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A Library of Monoclonal Antibodies to *Escherichia coli* K-12 Pyruvate Dehydrogenase Complex

A BIOCHEMICAL ANALYSIS AND THEIR ABILITY TO INHIBIT THE ENZYME COMPLEX*

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A library of monoclonal antibodies to K-12 *Escherichia coli* pyruvate dehydrogenase complex (PDHc) and its pyruvate decarboxylating (EC 1.2.4.1; E1) subunit is reported. 21 monoclonal antibodies were generated, and 20 were investigated, of which 9 were elicited to PDHc and 11 to pure E1 subunit; 19 were of the IgG1 isotype and one of the IgG3 isotype. According to an enzyme immunoassay, all 20 of the monoclonal antibodies bound the PDHc, and 17 bound the E1 subunit. According to Western blot analysis, 14 of the 19 monoclonal antibodies bound to the E1 subunit. The monoclonal antibodies inhibited PDHc from 0 to >98%. The six monoclonal antibodies that displayed greater than 30% inhibition of *E. coli* PDHc were unable to inhibit porcine heart PDHc nor did they bind porcine heart PDHc according to dot blot analysis. Radiolabeling gave binding constants ranging from 5 to 10 × 10^8 M^{-1} on these six monoclonal antibodies, with greater than 80% of maximal inhibition achieved in less than 1 min. One of the six, 18A9, gave >98% inhibition, required two antibodies/E1 subunit for maximum inhibition, and was shown to be a non-competitive inhibitor. Monoclonal antibody 15A9 was shown to counteract GTP-induced inhibition, while 1F2 influenced the conformation of E1, allowing two antibodies, which did not previously bind E1, to bind to it. A new mechanism-based kinetic assay is presented that is specific for the E1 component of 2-keto acid dehydrogenases. This assay confirmed that the three most strongly inhibitory monoclonal antibodies specifically inhibited the E1 function while antibody 1F2 led to enhanced activity, suggesting an induced conformational change in PDHc or in E1.

Pyruvate dehydrogenase (PDHc)[1] is a multienzyme complex that is found in both prokaryotic and eukaryotic cells. The enzyme complex catalyzes the oxidative decarboxylation of pyruvate in the following overall reaction (Ref. 1)

$$\text{pyruvate} + \text{CoA} + \text{NAD}^+ \rightarrow \text{acetyl-CoA} + \text{CO}_2 + \text{NADH} + \text{H}^+$$

REACTION 1

In *Escherichia coli*, three different enzyme components are involved in the above reaction: pyruvate dehydrogenase, utilizing thiamin diphosphate (ThDP) as a cofactor (EC 1.2.4.1; E1); dihydrolipoamide transacetylase, which contains covalently linked lipoic acid residues (EC 2.3.1.12; E2); and dihydrolipoamide dehydrogenase, containing tightly bound FAD (EC 1.8.1.4; E3). The multienzyme complex performs the following series of reactions (Refs. 2–4)

$$\text{pyruvate} + \text{E1} - \text{ThDP} - \text{Mg}^{2+} \rightarrow \text{E1} - \text{hydroxyethylidene} - \text{ThDP} - \text{Mg}^{2+} + \text{CO}_2$$

REACTION 2

$$\text{E1} - \text{hydroxyethylidene} - \text{ThDP} - \text{Mg}^{2+} + \text{E2} - \text{lipoamide} \rightarrow \text{E1} - \text{ThDP} - \text{Mg}^{2+} + \text{E2} - \text{acetyldihydrolipoamide}$$

REACTION 3

$$\text{E2} - \text{acetyldihydrolipoamide} + \text{CoA} \rightarrow \text{E2} - \text{dihydrolipoamide} + \text{acetyl} - \text{CoA}$$

REACTION 4

$$\text{E2} - \text{dihydrolipoamide} + \text{E3} - \text{FAD} \rightarrow \text{E2} - \text{lipoamide} + \text{E3} - \text{FADH}_2$$

REACTION 5

$$\text{E3} - \text{FADH}_2 + \text{NAD}^+ \rightarrow \text{E3} - \text{FAD} + \text{NADH}$$

REACTION 6

The complex consists of multiple copies of each subunit: 24 E1, molecular weight 99,474 (Ref. 5); 24 E2, molecular weight 65,959 (Ref. 6); and 12 of E3, molecular weight 50,554 (Ref. 7) for a total calculated molecular weight of 4.57 × 10^6 daltons.

Metabolic inhibitors of PDHc include nicotinamide adenine dinucleotide (NADH), acetyl-CoA, and guanosine triphosphate (GTP) (8–11). Mammalian PDHc is regulated in a more complex fashion by phosphorylation and dephosphorylation of the α subunit of E1 (4). Other studies were directed at the investigation of various substrate analogues and their ability to inhibit PDHc. Small molecule inhibitors of PDHc include bromopyruvate (12), fluoropyruvate (13, 14), phosphonate analogues of pyruvate (15, 16), mono- and bifunctional arsenoxides (17–19), branched chain keto acids (20), and tetrahydrothiamin pyrophoshate (21).

Within the last decade, there has been significant interest in the role of PDHc in various clinical disorders. In some of these studies, there were reported polyclonal rabbit antibodies being made against various species of PDHc and its subunits. Two

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[1] The abbreviations used are: PDHc, pyruvate dehydrogenase multienzyme complex; MAb, monoclonal antibody; ThDP, thiamin diphosphate; MES, 2-(*N*-morpholino)ethanesulfonic acid; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; FBS, fetal bovine serum; PBS, phosphate-buffered saline; HPLC, high pressure liquid chromatography.

studies reported that these rabbit antibodies have the ability to inhibit various species of PDHc activity to some extent (22, 23). Although immunochemical inhibition of PDHc was not the major emphasis of these reports, they do constitute the first reported immununochemical inhibition of PDHc. Interest in another clinical disorder, primary biliary cirrhosis, has resulted in monoclonal antibodies being generated to the human E2 component of PDHc (24, 25). These antibodies have been reported to inhibit the PDHc but are directed specifically at the E2 subunit.

Reported here is a description of a library of monoclonal antibodies (MAb) recently generated to aerobic *E. coli* PDHc and its E1 subunit. 21 MAbs were cloned to 100% homogeneity. 20 of the 21 MAbs were studied for their ability to bind and inhibit PDHc. In addition, further biochemical characterization was done on six that displayed high levels of inhibition. A new chromophoric assay has been developed for measuring E1 activity in the PDHc, and its application provides complementary support for the conclusions. These findings 1) represent the first reported MAbs to aerobic K-12 *E. coli* pyruvate dehydrogenase and its E1 subunit, 2) represent the first MAbs to bind and inhibit the E1 subunit, and 3) provide relevant information for future studies that will continue to define the E1 active center and the thiamin diphosphate binding site, since no high resolution x-ray studies have yet been reported for this enzyme.

EXPERIMENTAL PROCEDURES

Materials—Female Balb/c mice, 6–8 weeks of age, were obtained from Jackson Labs. Mouse myeloma cells (NSO) were donated by Ellyn Fischberg (Roche Diagnostic Systems). Goat anti-mouse Kynar was obtained from Roche Diagnostic Systems. Freund's adjuvants, complete and incomplete, were purchased from Difco Laboratories; alkaline phosphatase-conjugated goat or rabbit anti-mouse immunoglobulins were from Zymed; rabbit anti-mouse IgG and goat anti-mouse IgG were from ICN Immunobiologicals; Bovine serum albumin (BSA), Fraction V, was from Miles; Fetal bovine serum (FBS) was from HyClone Laboratories; SeaPlaque agarose and polyethylene glycol were from ACE Scientific. The β-mercaptoethanol, 8-azaguanine phosphatase substrate tablets (*p*-nitrophenyl phosphate, disodium salt), Pristane, sodium azide, and trypan blue were all from Sigma. Diethanolamine and Tween 20 were from Fisher Scientific. Hanks' balanced salt solution, L-glutamine, NCTC 109, non-essential amino acids, and trypsin-EDTA were obtained from Life Technologies, Inc. Hypoxanthine/aminopterin/thymidine, hypoxanthine/thymidine, and penicillin/streptomycin were purchased from Flow Laboratories. Iscove's modified Dulbecco's medium was purchased from Hazelton Biologics. $Na^{125}I$ was from Amersham Corp. ELISA plates were purchased from Costar; removable microtiter plate wells were from Dynatech Laboratories Inc.; Falcon T25 flasks, Falcon Petri plates, Falcon Microtest III 96-well plates, and Falcon 6- and 24-well Multiwell plates were from Becton Dickinson Labware. EDTA, phenylmethanesulfonyl fluoride, leupeptin, and pepstatin were from Boehringer Mannheim; MES was from Calbiochem; Paragon high resolution electrophoresis gels were from Beckman. The PDHc was purified from *E. coli* K-12 cells purchased from Grain Processing Corp. (Muscatine, IA). The PDHc complex was purified (26, 27) with the modifications outlined below. During the extraction step, a French press was used with an extraction pressure of greater than 20,000 p.s.i. Throughout the entire purification, all buffers had a constant concentration of 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 1 mg/liter leupeptin, and 2 mg/liter pepstatin to minimize proteolysis. Finally, Bio-Rad DNA grade hydroxylapatite (28) was substituted for the calcium phosphate gel. Resolution of the E1 subunit from the complex was done according to Angelides *et al.* (29). The purified complex was dialyzed at pH 9.5 and placed over a Sepharose 6B column monitored at 280 and 450 nm. The two peaks were pooled and dialyzed against pH 7.0. At 4 °C, 0.36 g/ml of $(NH_4)_2SO_4$ was added to the second peak pool, which was then centrifuged. An additional 0.1 mg/ml $(NH_4)_2SO_4$ (70%) was added to the supernatant and centrifuged. The pellets were redissolved at pH 7.0, and the protein concentration was determined by the Bio-Rad protein assay kit (30). The second pellet was used for all experiments involving E1. According to SDS-polyacrylamide gel electrophoresis (31), both the complex and the E1 subunit were more than 95% pure.

Immunization and Inhibition Procedures for Monoclonal Antibodies—Female, BALB/c, 4–6-week-old mice were injected intraperitoneally with either 100 μg of PDHc or E1 antigen in complete Freund's adjuvant and with incomplete Freund's each month thereafter. Once an orbital test bleed inhibited 1000 units/liter of PDHc activity, greater than 95% fusion took place. The activity of PDHc was assayed (32) and was adapted to the Cobas Bio centrifugal analyzer instrument (Roche Diagnostic Systems). Mouse sera were added to the PDHc enzyme, and after a 1-h incubation, inhibitory activity was measured. This method was also used to determine the type of inhibition caused by monoclonal antibody 18A9.1.3PDHc when generating a Lineweaver-Burk plot.

Production of Monoclonal Antibodies—The hybridomas were produced by a modification of the protocol of Kohler and Milstein (33). 3 days before the fusion, the selected mouse was boosted intraperitoneally with 100 μg of antigen. The spleen cells were fused with mouse myeloma (NSO) cells at a 5:1 ratio (spleen:NSO) using 50% polyethylene glycol (M_r 4000). Fused cells were plated in hypoxanthine/aminopterin/thymidine (0.1 mM sodium hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine), supplemented with Iscove's modified Dulbecco's medium containing 20% FBS. After 10 days, supernatants from the wells were screened for antibodies specific to the antigen by the described ELISA and the inhibition assay.

ELISA—All hybridoma supernatants from wells that contained 50–60% cell growth were screened for specific binding to PDHc initially, and those positives were then screened for E1 binding by employing an indirect solid-phase microtiter plate ELISA. Microtiter plates were coated with 12 μg/ml PDHc or E1 antigen incubated and washed with a PBS Tween washing buffer. The plates were blocked with 1% BSA and 0.1% sodium azide and stored at 4 °C for use. For assay, the respective hybridoma supernatant was added to the well, incubated at 37 °C, and washed. An alkaline phosphatase-conjugated goat anti-mouse IgG, IgA, and IgM mixture was added, incubated, and washed, and then substrate was added. The substrate was a 5-mg tablet of *p*-nitrophenyl phosphate disodium salt dissolved in 5 ml of diethanolamine buffer, pH 9.8, containing 0.2 mM $MgCl_2$. Plates were incubated, the reaction was terminated by the addition of 3 N NaOH, and absorbances were read at 405 nm.

Cloning—Selected hybridomas were switched to Iscove's modified Dulbecco's medium containing 10% FBS and hypoxanthine/thymidine (0.1 mM sodium hypoxanthine and 16 μM thymidine). Those cell lines that continued to produce the desired antibodies were cloned by the soft agar technique. The colonies were picked and plated into a 96-well plate, and the procedure was repeated until a 100% clone was obtained. Cells that were 100% cloned were grown up and frozen (−80 °C) in 10% Me_2SO, 90% FBS at 1×10^6 cells/ml/vial.

Monoclonal Immunoglobulin Subclass—Antibody subclass was determined using the ELISA described above and using subclass-specific alkaline phosphatase-conjugated enzymes. Antibody subclasses were determined from culture supernatants prior to injecting cells for ascites production.

Ascites Production—Balb/c mice, 3 months old, pretreated 7 days earlier with Pristane (2,6,10,14-tetramethyl-pentadecane) were injected intraperitoneally with 1×10^6 cells in PBS. The ascites fluid was collected approximately 2 weeks after injection by tapping the mice two to four times at 2-day intervals. All ascites taps were centrifuged at 15,000 × *g* for 10 min. The ascites from each respective clone were pooled and frozen at −70 °C.

Inhibition of PDHc by Antibodies—The activity of PDHc was assayed according to Maldonado *et al.* (32) and was adapted to the Cobas Bio centrifugal analyzer instrument (Roche Diagnostic Systems). Mouse sera or dialyzed tissue culture supernatants from positive PDHc ELISA-screened clones were first diluted serially with 20 mM potassium P_i buffer, pH 7.0, containing 0.1% BSA. These diluted solutions were then added to an equal volume of the same buffer containing 2000 units/liter of PDHc enzyme. After mixing, the solution was incubated at 4 °C for 1 h. At the end of the incubation, the enzyme activity was measured. Percent inhibition was calculated by dividing the activity remaining by the total activity placed into the mixture minus 1, and times 100. This method was also used to determine the activity in the presence of MAbs.

Purification of the Monoclonal Antibodies—The method of Ey *et al.* (34) was adapted to the Waters HPLC to purify the MAbs using the Bio-Rad Affi-Prep protein A MAPS Prep HPLC column. In the reported work, only IgG1 antibodies were purified. The column was first washed with 0.02 M PBS, pH 7.5, and then equilibrated with 1 M glycine, 3 M NaCl buffer, pH 9.0. 3 ml of ascites fluid was diluted 1:2 with the 1 M glycine, 3 M NaCl buffer, filtered through a 0.22-μm Millipore membrane, loaded on the column, and eluted until the protein peak appearing at 280 nm was resolved to less than 0.05 absorbance units. Next, 0.1

M sodium citrate, pH 6.0, was applied, and the protein peak of interest was eluted. The column was then stripped with 0.1 M sodium citrate, pH 3.5. The pH 6.0 fraction pool was dialyzed against 0.1 M sodium phosphate with 0.1 M NaCl, pH 7.5, and the concentration of the IgG was determined at 280 nm using an extinction coefficient of 1.5 g^{-1} liter cm^{-1} (35).

Native Gel Electrophoresis—To assess the purity of the MAbs, nondenaturing electrophoresis was performed on the Beckman Paragon gel electrophoresis system according to the product package insert. Briefly, the sample was diluted in the manufacturer's 0.075 M barbital buffer, pH 8.6, applied to the high resolution electrophoresis gels, and then electrophoresed at 200 V for 55 min. The gel was then stained with the manufacturers Paragon blue stain, destained in acetic acid, and dried in the Beckman Paragon gel dryer.

Western Blotting—All Western blots were performed according to standard procedures with the following exceptions. In place of the nitrocellulose membrane, polyvinylidene difluoride (Bio-Rad), was used. Milk diluent (casein) from Kirkegaard and Perry was used as a blocking buffer. The TMB membrane peroxidase substrate system was used as the substrate, and the wash buffer was PBS Tween. In addition, 0.5-mm mini-gels were used instead of 1.5-mm slab gels (Bio-Rad). 1 mg of purified PDHc or E1 was run on the mini-gel, and the gel was transblotted onto polyvinylidene difluoride paper. Each ascites fluid was diluted 1:2000, and the second antibody (Goat anti-mouse horseradish peroxidase) was used at 1:5000 dilution.

Radiolabeling PDHc—Radiolabeling the PDHc antigen was performed using the Bio-Rad Enzymobead™ radioiodination reagent according to Morrison and Bayse (36). To 50 μl of enzymobeads, pH 9.5, was added 50 μg of PDHc or E1, 30 μl of 1% β-D-glucose (all in 0.05 M potassium P_i, pH 7.5), and 1 mCi of $Na^{125}I$. The reaction was incubated for 25 min at room temperature, and then 150 μl of 5% BSA in 0.05 M potassium P_i, pH 7.5, was added. The material was next chromatographed on a 1 × 30-cm Sepharose G-100 column eluting with 0.1 M Tris-HCl, pH 7.5, with 0.02% NaN_3. The first radioactive peak was pooled and 1% BSA was added. Specific activities were determined to range from 2.0 to 3.0 μCi/μg.

Solution Phase Radiolabel Binding Assay—Concentrated radiolabeled PDHc or E1 stock was diluted in 20 mM potassium P_i, pH 7.0, containing 1% BSA, 0.1% NaN_3, to 20,000 cpm per 50 ml of buffer. Each MAb ascites was diluted 1:500 in the same buffer. 50 μl of respective label and 100 μl of a respective MAb were added to each test tube. All tubes were vortexed and allowed to incubate overnight at 4 °C. To each tube, 25 μl of a 10% solution of goat anti-mouse Kynar was then added. The tubes were vortexed and allowed to incubate for 15 min at room temperature. 4 ml of 150 mM NaCl was then added, and the suspension was centrifuged at 4000 × *g* for 15 min. The liquid was decanted, and the contents of the tubes were counted.

Effect of Monoclonal Antibodies on the GTP-regulating Site—Experiments were conducted to determine if any of the monoclonal antibodies could interfere with the inhibition of PDHc by GTP. Assay conditions were the same as in Schwartz and Reed (10) except 50 mM Tris, pH 7.8, was used in place of potassium P_i. All MAb ascites were diluted 1:10 in 0.1% BSA in 20 mM potassium P_i, pH 7.0, and were incubated with 20–30 μg of PDHc enzyme, approximately 600 IU/liter, for 1 h at 4 °C. The mixture was then assayed in the presence and absence of 0.5 mM GTP. A nonspecific MAb was also included to show the effect of ascites. In addition, the total activity of the enzyme was determined with and without GTP present. The inhibitory effect of GTP was calculated for each MAb. MAbs 21C3 and 18A9 were not included in this study because of the high degree of inhibition they exhibited in the absence of GTP. MAb 15A9 was then purified on a protein A column. Known amounts of purified 15A9 were incubated with 30 μg of PDHc, as described above, beginning with the highest concentration of 6.25 μM and titrated to a final concentration of 0.02 μM 15A9.

Cooperative Binding Assay—HPLC-purified MAb 1F2 was coated on microtiter plates at 10 μg/ml in 20 mM NaP_i, pH 7.5, with 0.065% sodium azide (50 μl/well). The plates were washed and blocked as previously described. To each separately coated antibody plate well, 50 μl of radiolabeled E1, approximately 10,000 cpm, and 50 μl of one purified MAb (either 7C9 or 13A8) at various stated concentrations was added. Both the label and the purified MAb were diluted in 0.1% BSA, 20 mM sodium P_i, pH 7.5. Competition between the bound and the soluble antibody for the labeled E1 was allowed to proceed overnight at 4 °C. The plates were then washed three times with PBS Tween. The contents of each well were removed, placed in a test tube, and counted in a γ counter.

Dot Blot Assay—A Schleicher and Schuell Minifold 96-well filtration system for dot-blot assays was used. Nitrocellulose (0.45 mm) was soaked in 20 mM potassium P_i, pH 7.0, and placed in the apparatus along with a blotting pad. A vacuum was applied, and 500 μl of 20 mM potassium P_i was added to all 96 wells. Porcine heart (Sigma) and purified *E. coli* PDHc were used as antigens to be blotted. The specific activity of the porcine heart enzyme was 0.45 IU/mg and of the *E. coli* PDHc was 30 IU/mg. The porcine heart PDHc was diluted to 1 mg/ml (0.45 IU) and serially diluted in 20 mM potassium P_i to 115 ng/ml total protein. The *E. coli* PDHc was diluted to 15 μg/ml (0.45 IU) and then serially diluted to 0.92 ng/ml. To each respective Minifold well, 1 ml of the respective protein was added and vacuum applied, and 2 ml of buffer was run through each of the minifold wells. The nitrocellulose paper was removed, blocked with casein blocking diluent overnight at 4 °C, and washed three times with PBS Tween. Each blot set was incubated with 1 mg/ml of purified respective MAb diluted in casein diluent overnight at 4 °C and washed. The strips were then incubated with goat anti-mouse horseradish peroxidase for 2 h at 25 °C, washed, and then added to the TMB substrate described in the Western blotting section. The strips were incubated for 10 min at 25 °C and then washed in water to stop the reaction.

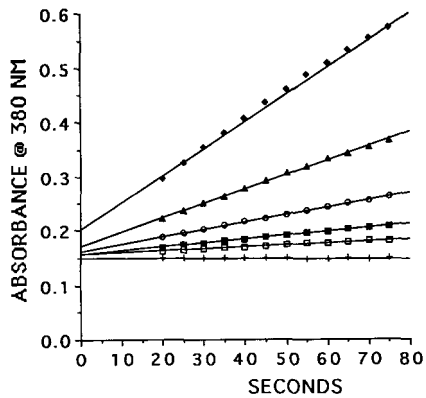


FIG. 1. **Best fit linearity for the E1 assay.** ♦, 2 mg/ml PDHc; ▲, 1 mg/ml PDHc; ○, 0.5 mg/ml PDHc; ■, 0.25 mg/ml PDHc; □, 0.125 mg/ml PDHc; +, BSA. This assay was run at 20 °C with a 20-s lag time. Other components in the assay were 5.0 mM pyruvate, 12 mM $MgCl_2$, 0.1 mM ThDP, 1.25 mM 4,4′-dithiodipyridine, and 1.25 mM 4-mercaptopyridine in 50 mM Tris, pH 7.7. See "Experimental Procedures" for details.

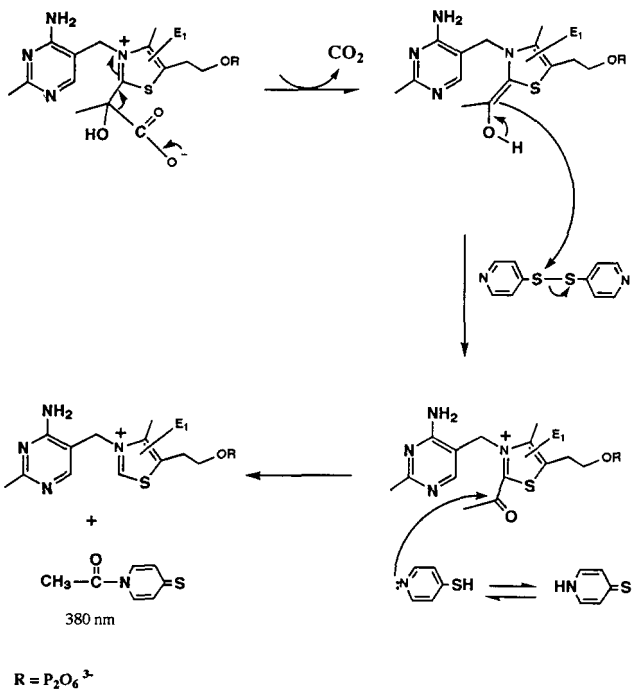
E1 Assay—The E1 assay was developed on the Cobas FARA™ centrifugal analyzer (Roche Diagnostics) with the following final concentrations of substrate reagents: 5.0 mM pyruvate, 12 mM $MgCl_2$, 0.1 mM ThDP, 1.25 mM 4,4′-dithiodipyridine, and 1.25 mM 4-mercaptopyridine in 50 mM Tris, pH 7.7. To 80 μl of enzyme sample was added 150 μl of substrate reagents and 15 μl of a water flush. A blank was recorded, and the reaction was monitored at 380 nm. A 15-s lag time was used, and 16 readings were taken at 5-s intervals at a reaction temperature of 20 °C. An ϵ_{380} = 72,200 was used to monitor the formation of the product of the reaction, *N*-acetyl-4-thiopyridine (37). Ascites fluid or purified MAbs were diluted in 20 mM potassium P_i, pH 7.0, with 0.1% BSA, added to various concentrations of purified PDHc or E1, and then incubated for 1 h at 4 °C. The solutions were then run according to the E1 assay. Fig. 1 illustrates the linearity of the assay under the optimal conditions, while Scheme I shows the chemistry (see Ref. 38 for details).

RESULTS

Two separate fusions, one using a mouse injected with the PDHc antigen and the other with the E1 antigen, were performed. Out of these two fusions, 21 MAbs were selected and cloned to homogeneity. 11 antibody clones resulted from the E1 fusion and were assigned an E1 suffix. The remaining 10 resulted from the PDHc fusion and were assigned the suffix PDHc. Isotyping studies done on tissue culture supernatants showed 20 of the monoclonal antibodies to be IgG1 and 1 to be an IgG3.

Experiments were conducted to determine the maximum inhibition produced by each MAb. All 21 ascites fluids were first titered in a microtiter plate with PDHc bound to the wells, and titers were expressed as one-half of the antibodies' maximum binding capacity. Each ascites had to have a titer of at least 2.5 × 10^3 to be used (one MAb, 12A9PDHc, failed to meet

this criterion and was rejected). The remaining 20 ascites fluids were diluted, and the inhibition was determined as described under "Experimental Procedures." Although it is known that the concentration of MAbs in ascites fluid can vary from one ascites to the next, the inhibition and radiolabel data were obtained under saturating conditions, as demonstrated by the stability of the results subsequent to more than one dilution. In addition, six of the MAbs (18A9, 21C3, 15A9, 1F2, 7C9, and 13A8) that exhibited greater than 30% inhibition were purified, and the maximum inhibition data summarized in Table I were verified by using greater than 700-fold molar excess of MAb to enzyme. To ensure that the inhibition was specific, ascites fluid from a non-relevant MAb was titered and demonstrated to give no inhibition. The extent of inhibition of PDHc by the remaining 20 MAbs is in the range from 0 to 99% (Table I). Those monoclonal antibodies, which gave greater than 60% inhibition, were all generated from the PDHc immunogen, whereas those generated from the E1 antigen gave a maximum of 50% inhibition.



SCHEME I. **Mechanism of new E1 assay.**

After all of the characterizations of the MAbs summarized in Table I were completed, 19 of the 20 monoclonal antibody ascites fluids remained. The remaining 19 antibodies were further evaluated by Western blot analysis. 14 of these MAbs were observed to bind the E1 subunit of the PDHc antigen of *E. coli* (data not shown). However, five of the MAbs (23H4, 8G10, 7C9, 8C9, and 13A8) did not show binding in Western blot analysis with PDHc or E1 from *E. coli*. Those five MAbs were reblotted at a higher concentration of ascites fluid and still did not demonstrate binding to *E. coli* PDHc or E1.

Six MAbs (18A9, 21C3, 15A9, 1F2, 7C9, and 13A8) that exhibited greater than 30% inhibition were also tested against porcine heart PDHc purchased from Sigma (specific activity, 0.45 IU/mg). Because of the low specific activity, 100 IU/liter of enzyme activity for both *E. coli* and porcine heart PDHc was used with 100 μg/ml of purified MAb. The data show no cross-species inhibition with porcine heart PDHc.

Additional studies were conducted to determine if these six MAbs (18A9, 21C3, 15A9, 1F2, 7C9, and 13A8) bound the mammalian PDHc antigen. Dot blot analysis was performed as described under "Experimental Procedures." The control antigen, *E. coli* PDHc, was matched to the mammalian total IU of enzyme added. The qualitative results illustrated that these six MAbs show binding to as low as 29 ng of *E. coli* PDHc but show no binding to mammalian PDHc when accounting for nonspecific binding signals.

These six MAbs were also chosen for further study, three because they exhibited the greatest inhibition (18A9, 21C3, 15A9) and three because they were generated from an E1 immunogen and displayed relatively high inhibition (7C9, 1F2, 13A8). All six MAbs were purified to homogeneity by HPLC protein A and were shown to be distinct and separate entities by their different migration patterns (data not shown).

TABLE I
Characteristics of the monoclonal antibody library

In these experiments, ascites were used.

Monoclonal antibody	Isotype	Average max inhibition[a] Ascites	Plate assay[b] ($A_{492\ nm}$)		Radiolabel binding PDHc
			PDHc	E1	PDHc
		%			%
18A9.1.3PDHc	IgG1	99	+	+	49
21C3.3.1.1PDHc	IgG1	85	+	+	47
15A9.1.3.2PDHc	IgG1	63	+	+	47
8C9.2.2E1	IgG1	53	+	−	34
7C9.3.3E1	IgG1	43	+	−	42
13A8.1.3.3E1	IgG1	42	+	−	32
3G1.2.2.1E1	IgG1	40	+	+	52
1F2.1.3E1	IgG1	34	+	+	52
23H4.2.2.1PDHc	IgG1	27	+	+	45
8G10.3.1.2E1	IgG1	21	+	+	32
6C5.1.2.1.1PDHc	IgG3	19	+	+	27
15E2.1.1.1E1	IgG1	10	+	+	46
11D9.2.3E1	IgG1	9	+	+	32
1G5.3.3PDHc	IgG1	0	+	+	31
12A12.2.3.2E1	IgG1	0	+	+	33
10E1.2.1.1E1	IgG1	0	+	+	30
3C6.3.1E1	IgG1	0	+	+	20
4F11.2PDHc	IgG1	0	+	+	29
3F11.1.2PDHc	IgG1	0	+	+	25
15A3.1.1PDHc	IgG1	0	+	+	28

[a] Inhibition of overall reaction according to the NADH assay.
[b] + is greater than 0.3 OD or 6 times background.

TABLE II
Summary of the binding constants of the purified monoclonal antibodies to PDHc

Binding constants were obtained by using a soluble phase PDHc radioimmunoassay. A competitive binding system was set-up between PDHc label, various concentrations of antigen, and the monoclonal antibodies. K values were determined using the molar PDHc inhibitor concentration giving 50% inhibition of tracer-antibody binding under equilibrium conditions. The percent binding of the tracer antibody without the inhibitor and the molar concentration of the PDHc label are used in the calculation.

Monoclonal antibody	K association
	liter/mol
18A9	1.0×10^9
21C3	5.3×10^8
15A9	6.4×10^8
7C9	8.6×10^8
1F2	9.3×10^8
13A8	8.3×10^8

The binding constants of these six MAbs to the PDHc were determined (39) by a competitive radioimmunoassay (Table II). The binding constants for these MAbs are similar, but MAb 18A9, which exhibits greatest inhibition, also exhibited the strongest binding constant.

The time course for attaining maximum inhibition by the six MAbs was also determined as described earlier. Greater than 85% of the maximum inhibition was achieved after 1 min, and by 10 min, maximum inhibition was essentially achieved (data not shown).

The stoichiometry of the interaction of MAb 18A9 with PDHc was also determined. Antibody concentrations ranging from 6.25×10^{-9} M to 6.25×10^{-6} M were added to 1000 IU/liter of enzyme, the mixtures were incubated for 1 h, and then the activity was assayed and a titration curve was drawn. Fig. 2*A* shows the titration curve for inhibition of the PDHc by MAb 18A9, and Fig. 2*B* illustrates the determination of the stoichiometry. Initial velocity was determined in the presence of MAb 18A9 (Fig. 3); the double reciprocal plot was suggestive of linear non-competitive inhibition with a K_i of 2.50×10^{-8} M. This behavior would be consistent with the MAb binding near or over but not within the active center pocket. Alternatively, it could be binding in a region distant from the active center and changing the conformation of the enzyme, thus changing its turnover number.

PDHc from *E. coli*, unlike its mammalian counterparts, is allosterically inhibited by GTP (10, 11). Experiments were conducted, as described under "Experimental Procedures," to determine if any of the monoclonal antibodies could interfere with the inhibition of PDHc by GTP. The mixture was assayed in the presence and absence of GTP. A ratio of activity in the absence of GTP to that in the presence of GTP was then calculated for each MAb. A nonspecific MAb was also included to show the effect of ascites on the ratio. If a MAb altered the regulatory binding site of the GTP, one would expect the regulating ability of GTP on the PDHc enzyme to be affected. When the ratio of activities in the absence to that in the presence of GTP approaches unity, inhibition due to GTP is blocked. Several MAbs may have some limited effect (data not shown), but purified MAb 15A9 at a concentration of 6.25 μM totally shuts down the regulation of the enzyme by GTP (Fig. 4).

Radiolabeling experiments were also conducted with MAbs 18A9, 21C3, 15A9, 1F2, 7C9, and 13A8. It was observed that MAbs 7C9 and 13A8, although elicited to the E1 antigen, did not bind the radiolabeled E1. This was in agreement with the plate binding E1 assay (see Table I) and Western blot analysis. A cooperative binding experiment was done, as described under "Experimental Procedures." The data in Fig. 5 suggest a conformational change induced in the E1 antigen when MAb 1F2 is bound to the radiolabeled E1, a conformational change that enables MAbs 7C9 and 13A8 to bind the E1. This could be deduced from Fig. 5 based on the increasing $[B]/[B_o]$ ratio for the binding of the radiolabeled E1 to the solid phase MAb 1F2 in the presence of 7C9 and 13A8. The control for this experiment was the unbound 1F2 in the absence of the other two MAbs competing for radiolabeled E1 at increasing concentrations of MAb 1F2.

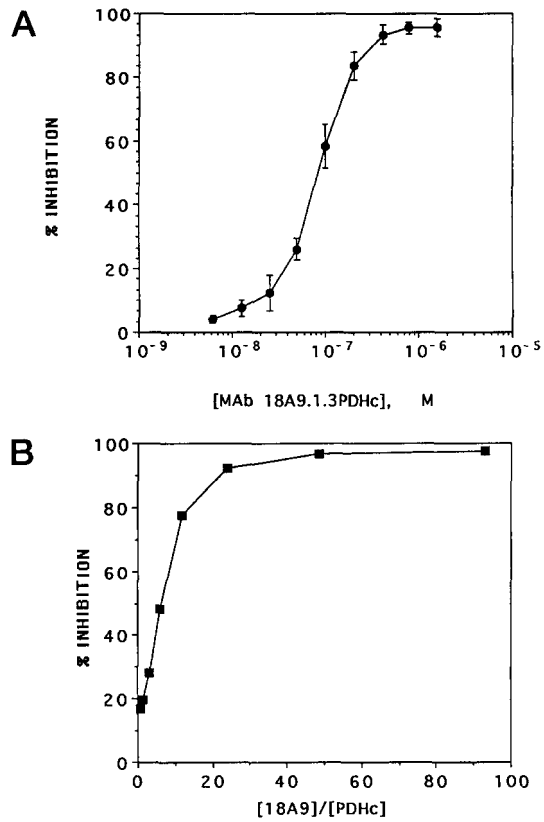


FIG. 2. *A*, Inhibition of PDHc with purified monoclonal antibody 18A9. *Error bars* represent ± 3 standard deviations. Antibody concentrations ranging from 6.25×10^{-9} M to 6.25×10^{-6} M were added to 2000 IU/liter of PDHc having a specific activity of 25 IU/mg and incubated for 1 h. The activity was assayed, and a titration curve was drawn. *B*, stoichiometry of interaction of monoclonal antibody 18A9 with PDHc. 2000 IU/liter total PDHc activity was used having a specific activity of 25 IU/mg.

We hypothesized that if one of the MAbs could inhibit the reaction monitored by the E1-specific assay, this would provide further evidence that the MAbs were specifically bound to the E1 subunit and blocked the transfer of the acetyl group to the nitrogen of the 4-mercaptopyridine. Fig. 6 demonstrates that according to the E1-specific assay, both PDHc and E1 (latter data not shown) were indeed inhibited by MAbs 18A9, 21C3, and 15A9, the best inhibitors. Next, purified MAb 18A9 was titrated against a constant concentration of purified E1 or PDHc at 1 mg/ml. Fig. 7 illustrates that the inhibition can be reversed when less than saturating amounts of antibody are used as demonstrated by the increased E1 activity, thus suggesting it to be specific. When titering the MAb 18A9 against PDHc and E1, at 0.25 mg/ml MAb 18A9 concentration, there is more activity observed with E1 than with PDHc. This is simply explained by an increased ratio of E1 to antibody with the purified E1, resulting in unbound E1 giving rise to enzyme activity.

According to Fig. 6, there is an apparent enhancement of E1 activity in the presence of MAb 1F2. This stimulation or en-

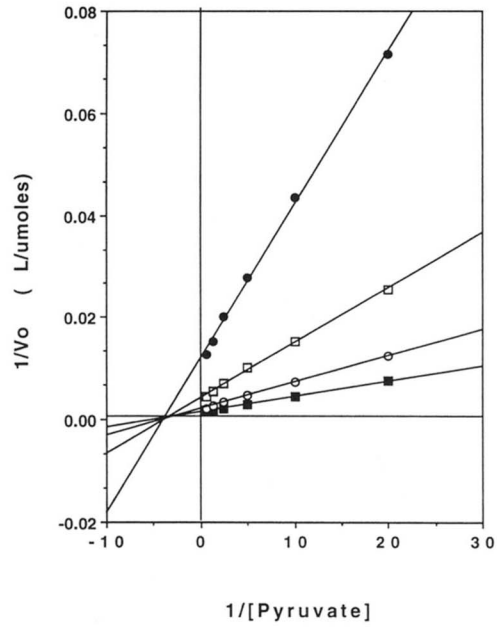


FIG. 3. **Lineweaver-Burk kinetic plot for monoclonal antibody 18A9 interacting with PDHc.** the K_i was determined to be 2.50×10^{-8} M. ■, no 18A9 inhibitor; ○, 7.5 μg/ml 18A9 inhibitor; ●, 30 μg/ml 18A9 inhibitor; □, 15 μg/ml 18A9 inhibitor. The PDHc 500 IU/liter enzyme and monoclonal antibody were diluted in 20 mM potassium P_i buffer, pH 7.0, with 0.1% BSA to the above stated final inhibitor concentrations. The mixtures were allowed to incubate 1 h at 4 °C. The various inhibitor concentrations were then run in the NADH assay. The various substrate concentrations were 5.0×10^{-5} M, 1.0×10^{-4} M, 2.0×10^{-4} M, 4.0×10^{-4} M, 8.0×10^{-4} M, and 1.6×10^{-3} M. The complete reaction mixture also consisted of 50 mM Bicine buffer, pH 8.1, 1.0×10^{-4} M CoASH, 3.0×10^{-3} M cysteine, 2.33×10^{-3} M NAD^+, 2.0×10^{-4} M ThDP, and 1.0×10^{-3} M $MgSO_4$. The production of NADH was continuously monitored at 340 nm at 27 °C. 1 unit of activity produced 1 μmol of NADH per min under these conditions.

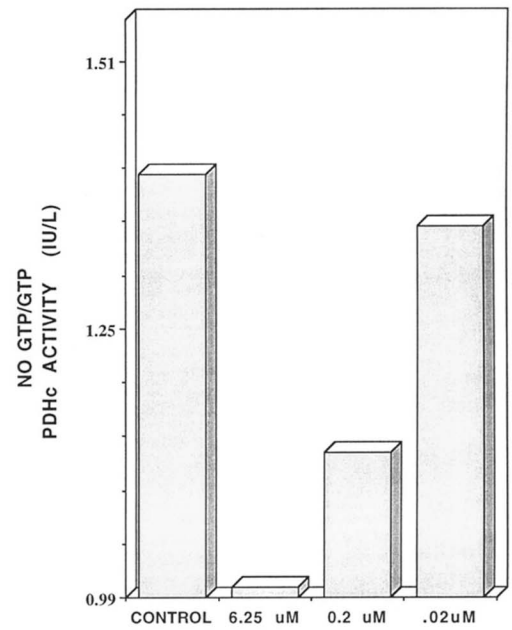


FIG. 4. **Effect of increasing monoclonal antibody 15A9 concentration on GTP-induced inhibition of PDHc.** With greater than two antibodies per E1 subunit (0.78 μM 15A9), the effect of GTP inhibition is blocked. At 6.25 μM, concentration of 15A9 the effect of 0.5 mM GTP is completely blocked. 600 IU/liter of PDHc enzyme was used. The error of measure is similar to that in Fig. 5. The fractional enhancement of the activity in the absence of GTP compared to the activity in the presence of GTP is plotted for the indicated levels of MAb 15A9.

hancement by MAb 1F2 was shown to be statistically significant.

DISCUSSION

20 of the 21 MAbs generated to PDHc and its E1 subunit have been studied. Western blot analysis revealed that 14 of 19 MAbs analyzed bound to the E1 subunit of PDHc. The observation that two of the five remaining MAbs, 23H4 and 8G10, bind E1 on the microtiter plate assay (Table I) but not according to Western blot analysis is thought to be due to the fact that Western blotting conditions destroy (while plate coating conditions preserve) these epitopes. It is implied, therefore, that these epitopes are non-continuous in nature.

The question of possible cross-species inhibition by selected MAbs was addressed, and no binding or inhibition of these MAbs to mammalian PDHc was observed. In view of the structural dissimilarity shown by genetic mapping, the lack of cross-reactivity of these MAbs to mammalian PDHc is not surprising.

Of the six MAbs exhibiting greater than 30% inhibition of PDHc, four (18A9, 21C3, 15A9, and 1F2) bind the E1 subunit of PDHc. Surprisingly, the other two, 7C9 and 13A8, although elicited to the E1 immunogen, appeared not to bind E1 at all. The possibility that the E1 antigen was contaminated with E2-E3 can never be totally ruled out; however, SDS-electrophoresis of the E1 antigen showed no other bands (data not shown), nor did Western blot analysis show any evidence of blotting to the E1 or to the PDHc antigen. If E2-E3 contaminant proteins did exist, since they would be present in such small percentages, they would have had to have been extremely immunodominant antigens. The possibility of other contaminating protein was also remote because these MAbs inhibited the enzyme quite effectively. We hypothesize that these MAbs are directed toward epitopes at a protein domain region where E1 contacts E2 or within some other domain within E1. Once the E1 antigen is resolved from PDHc, these regions may become distorted or are hidden from antibody recognition. During the process of producing these MAbs, it is assumed that peptide fragments that represented or conformed to the antigenic domain of the native E1 protein on the PDHc were exposed. This hypothesis is supported by the evidence that short peptide fragments from protein digests can represent domain region epitopes or discontinuous epitopes (40, 41). It is also believed that these MAbs are expressed to a conformational epitope that is only available on the holo-PDHc and not on the purified E1 subunit or on the SDS-denatured PDHc antigen. In the purified E1, the epitope must be buried according to the data in Fig. 5. MAbs 7C9 and 13A8 did not bind radiolabeled E1 by themselves, yet in the presence of monoclonal antibody 1F2 they bound E1. This suggests that 1F2 exposes the epitope that MAbs 7C9 and 13A8 can then bind. It is believed that 1F2 binds to the radiolabeled E1, causing a conformational change to occur. This then allows 7C9 or 13A8 to bind, which in turn stabilizes the complex. The 1F2-E1–7C9 complex has a higher binding constant than the 1F2-E1 complex, thus exhibiting higher B/B_0 values.

Enhancement of the E1 activity according to the E1-specific assay was observed in the presence of MAb 1F2, further supporting a conformational change induced in the E1 subunit when MAb 1F2 was bound. Such a conformational change would allow for a more efficient transfer of the acetyl group to the 4-mercaptopyridine. When looking at the overall reaction, MAb 1F2 inhibited the formation of NADH by decreasing the rate of transfer of the acetyl group to E2.

Due to the highest level of inhibition exhibited by MAb 18A9,

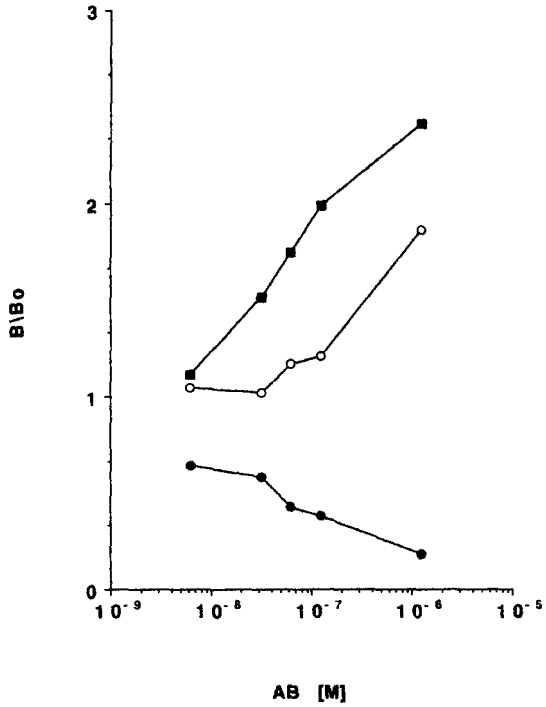


FIG. 5. **An induced conformational change of radiolabeled E1 antigen by monoclonal antibody 1F2.** ■, monoclonal antibody 7C9; ○, monoclonal antibody 13A8; ●, monoclonal antibody 1F2. B/B_o binding of the E1 radiolabel in the presence of 7C9 or 13A8/binding of E1 label in the absence of 7C9 or 13A8. Monoclonal antibody 1F2 was bound to the microtiter plate and was incubated in the presence of radiolabeled E1 and 7C9 or 13A8. This figure demonstrates that once 1F2 binds radiolabeled E1, 7C9 and 13A8 are then able to bind, increasing uptake of E1 antigen.

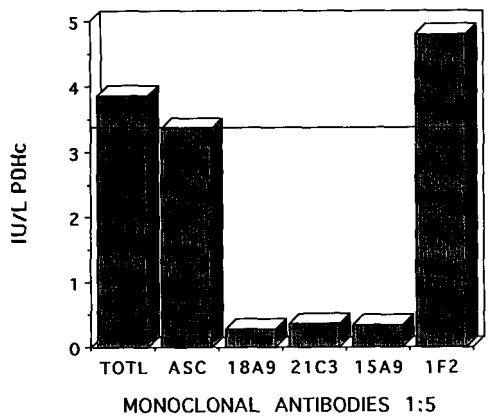


FIG. 6. **Inhibition of the E1 of PDHc enzyme with monoclonal antibody ascites diluted 1:5, monitored by the E1-specific assay.** *TOTL* refers to total PDHc activity without monoclonal antibody inhibition. *ASC* refers to a nonspecific ascites fluid diluted 1:5. All other *bars* represent specific monoclonal antibodies in the presence of the enzyme. See "Experimental Procedures" for details.

one would expect a very high level of specificity for this MAb to a distinctly defined epitope. Theoretically, only one MAb should bind each E1 subunit to obtain maximal inhibition. The structural evidence appears to show that E1 binds along the 12 edges of an octahedral E2 core, with E3 binding or attaching on the six faces (42–44). The inhibitor, rather than being a classical low molecular weight substrate analog, is an antibody with an M_r of about 150,000. If the antibody's effector antigen is E1, theoretically 24 E1 subunits must be bound by 24 MAbs to achieve maximum inhibition. The first antibodies binding the E1 subunits have binding constants similar in magnitude to the constants measured in Table II. Depending on the accessibility of the particular epitope, binding of each additional 18A9 antibody to the other E1 subunits within the complex could become increasingly more difficult due to increasing steric problems. This means that the binding constants for the last antibodies to be bound would be lower than the initial binding constants, resulting in ratios of greater than one antibody per E1 subunit needed to achieve maximal inhibition at 1 h. In viewing the stoichiometry of inhibition by 18A9 (Fig. 2*B*), greater than 90% inhibition is achieved with a ratio of [18A9]/[E1] of 1:1 (24 copies of E1 per PDHc), but to achieve that last 5–8% inhibition, a ratio of 2:1 is required. Observation of such a high level of inhibition for such a low ratio of antibody to enzyme antigen is nearly unprecedented. This can be explained by the allosteric behavior described above, as can the sigmoidal kinetic curve observed in the inhibition by 18A9 (Fig. 2*A*).

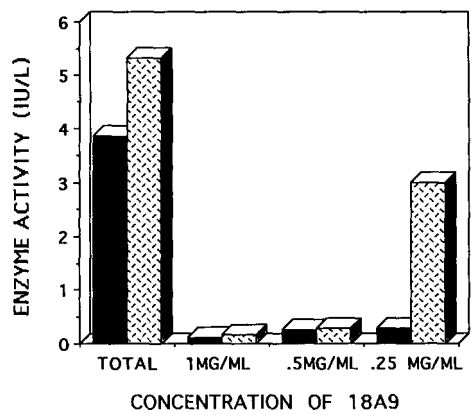


FIG. 7. **Titration of monoclonal antibody 18A9 in the E1 assay.** *TOTAL* refers to the PDHc or E1 subunit activity in the absence of monoclonal antibody 18A9. ■, PDHc enzyme, □ E1 subunit (both the PDHc and E1 were at 1 mg/ml). See "Experimental Procedures" for details.

It is very likely that MAb 18A9 directly affects the E1 component only, since isolated E1 component is also totally inhibited by it according to the E1-specific assay (data not shown). This would be in contrast to a polyclonal antibody elicited to an interlipoyl domain linker peptide, which interfered with the overall reaction without affecting the activities of the resolved components and therefore probably blocked the interaction between the E2 and E3 subunits (45).

The extent of maximal inhibition achieved by any MAb can be viewed analogously to the inhibitory efficacy of a small molecule inhibitor; the closer the inhibitor to the catalytic site, the greater the extent of inhibition. The linear non-competitive inhibition exhibited by MAb 18A9 is not surprising in view of this multisubunit and multicopy complex. This MAb clearly does not resemble the pyruvate substrate and hence should not exhibit competitive inhibition. It could act as a reversible "cap" over the active center, physically blocking entry to it but capable of interacting with PDHc both in the absence and in the presence of the substrate.

With some of the other purified MAbs (7C9 in particular), maximum inhibition is only achieved with molar ratios of MAb: PDHc greater than or equal to 700:1. In our earlier publication (46) we demonstrated that the the polyclonal antibodies generated to PDHc were to the three distinct subunits. In the immunoelectrophoresis we observed only three lines, suggesting three populations of antibodies (E1, E2, and E3). This evidence would suggest that the monoclonal antibodies should behave the same way.

Those MAbs (18A9, 21C3, and 15A9) displaying the greatest inhibition could be predicted to be directed at areas of the E1 subunit that are vitally important for enzyme activity. The E1

active site with its ThDP binding structural motif (47) is a good candidate. This binding region would likely constitute a discontinuous epitope (48) due to the nature of coenzyme or substrate binding sites in enzymes forming pockets. The motif is comprised of 30–40 amino acids that have common structural similarities among ThDP-dependent enzymes (47), is located on the E1 subunit, and is responsible for anchoring the Mg(II) ion and the diphosphate tail of the coenzyme. These three MAbs would likely bind over or near this active center. Epitope mapping studies (49) clearly demonstrate that the three MAbs have the same binding regions, whereas those MAbs derived from the E1 antigen have different binding regions, strongly supporting epitope dissimilarity.

Finally, it has been demonstrated that MAb 15A9 (Fig. 4) can block the allosteric inhibitory effect of GTP. This MAb could be bound at or near the GTP binding locus, or it could have caused a conformational change that distorted the binding locus for GTP, thus rendering it ineffective as an allosteric regulator. The behavior of MAb 15A9 suggests it to be a concentration-dependent inhibitory (negative heterotropic) effector molecule.

In summary, a MAb library has been presented that contains antibodies that bind as well as inhibit the *E. coli* PDHc and its decarboxylating E1 subunit. Of the MAbs characterized, 18A9 inhibits the enzyme to greater than 98%, and 15A9 can block the effect of GTP regulation of *E. coli* PDHc. This MAb should provide an excellent tool in studying the GTP binding site or its effector site. Lastly, conformation-dependent epitopes on the E1 subunit have been identified. MAbs 7C9 and 13A8, which could not bind the purified E1 subunit and which were elicited to this purified E1 antigen, were shown to bind E1 antigen in the presence of MAb 1F2.

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