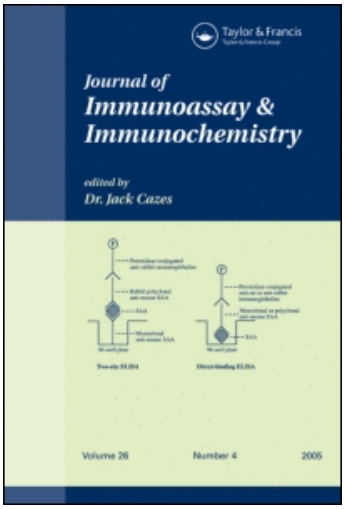

Journal of Immunoassay and Immunochemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Monoclonal Antibody Based Immunoassay for Human Aminoacylase-1

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To cite this Article Miller, York E. and Kao, Betty(1989) 'Monoclonal Antibody Based Immunoassay for Human Aminoacylase-1', Journal of Immunoassay and Immunochemistry, 10: 2, 129 — 152
To link to this Article: DOI: 10.1080/01971528908053232
URL: http://dx.doi.org/10.1080/01971528908053232

JOURNAL OF IMMUNOASSAY, 10(2&3), 129-152 (1989)

MONOCLONAL ANTIBODY BASED IMMUNOASSAY FOR HUMAN AMINOACYLASE-1

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ABSTRACT

Aminoacylase-1 is expressed in a wide variety of cell types. Although the exact role of this enzyme in cellular physiology remains unclear, it has been postulated to function in the salvage of acylated aminoacids. The enzyme is also of interest due to its gene map location on chromosome 3p21, a region deleted in several neoplasms. We have produced mouse monoclonal antibodies and rabbit antisera which recognize human aminoacylase-1. A sandwich ELISA has been developed which allows the measurement of human aminoacylase-1.

INTRODUCTION

Aminoacylase-1 (N-acylaminoacid aminohydrolase, ACY-1, E.C. 3.5.1.14) is a cytosolic enzyme widely distributed in mammalian tissues. The reaction catalysed by ACY-1 is the hydrolysis of acylated aminoacids into L-amino-acids and acyl groups. It has been postulated that ACY-1 is important in the catabolism of amino-terminally acylated proteins. (1)

The gene encoding human ACY-1 has been assigned to chromosome 3p21. (2) This region of chromosome 3 is deleted in a variety of disorders, including renal cell carcinoma, small cell lung cancer, non-small cell lung cancer and Von Hippel-Lindau Syndrome. (3,4,5,6) An immunoassay for ACY-1 would be of use both for measuring gene expression and for enzyme purification.

We have produced mouse monoclonal antibodies which are highly specific for human ACY-1 and combined these with a rabbit antiserum in a sandwich-type ELISA which allows the reproducible measurement of human ACY-1.

MATERIALS AND METHODS

Preparation of Human ACY-1

A crude preparation of human ACY-1 was produced by a modification of a published technique. (7) Briefly, a human kidney was obtained at autopsy, minced, suspended in distilled H_2O, homogenized using a Dounce homogenizer (Wheaton Scientific, Millville, NJ), then centrifuged at 1000 x g for 20'. The supernatant was heated to 70° C for 20', cooled to 4° C and adjusted to a concentration of 35% ammonium sulfate by the addition of an 100% ammonium sulfate solution. After overnight incubation at 4° C, the supernatant was harvested by decantation and the concentration of ammonium sulfate was then adjusted to 40%. After 16 hours incubation at 4° C, the precipitate was obtained by centrifugation at 2000 x g for 20', then dialysed exhaustively against phosphate buffered saline (PBS) and frozen at -70° C.

ACY-1 Assay

The electrophoretic ACY-1 assay originally described by Voss and colleagues was performed as modified by Quavi and Kit. (8,9)

Hybridoma Preparation and Screening

Two Balb/c mice were immunized intraperitoneally and subcutaneously with 100 ug of crude ACY-1 in 0.5 ml of 1:1 PBS:complete Freund's adjuvant (CFA). Four weeks later, a booster immunization of 100 ug ACY-1 in 1:1 PBS:incomplete Freund's adjuvant (IFA) was administered by the same routes. Six weeks thereafter, a final immunization of 100 ug crude Acy-1 was given intravenously and intraperitoneally. Four days later, mice were sacrificed, spleens removed and fusion with NS-1 myeloma cells performed, using polyethylene glycol, MW 6000. Hybrid myelomas were cultured in RPMI 1640 supplemented with 12% fetal bovine serum, glutamine (final concentration 600 $mg/_{ml}$), glucose (final concentration 4000 $mg/_{ml}$), hypoxanthine (1×10^{-4} M), thymidine (1.6×10^{-5} M) and aminopterin (4×10^{-7} M).

Initial screening was performed using a direct ELISA with target antigen of 1 ug crude ACY-1 incubated overnight in each well of 96 well microtiter plates (Immulon II, Dynatech Laboratories, Alexandria, VA).

Plates were blocked for two hours with PBS containing 1% bovine serum albumin and 0.05% Tween-20 (Sigma Chemical, St. Louis, MO), then washed thrice with PBS before a two hour incubation with 50 ul hybridoma supernatant. After three PBS washes, bound antibody was detected using affinity-purified goat anti-mouse immunoglobulins conjugated with horseradish peroxidase (Kirkegaard-Perry, Gaithersburg, MD) diluted 1/5000 in PBS containing 1% bovine serum albumin and 0.05% Tween-20. Plates were washed thrice, then developed using the chromogenic substrate 2,2'-azino-bis-3'-ethylbenz-thiazoline sulfonic acid (ABTS), 0.8 mM in 0.1 M sodium citrate, pH 5.0, with 0.001% H_2O_2. Optical density (OD) at 414 nm was determined on an MR 600 automated microtiter plate reader (Dynatech Laboratories). Supernatants producing an OD of greater than twice background were deemed positive and subjected to further analysis.

Secondary screening was based on the ability of hybridoma supernatants to retard electrophoretic

migration of crude ACY-1 after overnight coincubation at 4^{o} C. Hybridomas yielding supernatants positive in this assay were cloned twice by limiting dilution, then expanded into 75 cm^2 flasks. Ascites was produced by injecting hybridomas (10^6 to 10^7) intraperitoneally into pristane-primed Balb/c mice. MAb isotype was determined by double immunodiffusion against isotype specific goat anti-mouse immunoglobulins (Miles Scientific, Naperville, IL).

MAb Purification

24 mg crude ACY-1 was conjugated to 5 gm cyanogen bromide activated Sepharose 4B (Pharmacia, Piscataway, NJ) and washed according to manufacturer's instructions. This material was placed into a 15 ml column and further washed with alternating 0.1 M Tris, 1.0 M NaCl, pH 4.1 and 0.1 M Tris, 1.0M NaCl, pH 8.6 twice then with 20 mM Tris, pH 7.2. 1.0 ml of ascites was diluted by the addition of 9.0 ml 20 mM Tris, pH 7.2 and pumped

continuously through the column at 4^{o} C for five hours. The column was then washed overnight with 20 mM Tris, pH 7.2 and eluted with 0.1 M citric acid, pH 4.0. Fractions were immediately neutralized with 1.0 M NaOH, then dialysed exhaustively against PBS.

Preparation of Rabbit Anti-ACY-1 Serum

A New Zealand white rabbit was initally immunized subcutaneously with 100 ug of porcine ACY-1 (Sigma Chemical) in 1.0 ml 1:1 PBS:CFA and boosted three weeks later with 50 ug porcine ACY-1 in 1.0 ml 1:1 PBS:IFA. For four subsequent monthly immunizations, 100 ug porcine ACY-1 was subjected to sodium dodecyl sulfate polyacrilamide gel electrophoresis (SDS-PAGE) under reducing conditions, the Coomassie brillant blue stained 40,000 MW band cut out, minced finely in PBS, and injected subcutaneously in multiple sites.

Immunoblotting

Immunoblotting with either anti-ACY-1 MAbs (10% ascites) or rabbit antisera (1/50) was performed by a

previously described method. (10) In all immunoblots, 18.5 ug of crude ACY-1 was loaded per lane prior to SDS-PAGE.

Cell Lines

The K1, 314-2 and A549 cell lines were propagated in Ham's F-12 media supplemented with 5% fetal bovine serum. (11) Prior to assay, cells were harvested by trypsinization, washed twice in PBS, resuspended in a equal volume of distilled H_2O, then freeze-thawed three times. After centrifugation for five minutes in a high-speed microfuge, (Eppendorf Model 5412, West Germany), supernatants were removed and protein determined. (12)

Human Tissue

Human tissues were obtained either at autopsy (within 18 hours of death) or at the time of surgery.

Tissues were minced finely, suspended in distilled H_2O, homogenized in a Dounce tissue grinder (Wheaton Scientific), then prepared as cell lines were.

Immunoassay for Human ACY-1

A sandwich type immunoassay was devised, using the anti-ACY-1 Mab, 3D12, as a "capture" antibody. Microtiter plates (Immulon II, Dynatech) were incubated overnight with 0.125 ug/well immunoaffinity purified 3D12 in PBS. Plates were subsequently blocked for at least 24 hours with PBS containing 0.1% bovine serum albumin and 0.05% Tween-20. After washing twice with PBS, 50 ul of various dilutions in PBS of either crudely purified human kidney ACY-1 or unknown samples were incubated overnight in microtiter wells, then washed twice with PBS. Plates were then incubated with a 1/250 dilution of rabbit anti-porcine ACY-1 serum for two hours, washed twice, then incubated with a 1/600 dilution of horseradish peroxidase conjugated swine anti-rabbit immunoglobulin serum (Accurate Chemicals,

Westbury, NY) for one hour. Plates were washed with PBS, then incubated with ABTS chromogenic substrate for 1 hour. Absorbance (414 nm) was read on an automated microplate reader (MR 600, Dynatech). The background absorbance of wells incubated with PBS, rather than antigen, was subtracted from all absorbances. Standard curves were constructed using various dilutions of crude ACY-1 (1.0, 0.5, 0.1, 0.05, 0.01 and 0.005 ug/well). The slope of linear portions of unknown sample determinations was normalized to linear portions of this standard curve, with results expressed as ng standard/ug sample. All samples were run in triplicate and standard deviations calculated. For determination of recovery, 800 ug/ml of a sample with low ACY-1 content (lung) was mixed with an equal volume of 5 ug/ml standard crudely purified ACY-1 and assayed as an unknown sample. Recovery was calculated as the ratio of measured ACY-1 to the sum of the ACY-1 content contributed by lung and standard.

RESULTS

MAb Production and Characterization

Viable hybridomas were present in approximately 90% of the 384 microtiter wells into which cells were plated after fusion. Twenty wells were positive by the initial screening ELISA. Of these, four (2C1, 2C3, 3D12, 4F8) contained supernatants which retarded the electrophoretic migration of ACY-1. (Figure 1) All were of the IgG1 isotype.

As an independent test of ACY-1 specificity, immunoblotting was performed against crude ACY-1 reduced, separated by SDS-PAGE and transferred to nitrocellulose. All MAbs specifically recognized a protein of MW 40,000, similar to the published MW of bovine ACY-1. (1) This protein was not recognized by either second antibody alone or a first MAb of IgG1 isotype which is specific for an irrelevant antigen, human transferrin. (10) (Figure 2)

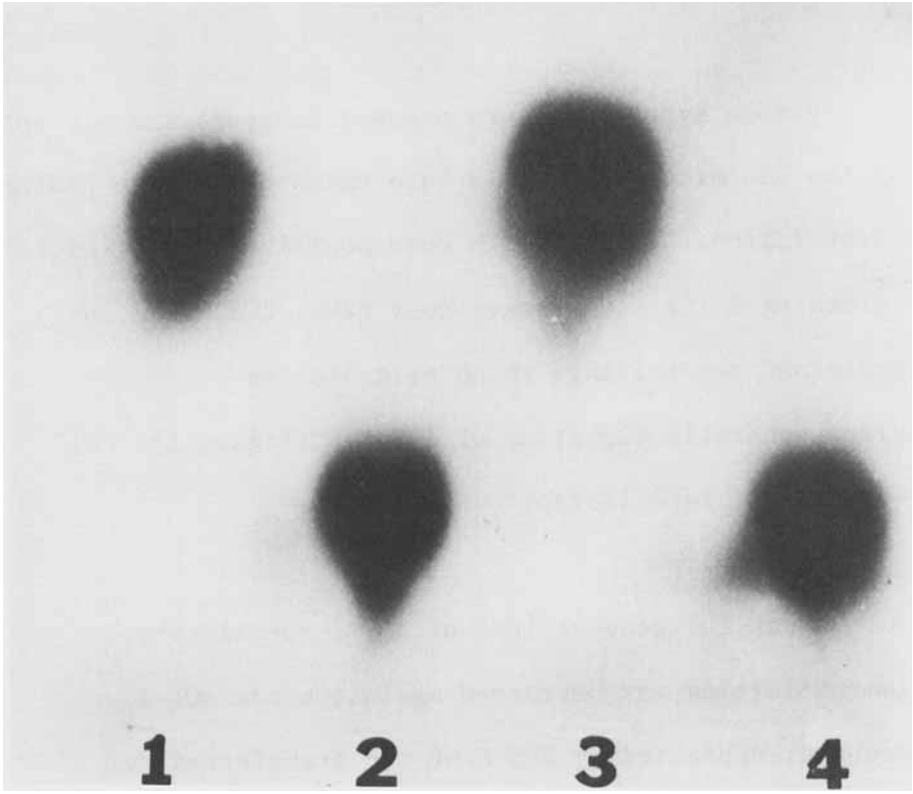

Figure 1. MAb retardation of electrophoretic migration of human ACY-1. Lane 1, enzyme preincubated with RPMI 1640, 12% fetal bovine serum, lane 2, preincubation with 3D12 containing supernatant; lane 3, preincubation with irrelevant MAb, Tf-1; lane 4, preincubation with 4F8.

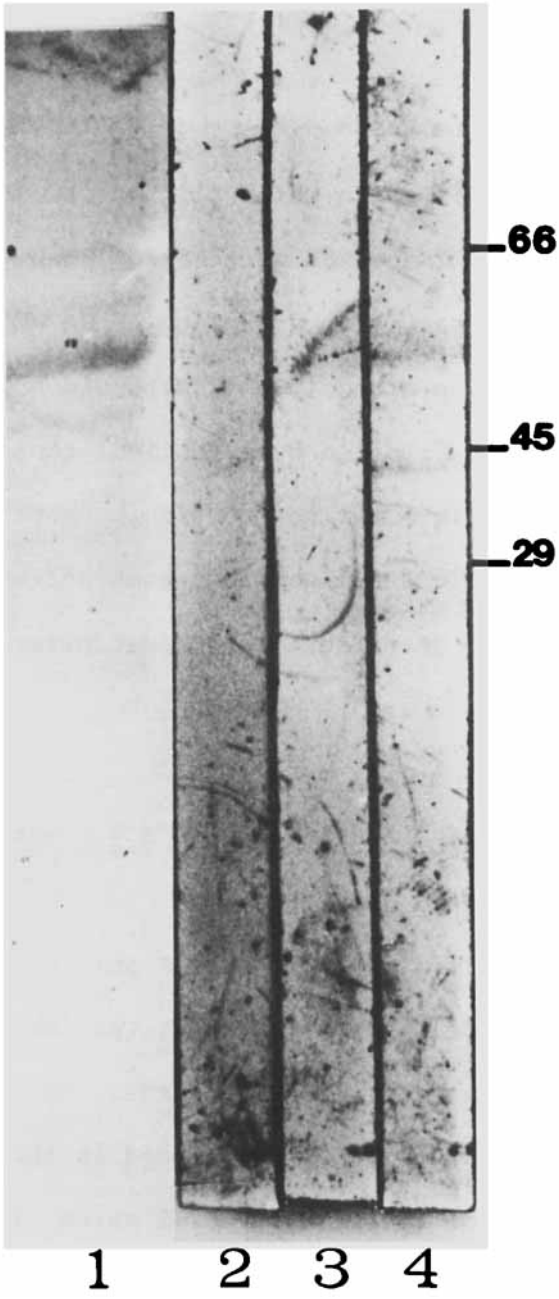

Figure 2. Immunoblot demonstrating recognition of an 40,000 MW antigen by 3D12. Lane 1, Coomassie stained crude ACY-1 preparation; lane 2, immunoblot reacted with second antibody (horseradish peroxidase conjugated goat anti-mouse) alone; lane 3, immunoblot reacted with monoclonal antibody Tf-1, which recognizes the irrelevant antigen, human transferrin; lane 4, immunoblot reacted with 3D12. The high MW band recognized by both 3D12 and Tf-1 is a major contaminating protein in the crude ACY-1 preparation and is recognized by all IgG1 subclass MAbs tested.

Direct ELISA's in which all possible combinations of 2C1, 2C3, 3D12 and 4F8 were mixed, then bound to microtiter plates coated with 1 ug crude ACY-1 revealed that the presence of additional MAbs did not augment the optical density produced by an optimal concentration of a single MAb. In addition, double immunodiffusion of crude ACY-1 against all possible combinations of MAbs did not produce detectable immunoprecipitation bands.

Evaluation of Rabbit Anti-porcine ACY-1 Serum

The commercial preparation of porcine ACY-1 with which the New Zealand white rabbit was immunized was not pure, as evaluated by SDS-PAGE. Cross-reactivity of the serum to human ACY-1 was established in that preincubation of rabbit anti-ACY-1 serum with human ACY-1 retarded electrophoretic migration of the enzyme (data not shown). Immunoblotting of porcine ACY-1 and human ACY-1 reduced, separated by SDS-PAGE, and

transferred to nitrocellulose revealed that the rabbit antiserum recognized multiple bands in the porcine ACY-1 preparation, but predominantly recognized a MW 40,000 protein in the human ACY-1 preparation. (Figure 3)

Immunoassay for Human ACY-1

A standard curve of crude human ACY-1 was analysed on each microtiter assay plate. A near-linear portion of this curve typically existed between 5 and 100 ng protein. (Figure 4) Unknown samples were initially assayed at concentrations of 0.05, 0.1, 0.5, 1.0 and 5.0 ug/well. If these concentrations failed to yield OD's which decreased in near-linear fashion with increasing dilutions, higher concentrations of 1.0, 5.0, 10, 20 and 40 ug/well were tested. (Figure 5) Depending on ACY-1 content, limits of linearity varied in unknowns. The slope of a near-linear portion of the dilution curve were used to normalize unknown samples to the standard preparation of crude ACY-1, with the unit of measurement ng standard/ug sample.

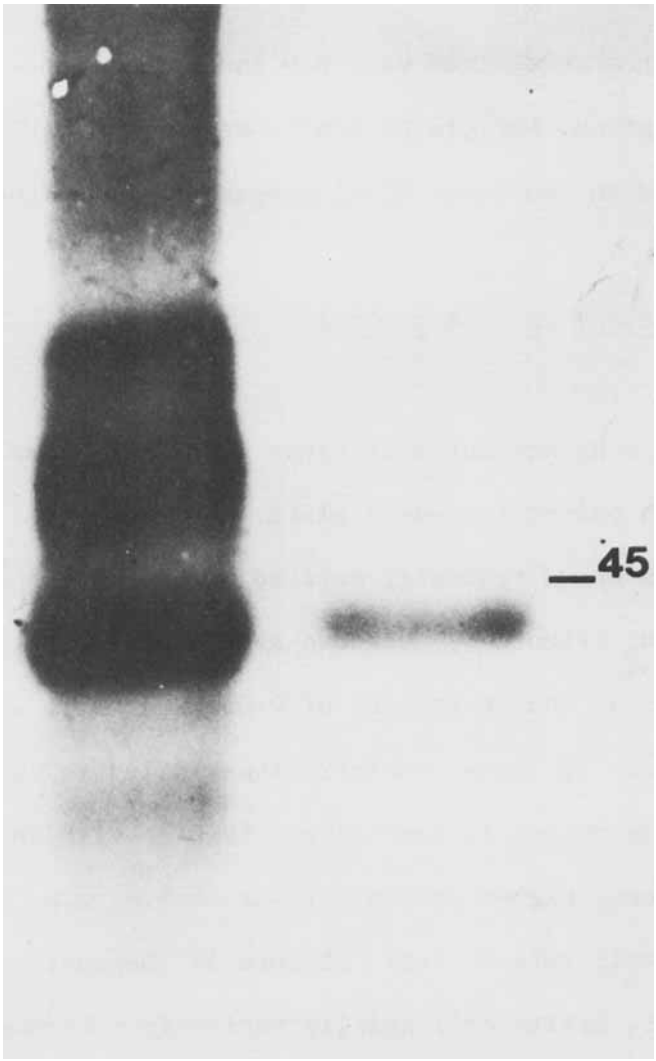

Figure 3. Rabbit anti-ACY-1 serum staining of immunoblots of porcine and human ACY-1 preparations. Left lane, porcine ACY-1; right lane, human ACY-1. Cross-reactivity of serum to human ACY-1 is relatively specific.

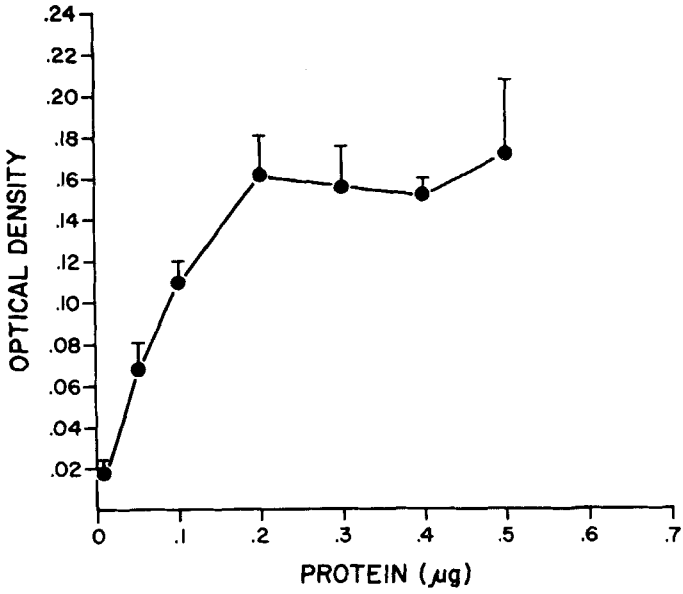

Figure 4. Standard curve for ACY-1 ELISA, demonstrating near-linearity between 5 and 100 ng. Bars represent standard deviations.

Individual samples from either tissues or cells were stable for at least four freeze-thaw cycles after initial preparation, giving similar results, generally within 20% when compared to frozen aliquots of the standard ACY-1 preparation.

Recovery averaged 102%. The precision of the assay is reflected by a coefficient of variation for the

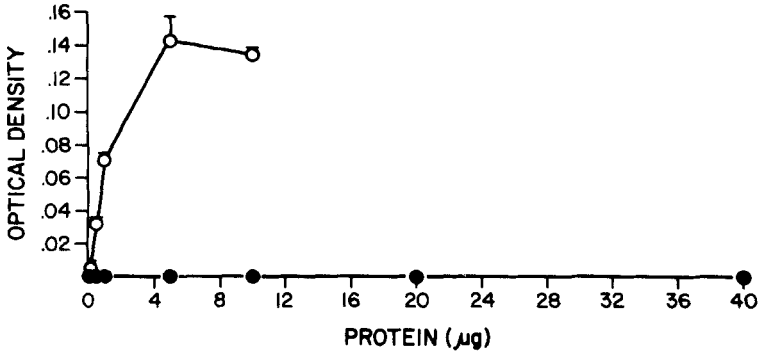

Figure 5. ACY-1 ELISA curves for human liver (o—o) and human erythrocytes (●—●), demonstrating that erythrocytes lack ACY-1 antigen. Bars represent standard deviations.

TABLE 1

ACY-1 CONTENT OF HUMAN TISSUES

Brain	19.5 ng standard/ug sample
Lung	6.2
Heart	5.6
Liver	68.5
Kidney	562.5
Adrenal medulla	4.1
Erythrocytes	0

standard curve of 15%, with similar values for unknown samples.

Chromosomal Localization of Antigen Recognized by Immunoassay

As an independent test of the specificity of the immunoassay, a Chinese hamster cell, K1, a human-Chinese hamster somatic cell hybrid containing chromosome 3 as its only human genetic material, 314-2, and a human cell line, A549, were assayed for ACY-1 antigen, with the following results: K1, 1.8 ng standard/ug protein; 314-2, 12.8 ng standard/ug protein; A549, 4.2 ng standard/ug protein. The expression of an antigen recognized in the ELISA by the somatic cell hybrid containing chromosome 3 as its only human genetic material is consistent with the human gene map assignment of ACY-1. (2)

Tissue Expression of ACY-1

A variety of human tissues obtained at autopsy, including kidney, liver, lung, brain and adrenal medulla,

as well as erythrocytes (obtained by venipuncture), were assayed for ACY-1 antigen. All but erythrocytes expressed ACY-1, with kidney having the highest expression. (Figure 5 and Table 1) All tissues which expressed ACY-1 antigen were positive by the electrophoretic activity assay. Erythrocytes were negative by this assay in addition to the ELISA. When autopsy and surgical specimens of the same tissue from different individuals were compared, autopsy specimens expressed approximately half the ACY-1 as did fresh surgical specimens. This is consistent with previous reports in which ACY-1 has been described as relatively stable. (1,7)

DISCUSSION

MAbs which recognise human ACY-1 have been produced. Several independent criteria support the specificity of these MAbs: ability to retard the electrophoretic mobility of human ACY-1, recognition of an 40,000 MW protein in immunoblots and recognition of an antigen

encoded by human chromosome 3. The MAbs are not additive in a direct ELISA nor do mixtures of the MAbs cause immunoprecipitation bands in double immunodiffusion against a crude ACY-1 preparation. Thus, although we have not performed detailed analysis of epitopic recognition by these MAbs, we have no evidence to suggest that they recognize more than one epitope. Therefore, we have chosen one MAb, 3D12, for use in additional studies.

We theorized that a sandwich-type immunoassay, using 3D12 as the capture antibody and polyclonal antibodies to detect immunoimmobilized antigen, might provide more sensitivity than a competition immunoassay. Rabbit antiserum to porcine ACY-1 was demonstrated to be relatively specific when immunoblotted against crude human ACY-1. It is likely, however, that the majority of specificity in the immunoassay is provided by the monoclonal immunologic reagent, 3D12. The specificity of the immunoassay has been demonstrated in that it does not measure human ACY-1 in erythrocytes. The assay is sensitive enough to measure human ACY-1 in small samples of human tissue.

The ACY-1 immunoassay can now be applied to the analysis of ACY-1 expression in disorders involving deletions of chromosome 3p, as well as to the purification of the enzyme, with the eventual goal of the development of nucleic acid probes for studying ACY-1 expression.

ACKNOWLEDGEMENTS

Supported by Career Development and Merit Review grants from the Veterans Administration and by a grant from the Milheim Foundation. We thank Roxie Kight for assistance in manuscript preparation. This is contribution number 939 from the Eleanor Roosevelt Institute for Cancer Research.

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