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Histoenzymological Study of Selected Dehydrogenase Enzymes in *Pneumocystis carinii*

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The metabolic activity of *Pneumocystis carinii* cysts was studied histochemically by a tetrazolium dye technique to assess substrate-specific dehydrogenase activity. Lactate dehydrogenase, succinate dehydrogenase, and glutamate dehydrogenase produced moderate-to-strong reactions in the cysts, whereas glucose-6-phosphate dehydrogenase had little if any reactivity. These results suggest that pneumocystis cysts have some of the enzymes necessary for glycolysis, Krebs cycle activity, and intermediary protein metabolism. These studies provide a method of directly assessing metabolic pathways in *P. carinii* which circumvents the uncertainties of specificity inherent in previous investigations with partially purified suspensions.

Pneumocystis carinii is a major pathogen in patients with a wide variety of cellular and humoral deficiencies, particularly the acquired immunodeficiency syndrome (7). Despite the major clinical importance of *P. carinii*, very little is known about its metabolism. Studies of the basic biology and biochemistry of this organism have been hampered by an inability to culture the organism in vitro in large quantities or to obtain highly purified organisms from lung preparations (3, 9, 17, 18). Previous studies with partially purified suspensions of pneumocystis obtained from rat lungs suggest that pneumocystis consumes oxygen, metabolizes glucose to CO_2, synthesizes proteins and RNA from precursors, and has modest levels of superoxide dismutase (15, 16). These studies have been ambiguous, however, in that there is no certainty that the parameters measured truly reflect properties of pneumocystis rather than of other murine or microbial components of the lavage sample studied.

Light microscope histoenzymology with tetrazolium dyes permits topographic localization of enzymatic activity (4, 5, 10, 13). For the study of pneumocystis, such an approach provides the opportunity to localize enzymatic activity to the organism, thus demonstrating that the activity is related to the protozoan rather than to a concomitant murine cell, fungus, or bacteria. In the current investigation, *P. carinii* was shown to produce moderate-to-strong tetrazolium reactions from glutamate dehydrogenase (EC 1.4.1.2) (GDH), lactate dehydrogenase (EC 1.1.1.27) (LDH), and succinate dehydrogenase (EC 1.3.99.1) (SDH), while glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (G6PDH) reactivity was minimal.

Identification of the presence of these enzymes in pneumocystis cysts suggests that these organisms have the potential for anaerobic and intermediary protein metabolism and Krebs cycle activity. These studies are an initial step in elucidating the biology of this organism and developing an understanding of the growth requirements and metabolic processes of the organism. These insights may be useful for facilitating the development of new diagnostic procedures and new approaches to therapy.

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MATERIALS AND METHODS

Pneumocystis organisms were obtained from the lungs of eight Sprague-Dawley rats treated with Decadron and fed low-protein chow (12). The rats came from different lots over a 1-year period. Animals were sacrificed with carbon dioxide, and the lungs were removed and rinsed in phosphate-buffered saline. Lung imprints were made on microscope slides and stained with Diff Quik (Dade Diagnostics, Inc., Aguada, Puerto Rico) to rapidly assess the presence or absence of pneumocystis. If the organism was present, the lungs were immediately homogenized by passage through a tissue sieve with a no. 60 mesh screen (Bellco Glass, Inc., Vineland, N.J.), and smears of the homogenized lung were placed on microscope cover slips.

Tachyzoites of *Toxoplasma gondii* (Rh strain) were also studied to provide a comparison with the pneumocystis organisms. Toxoplasma tachyzoites were obtained from mouse peritoneal cavities as previously described (8). Pneumocystis cysts and toxoplasma tachyzoites were recognized in specimens on the basis of their typical morphology by bright-field and phase-contrast microscopy. Smears of homogenized lung and peritoneal fluid made on cover slips were allowed to air dry for 0.5 h at room temperature before staining with the tetrazolium dye.

Tetranitroblue tetrazolium dye (Sigma Chemical Co., St. Louis, Mo.) was used in the reactions. The tetrazolium experiments were done as described previously (10). SDH was studied in an aqueous incubation medium. The more soluble enzymes, LDH, GDH, and G6PDH, were studied in a viscous gel medium. The stock incubation solutions were prepared with 10 parts of 0.2 M Tris hydrochloride buffer (pH 7.4), 8 parts of distilled water, 4 parts of 1 or 2% magnesium chloride, 4 parts of 0.05 or 1% sodium cyanide, and 10 parts of 0.2 or 0.4% tetranitroblue tetrazolium dye. The lower concentrations of the reagents were used for the aqueous incubation medium; the higher concentrations were used when preparing the gel incubation medium. To prepare this medium, we added an equal volume of 20% polyvinyl alcohol (Sigma) at pH 7.4 to the stock solution just before incubation. Phenazine methosulfate (Sigma) was added to the reaction medium to circumvent any interference from diaphorases. The cyanide was added to the stock solution to prevent interference from cytochrome oxydase. Stock solutions (1 M) of sodium succinate, sodium and lithium lactate,

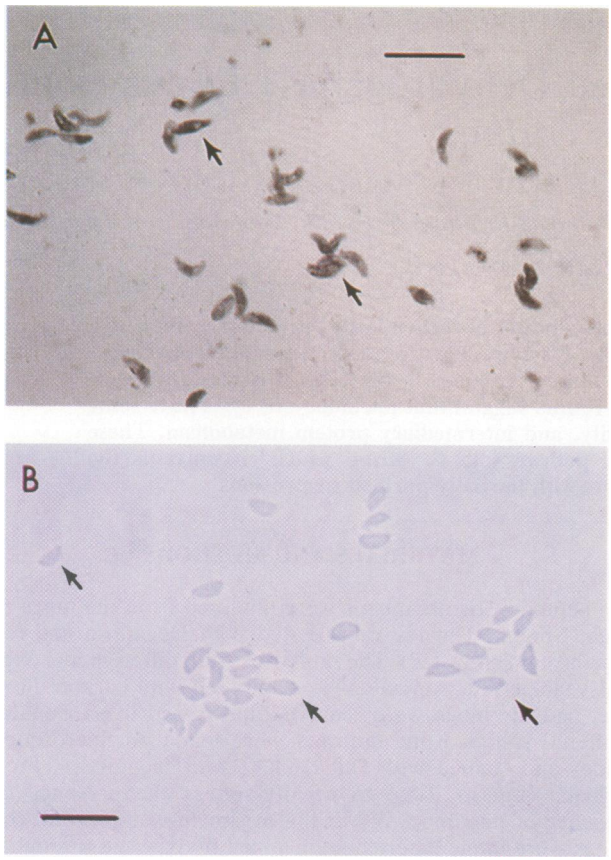

FIG. 1. G6PDH activity in *T. gondii* tachyzoites (A) and negative control run without substrate (B). Arrows indicate organisms. Bar equals 12.5 μm.

glucose-6-phosphate, and sodium glutamate were mixed, stored at −20°C, and thawed when used. NAD (Sigma) was added as coenzyme for the LDH and GDH reactions, while NADP (Sigma) was used for G6PDH. The incubation solutions were mixed fresh daily. The final incubation medium consisted of 6 ml of appropriate stock solution, 0.5 to 1.0 ml of 1 M substrate solution, 0.1 to 1.0 mg of phenazine methosulfate, and 2.0 to 5.0 mg of NAD or NADP. The amounts of substrate, phenazine methosulfate and coenzyme were titrated to prevent diffusion artifact and to help ensure negative controls for each set of experiments.

The reactions were done by incubating the cover slips with the dried lung imprints or smears in the medium in capped Columbia jars at 37°C in the dark. Controls were run in parallel by substituting distilled water for substrate in the final incubation medium. The time of incubation varied from 10 min to 1 h to produce maximum reaction intensity with a correspondingly negative control. The slides were then rinsed in distilled water, fixed in formol, and mounted in glycerol. Some slides were counterstained with Diff Quik to better identify the *P. carinii* cysts in the bright field. Indirect immunofluorescence staining of the pneumocystis after the tetrazolium reaction was done with hyperimmune rabbit antipneumocystis antibody produced by immunizing rabbits with isolated cysts and adsorbing the hyperimmune serum with normal rat lung (J. A. Kovacs, J. L. Halpern, J. C. Swan, J. Moss, J. E. Parrillo, and H. Masur, unpublished data). The second antibody was fluorescein-conjugated goat anti-rabbit immunoglobulin G (Cooper Biomedical, Inc., West Chester, Pa.).

RESULTS

The activity of the tetrazolium reaction was judged in a semiquantitative manner as being either 3+ (strong), 2+ (moderate), 1+ (weak), or 0 (absent), based on examination of the formazan precipitate. Tachyzoites of *T. gondii*, used for comparative purposes, were easily recognized in the peritoneal exudates (Fig. 1). The tachyzoites demonstrated moderate-to-strong activities of LDH, GDH, and G6PDH and weak-to-moderate activity of SDH (Table 1). Controls run without substrate or with heat-killed tachyzoites did not precipitate formazan. Figure 1 shows a toxoplasma trophozoite with positive reaction for G6PDH (A) and a negative control (B).

The pneumocystis cysts were found easily by phase-contrast microscopy. To confirm that the histochemical reactions indeed were occurring in pneumocystis cysts, we stained some smears by indirect immunofluorescence with antipneumocystis antibody and examined them for fluorescence. When a pneumocystis cyst was identified by both phase-contrast and immunofluorescence microscopy, the condenser was changed to bright field, and the formazan precipitate was assessed for intensity. No autofluorescence was noticed on untreated slides. Control cysts are shown in Fig. 2 by phase-contrast (A) and immunofluorescence (B) to demonstrate morphology and by bright field (C) to demonstrate the absence of formazan. Cysts identified by immunofluorescence (D) show a positive reaction with formazan precipitate seen in the bright field (E), indicating the presence of SDH. *P. carinii* cysts had moderate levels of SDH activity, moderate-to-strong activity of LDH and GDH, and absent-to-very-weak activity of G6PDH (Table 1). Controls run without substrate were negative. The results appeared reproducible in the pneumocystis cysts obtained from different individual rats from different lots.

DISCUSSION

This study of the enzymatic activity of *P. carinii* with a tetrazolium technique expands current understanding of the metabolism of this organism. Prior studies with partially purified suspensions of heavily infected lung have yielded indirect information concerning metabolic activity (15, 16). These results were equivocal since the contribution from contaminating bacteria and host cells could not be determined with certainty. Using the tetrazolium staining technique to study dehydrogenase activity, the reaction can be localized to organisms by direct bright-field visualization (5, 10). Thus, it can be ascertained whether enzymatic activity is attributable to pneumocystis as opposed to contaminating organisms or murine cells in the sample studied.

The histoenzymology of *T. gondii* has been previously reported (2, 11, 14). Our results concur with those studies

TABLE 1. Enzymatic activity in *P. carinii* and *T. gondii* tachyzoites

Enzyme	Intensity of tetrazolium reaction in:	
	P. carinii cysts	*T. gondii* tachyzoites
LDH	2+–3+	2+–3+
SDH	2+	1+–2+
G6PDH	0–1+	2+–3+
GDH	2+–3+	2+–3+

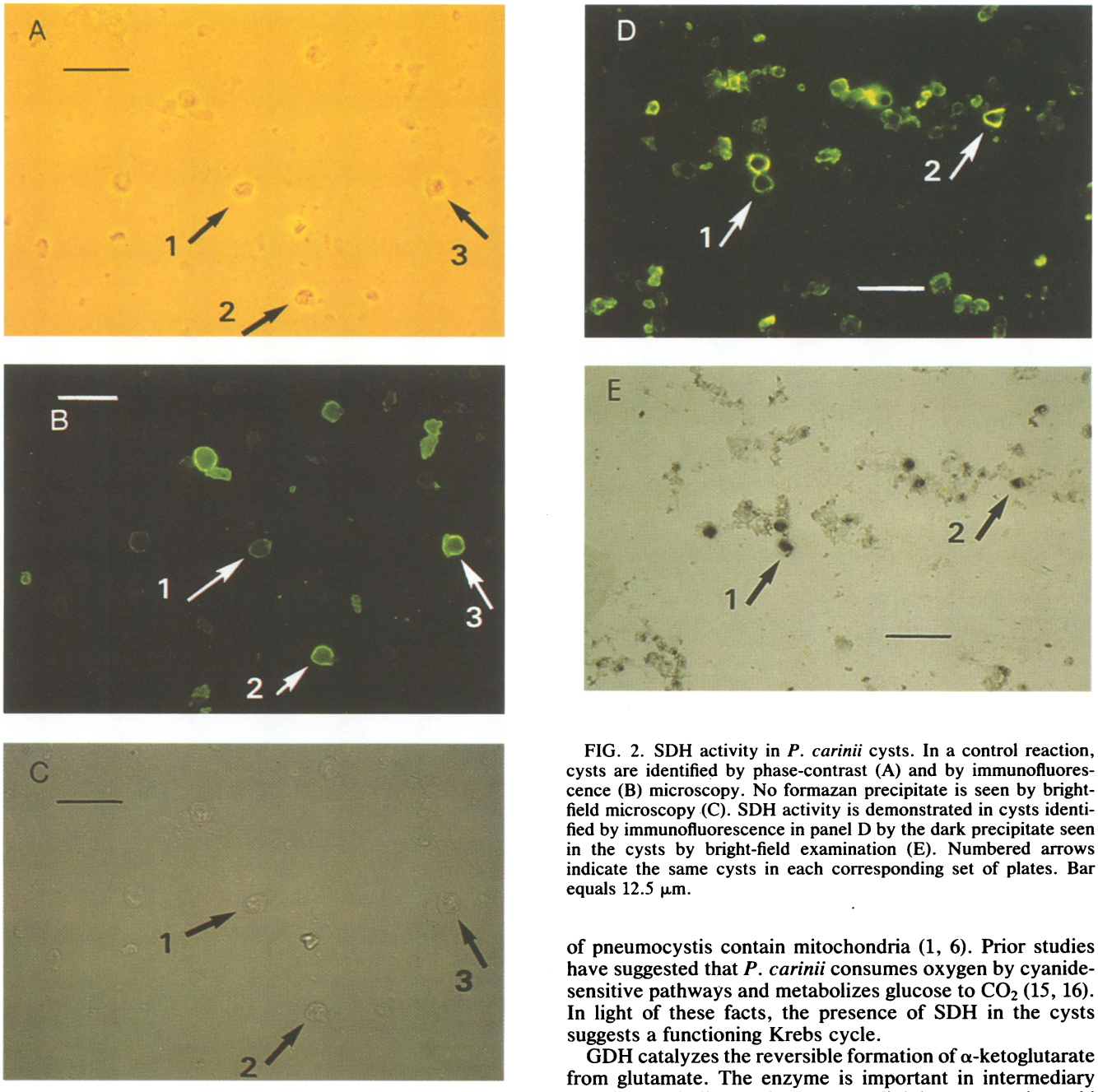

FIG. 2. SDH activity in *P. carinii* cysts. In a control reaction, cysts are identified by phase-contrast (A) and by immunofluorescence (B) microscopy. No formazan precipitate is seen by bright-field microscopy (C). SDH activity is demonstrated in cysts identified by immunofluorescence in panel D by the dark precipitate seen in the cysts by bright-field examination (E). Numbered arrows indicate the same cysts in each corresponding set of plates. Bar equals 12.5 μm.

and thus serve as a control for our experiments with *P. carinii*.

LDH catalyzes the formation of lactate from pyruvate with reoxidation of NADH to NAD^+. This allows glycolysis to proceed in anaerobic conditions and is important in lactic acid fermentation. Our studies indicate the presence of LDH in pneumocystis. In previous studies, *P. carinii* converted pyruvate and glucose to CO_2 at a measurable rate. Also, incubation of *P. carinii* organisms in an atmosphere without oxygen did not suppress the subsequent ability to convert glucose to CO_2 (16). Its viability in hypoxic conditions may be related to the presence of LDH activity.

SDH is a mitochondrial enzyme that functions in the tricarboxylic acid cycle. Both sporozoites and trophozoites of pneumocystis contain mitochondria (1, 6). Prior studies have suggested that *P. carinii* consumes oxygen by cyanide-sensitive pathways and metabolizes glucose to CO_2 (15, 16). In light of these facts, the presence of SDH in the cysts suggests a functioning Krebs cycle.

GDH catalyzes the reversible formation of α-ketoglutarate from glutamate. The enzyme is important in intermediary protein metabolism and serves as a link between amino acid metabolism and the Krebs cycle. Previous work has suggested that *P. carinii* incorporates amino acid precursors into protein (16). The presence of GDH in the organisms is further indirect evidence of intermediary protein metabolism and Krebs cycle activity.

G6PDH allows the deviation of glucose from glycolysis toward the pentose shunt, which is important in the formation of NADPH and pentose sugars. Prior metabolic studies, analyzing CO_2 generation from labeled glucose, have indirectly suggested pentose shunt activity in *P. carinii* (16). Our results indicate absent-to-weak G6PDH activity in the cysts. Several possibilities may explain this inconsistency. First, there may not be a good correlation between the very weak activity of the enzyme as revealed by staining and its in vivo activity. Alternatively, the enzyme may be absent, with the

organism using metabolic pathways other than the pentose shunt to use different labeled forms of glucose. Third, contaminating cells in partially purified suspensions of *P. carinii* may be responsible for the pentose shunt activity detected radiochemically. Finally, the activity noted in suspensions of organisms may have been due to trophozoites, not the cysts. Different forms of the organism may have different enzymatic profiles and metabolic potentials.

Studies of antioxidant enzymes in suspensions of *P. carinii* have suggested that superoxide dismutase is present while catalase and glutathione peroxidase are absent in the organisms. *P. carinii* was also found to be sensitive to superoxide and hydrogen peroxide oxidant stress, i.e., lethal oxygen-radical-generating systems (15). NADPH functions with glutathione reductase and glutathione peroxidase in protection against oxidant stress. Absent-to-low levels of G6PDH activity and subsequent low NADPH generation by this pathway may correlate with the absence of glutathione peroxidase and the apparent susceptibility of the organism to oxidant stress.

The enzymatic reactions in the trophozoites are not reported because of technical difficulties in definitively identifying trophozoites in the presence of the formazan precipitate.

In conclusion, this study suggests that *P. carinii* cysts have appreciable LDH, SDH, and GDH enzymatic activities as revealed histochemically. These results, in conjunction with previous studies, indicate that *P. carinii* cysts exhibit glycolytic activity, a functional tricarboxylic acid cycle, and a key enzyme of intermediary protein metabolism. The absent-to-weak activity of G6PDH may be consistent with the susceptibility of *P. carinii* to oxidant stress. The organism thus appears to be capable of using several different metabolic pathways. The use of histochemistry to assess other enzyme systems, and perhaps coupled with electron microscopy, should expand the understanding of this organism. Moreover, used as a metabolic marker, tetrazolium staining of organisms obtained by bronchoalveolar lavage may be potentially useful to assess organism viability and thus aid in monitoring the efficacy of drug therapy.

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