about 30 minutes. Longer periods of incubation usually resulted in the growth of the intracellular formazan deposits and a loss of cellular motility and viability. Controls were made in the same manner except that succinate was omitted or the enzyme inactivated with heat, iodoacetic acid, and ethyl maleimide.

Comparable studies were performed in test tubes using larger quantities of blood (heparinized) and incubating solutions in order to compare the effect of various buffers and suspending solutions, as well as the effect of enzymatic inactivation on the dehydrogenase reaction and sites of intracellular localization. Either vital films or air-dried films prepared from the above mixture of blood and media fixed with formalin vapor and mounted with glycerin were prepared and examined microscopically. 0.1 M tris buffer, pH 7.4, caused slight cellular damage; albumin (6 per cent) and polyvinylpyrrolidone (6 and 7.5 per cent) resulted in marked clumping and subsequent death and destruction of granulocytes and platelets. Sorensen's phosphate buffer, pH 7.4, was considered the best suspending medium and gave excellent preservation of cellular morphology, viability, and motility; produced no cellular clumping and gave reproducible histochemical reactions. Heat, iodoacetic acid, and ethyl maleimide completely inhibited the tetrazolium reaction.

Method B

This method differed from Method A only in the addition of a non-ionic surface active agent to the incubating solution. This agent served to increase cellular permeability permitting the access of the tetrazolium salt and/or substrate to the cell. The incubating solution consisted of 2.6 mg./ml. of sodium succinate, 1 mg./ml. of nitro BT in 0.1 M Sorensen's phosphate buffer, pH 7.4, and 1 drop/ml. of renex 20.[2] Maximal reactions developed between 30 and 60 minutes. Since these were accompanied by cellular damage, constant microscopic observation of the preparation was desirable. Control procedures included omission of succinate and/or the surface active agent, as well as enzymatic inactivation by heat, iodoacetic acid, and ethyl maleimide.

RESULTS

When Method A was used nearly all the

[1] Dajac Laboratories, Philadelphia. A highly purified sample of nitro BT prepared by and supplied through the courtesy of Dr. A. M. Seligman also was tested. Both preparations yielded similar results in these studies.

[2] Renex 20 was obtained from the Atlas Powder Co., Wilmington.

neutrophils of the blood exhibited a positive dehydrogenase reaction even in the absence of succinate (endogenous dehydrogenase activity), but these cells were somewhat more reactive when succinate was used in the incubating solution. The localization and distribution of enzyme activity indicated by the deposition of formazan showed pronounced variations in the neutrophils. Usually, the first appearance of formazan within the neutrophil was in the region of the cytocentrum where it showed as small blue-purple granules, as small rod-like deposits, or occasionally as a non-particulate diffuse blue-purple coloration. After 15 to 30 minutes' incubation formazan deposits appeared as moderate-sized granules or crystal-like deposits in some of the neutrophils (Figs. 1 and 2). The ameboid activity of the cells permitted the formazan deposits to assume a random distribution throughout the cytoplasm. Many neutrophils showed a blue-purple coloration localized in their specific (neutrophil) granules (Fig. 3). In some instances, all of the neutrophil granules were colored, while in other cells only a variable number of these granules were colored. In addition, some neutrophils exhibited a diffuse, non-particulate blue-purple coloration of their cytoplasm (hyaloplasm) with little or no particulate formazan localization in the neutrophil granules or hyaloplasm (Fig. 4).

Eosinophils usually were reactive and exhibited a diffuse, non-granular blue-purple coloration of their cytoplasm (Fig. 5) which made its first appearance in the region of their prominent cytocentrum. Few eosinophil granules exhibited dehydrogenase activity. Although monocytes were almost uniformly non-reactive for dehydrogenase activity with this technique, a few monocytes exhibited a blue-purple deposition of formazan around the cytocentrum (Fig. 6). These deposits were shown to be localized in the so-called segregation vacuoles that can be seen in monocytes stained by the supravital (neutral red and Janus green) technique, as well as in unstained vital films examined with the phase microscope. Mitochondria were non-reactive.

Only extremely rarely did lymphocytes exhibit a positive reaction with the nitro BT (Method A) reaction. In these instances, the lymphocytes appeared slightly damaged morphologically and the formazan coloration was localized in some of their mitochondria and in their "neutral red" vacuoles. Platelets and erythrocytes were consistently non-reactive with this technique.

In the bone marrow, a few neutrophil and eosinophil myelocytes (Fig. 7) exhibited a positive dehydrogenase reaction with the deposition of formazan localized near the cytocentrum. This reactivity appeared both as a local coloration of the specific granules and as a diffuse coloration of the cytoplasm (hyaloplasm) in this region. Developing erythroid elements occasionally showed a blue-purple staining of a small number of their cytoplasmic vacuoles which were shown to correspond to the neutral red staining vacuoles seen in routine supravital films. Mitochondria, identified by Janus green B and phase microscopy, were consistently unstained in both developing erythroid and myeloid elements. Megakaryocytes were usually non-reactive but occasionally exhibited a minimal reaction in the form of a finely granular deposition of formazan in their cytoplasm. Only rarely did plasma cells yield a positive dehydrogenase reaction and in these instances activity was localized to their cytoplasmic vacuoles rather than to their mitochondria.

About two-thirds of the lymphocytes present in normal human lymph nodes, and in nodes obtained from rats, exhibited a positive dehydrogenase reaction with sites of activity localized in their mitochondria. Lymphoblasts were positive but less reactive than the more mature, medium-sized, and small lymphocytes. Many lymphocytes were damaged during the preparation of the cell suspensions, but lymphocytes exhibiting a positive dehydrogenase reaction showed only minimal signs of morphologic damage. Lymphocytes with marked cellular alterations or damage, in most instances, failed to react following this technique.

Because of the possibility that the tetrazolium salt and/or succinate was unable to pass through the plasma and/or mitochondrial membranes, a non-ionic surface active agent was added to the incubating solution in order to increase membrane permeability (Method B). Freezing and thawing was attempted initially as a method to alter cellular permeability but proved to be very destructive with immediate cellular fragmentation and dissolution. With the surface active agent–nitro BT technique (Method B) it was possible to stain the mitochondria in cellular elements of the blood and bone marrow.

When Method B was employed, the lymphocytes of the blood exhibited a rather strong formazan production which was sharply localized in their mitochondria (Figs. 8, 9). The lymphocytes exhibited only minimal signs of morphologic

damage at the time they began to stain. However, the lymphocytes and their mitochondria soon became swollen indicating cellular damage. Monocytes showed a positive dehydrogenase reaction with this technique and the blue-purple coloration appeared to be localized in both their mitochondria and, perhaps also, in some of their cytoplasmic vacuoles. Blood platelets exhibited several fine formazan deposits which possibly corresponded to their mitochondria. Mature erythrocytes also were capable of exhibiting a positive dehydrogenase reaction with the formation of three or four small formazan deposits within the cell. Formazan deposits did not make their appearance until immediately prior to erythrocyte hemolysis. In most preparations, granulocytes were rounded, lacked ameboid activity and exhibited marked Brownian movement which indicated the loss of cellular viability. These cells were non-reactive for dehydrogenase activity in contrast with their strong reactivity and viability in preparations lacking the surface—active agent. Endogenous dehydrogenase activity was observed in the mitochondria of most of the

FIGURE 1

Neutrophil containing several dark cytoplasmic deposits of formazan. Nitro BT–succinate in Sorensen's phosphate buffer (Method A). 15 minute incubation. × 1250.

FIGURE 2

A group of neutrophils. Neutrophil (*A*) is non-reactive; neutrophil (*B*) shows a number of cytoplasmic deposits of formazan; neutrophil (*C*) exhibits a strong reaction with both formazan aggregates and crystal-like deposits. Nitro BT–succinate in 6 per cent polyvinylpyrrolidone. 60 minute incubation. × 1250.

FIGURE 3

Neutrophil (*A*) is non-reactive; neutrophil (*B*) shows a positive dehydrogenase reaction localized in the neutrophil granules. Nitro BT–succinate in 6 per cent human albumin. 30 minute incubation. × 1250.

FIGURE 4

Two neutrophils showing a positive dehydrogenase reaction in the hyaloplasm and near the cell membrane. The reaction appears essentially non-particulate. Nitro BT–succinate in Sorensen's phosphate buffer (Method A). 30 minute incubation. × 1250.

FIGURE 5

Eosinophil showing a diffuse non-particulate dehydrogenase reaction localized in the hyaloplasm. Nitro BT–succinate in Sorensen's phosphate buffer (Method A). 30 minute incubation. × 1250.

FIGURE 6

Monocyte with formazan deposition localized in the region of the cytocentrum. Nitro BT–succinate in Sorensen's phosphate buffer (Method A). Phase contrast microscope. 45 minute incubation. × 1250.

FIGURE 7

Eosinophilic myelocyte with a diffuse non-particulate dehydrogenase reaction in the prominent cytocentrum and also a coloration of the eosinophil granules near this region. Nitro BT–succinate in Sorensen's phosphate buffer (Method A). 30 minute incubation. × 1250.

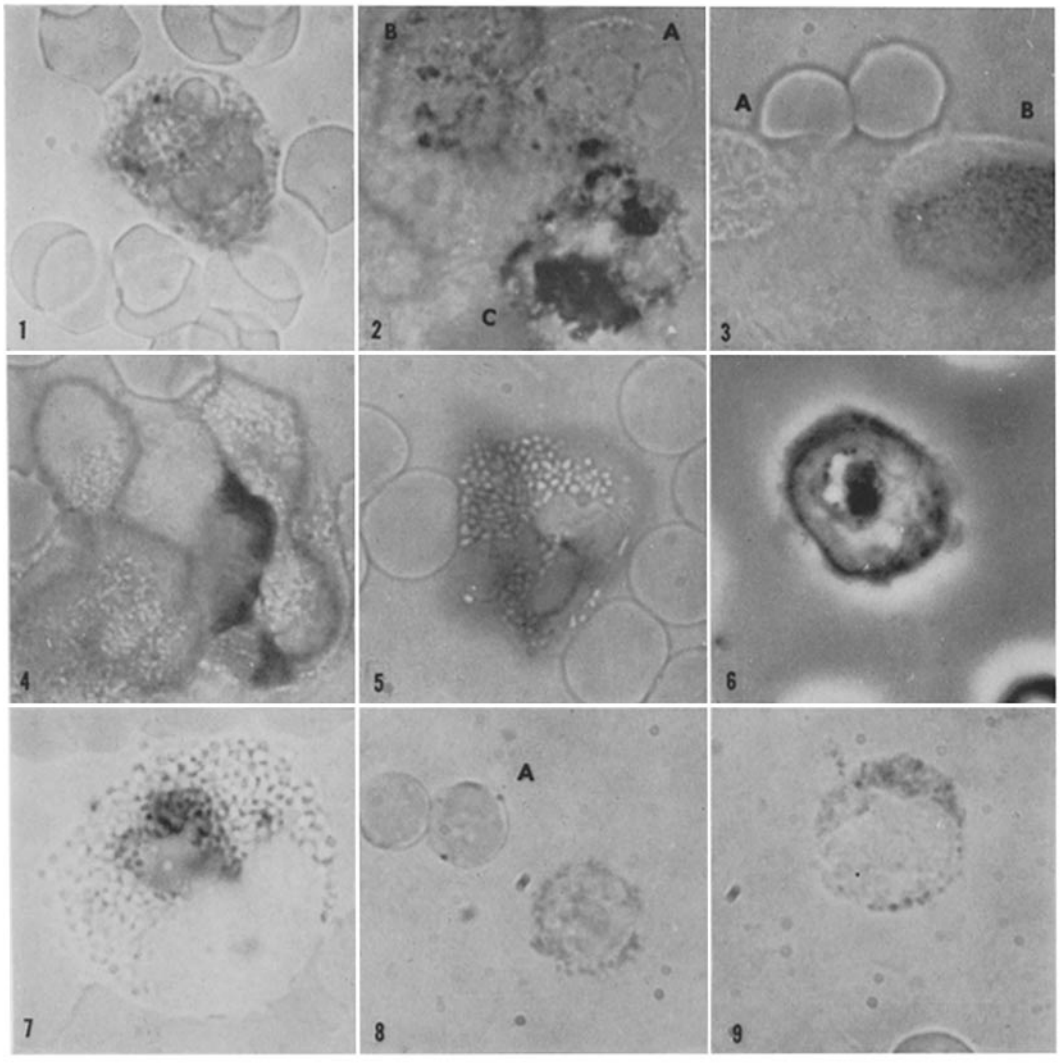
FIGURES 8 and 9

Lymphocytes exhibiting a positive dehydrogenase reaction localized to their mitochondria. Two small formazan granules may be seen in the erythrocyte (*A*). Nitro BT–succinate–renex 20 in Sorensen's phosphate buffer (Method B). 30 minute incubation. × 1250.

cells of the blood and bone marrow but the addition of succinate to the incubating solution definitely increased mitochondrial reactivity. Enzymatic inactivation by heat, iodoacetic acid, and ethyl maleimide inhibited the tetrazolium reaction.

DISCUSSION

The demonstration of endogenous and succinic dehydrogenase activity in the intact living cells of the blood and bone marrow obtained with the nitro BT–succinate (Method A) technique correspond to the results reported by other investigators employing either nitro BT (8, 17) or other tetrazoles (14, 15) in their incubating solutions. Our observations indicate that, in most instances, sites of dehydrogenase activity shown by the reduction of the tetrazole are non-mitochondrial; *e.g.*, cytoplasmic vacuoles, specific granules, or the diffuse hyaloplasm of the granular leukocytes. It seemed unlikely that the number of mitochondria present in the mature granulocytes could account for either the strong reactivity or the intracellular distribution of formazan observed in these cells. Except in certain instances when the cells were morphologically damaged, the mitochondria observed in the agranulocytes, developing myeloid, and erythroid cells were histochemically negative for dehydrogenase activity (Method A).



It has been recognized that the intact membrane of the mitochondrion acts as a barrier for the penetration of the tetrazolium salts and that this membrane must be damaged in order to obtain adequate histochemical reactions with nitro BT for the oxidative enzymes residing in the mitochondrion (13). Increased cellular and mitochondrial permeability was achieved in our studies by the addition of a non-ionic surface-active agent to the incubating solution (Method B). In such preparation the mitochondria present in most of the cellular elements of the blood and bone marrow consistently exhibited a positive dehydrogenase reaction. In addition to the agranulocytes, developing granulocytes, and erythroid cells, blood platelets and mature erythrocytes also exhibited a positive dehydrogenase reaction with this technique. However, granulocytes exposed to the surface-active agent–tetrazolium reaction exhibited both morphologic alteration and loss of motility and failed to show dehydrogenase activity in contrast with preparations lacking this agent. These observations suggest that in order to demonstrate successfully dehydrogenase activity in the cells of the blood and bone marrow with the tetrazolium methods, it is necessary to have increased cellular and/or mitochondrial permeability and partially viable cells with an essentially intact dehydrogenase system.

The localization of histochemically demonstrable dehydrogenase activity obtained in these studies, using both of the tetrazolium methods described, conforms with biochemical evidence that has established the presence of Krebs cycle activity in human leukocytes (5, 11, 16). Small amounts of dehydrogenase activity have also been demonstrated biochemically in mature erythrocytes (7). Succinic dehydrogenase, alpha glycerophosphate dehydrogenase activity (10, 16), and endogenous oxygen consumption appear high in both leukocytes and blood platelets (16). The addition of intermediate Krebs cycle substrates, in most instances, produces a greater metabolic response in damaged or broken cells than in intact cells and appears to be dependent upon cellular permeability (11). Although the oxidative activity of the leukocytes may not be quantitatively similar to that of other tissues, a final common pathway for oxidation is qualitatively similar to that present in other tissues (11).

Controlled histochemical studies indicate that the formazan deposition noted in the cells of the blood and bone marrow with both Methods A and B is the result of dehydrogenase activity since it is possible to inhibit cellular reactivity with heat, iodoacetic acid, and ethyl maleimide. The non-mitochondrial localization of dehydrogenase activity noted in these studies may perhaps be related to the results from biochemical studies of homogenates, which have suggested that the dehydrogenase and oxidative enzymes present in other cells and tissues of the body in some instances may be extramitochondrial as well as localized within the mitochondrion (6, 9).

Because of the presence of endogenous dehydrogenase activity in certain of the leukocytes of the blood and bone marrow, further studies designed to identify various members of the oxidative pathway (*e.g.* by employing various substrates) (13) may be difficult to interpret without marked differences in cellular reactivity, localization, or adequate methods to destroy endogenous dehydrogenase activity with minimal impairment of cellular integrity. This study indicates that to evaluate adequately dehydrogenase activity in the cellular elements of the blood and bone marrow, both dehydrogenase techniques described herein should be employed. Less drastic methods should be sought to reduce cellular damage resulting from the action of the surface-active agent and further to ensure adequate cellular preservation and better enzymatic reactivity and localization.

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