Human β-Glucuronidase Deficiency Mucopolysaccharidosis

IDENTIFICATION OF CROSS-REACTIVE ANTIGEN IN CULTURED FIBROBLASTS OF DEFICIENT PATIENTS BY ENZYME IMMUNOASSAY

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ABSTRACT An enzyme immunoassay for human β-glucuronidase was developed to determine the presence or absence of antigenically cross-reactive material (CRM) in patients with β-glucuronidase deficiency mucopolysaccharidosis. This assay provided a sensitive means of measuring the primary interaction between the enzyme molecule and antibody but required neither pure antigen nor monospecific antiserum, an important consideration, since neither of these was available. Goat antiserum to partially purified human placenta β-glucuronidase did not recognize differences in normal enzyme from human placenta, liver, fibroblasts, or blood platelets. CRM was identified in fibroblast extracts from all four of the unrelated β-glucuronidase-deficient patients studied, but titration patterns indicated genetic heterogeneity among these four mutant proteins. Fibroblast enzymes from two obligate heterozygotes were distinguishable immunologically from normal enzyme. The enzyme immunoassay was also used to compare human enzyme with liver enzyme from other mammalian species. CRM was present in liver extracts of all species tested, but the liver enzymes, except for the rabbit, were weakly cross-reactive. We conclude that despite certain limitations, the enzyme immunoassay for human β-glucuronidase is useful and that all four β-glucuronidase-deficient patients studied possess CRM.

INTRODUCTION

There has been much recent interest in the possible utility of enzyme replacement therapy for genetic disease (1–6). Deficiency of β-glucuronidase (EC 3.2.1.31) has several features that make it attractive as a model disease for the study of enzyme replacement therapy. This deficiency produces a mucopolysaccharide storage disease, several cases of which have been recently recognized (7–10). Cultured fibroblasts from these patients show exaggerated accumulation of ^{35}S-mucopolysaccharide, corrected by added β-glucuronidase (11). The human enzyme is remarkably stable and can be isolated with relative ease from a number of different organ sources (12). However, the corrective potency of the human enzyme for cultured β-glucuronidase-deficient fibroblasts varies widely, depending on the organ source (12). Thus, there appear to be different uptake forms of the enzyme.

It is not known whether or not β-glucuronidase-deficient patients produce a protein molecule that antigenically cross-reacts with normal β-glucuronidase. Low levels of β-glucuronidase catalytic activity have been detected in extracts of cultured fibroblasts of deficient patients (8, 13). While this could represent normal or mutant human β-glucuronidase, it could also represent nonspecific activity of another lysosomal hydrolase or activity of bovine β-glucuronidase taken up by deficient fibroblasts from fetal calf serum in the culture medium. The ability of deficient patients to produce antigenically cross-reactive material (CRM)[1] could be important with respect to the success and safety of enzyme replacement therapy in these patients (14). Patients with no CRM would be more likely to develop antibodies to infused enzyme that would limit

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[1] *Abbreviations used in this paper*: AS-Sepharose, antiserum-Sepharose; CRM, antigenically cross-reactive material; EIA, enzyme immunoassay; NS-Sepharose, normal serum-Sepharose; TSB buffer, 0.02 M Tris-HCl, 0.075 M NaCl, pH 7.2, containing 1.0% bovine serum albumin.

TABLE I
Protein and Enzymes in Fibroblast Extracts

Cell strain	Protein	β-Glucuronidase	β-Galactosidase	α-Galactosidase	α-Mannosidase
	mg/ml	*U/mg**	*U/mg*	*U/mg*	*U/mg*
Control (S235)	7.7	896	1697	96	688
Control (S236)	9.9	479	1427	43	352
Patient 1 (T.Y.)	7.7	8	1885	86	370
Patient 2 (J.E.)	12.2	11	782	60	366
Patient 3 (C.R.)	4.7	16	2515	140	450
Patient 4 (K.B.)	5.2	18	2154	82	561
Heterozygote (D.R.)	8.8	130	839	45	258
Heterozygote (C.S.)	13	206	1006	80	295

* U = nanomoles of 4-methylumbelliferone liberated per hour from the respective artificial substrate.

the effectiveness of further enzyme administration. Therefore, it seemed important to determine whether or not the available β-glucuronidase-deficient patients possessed CRM.

Neuwelt, et al. (15) reported an enzyme immunoassay that they used to detect and immunologically characterize CRM in arylsulfatase-A deficiency. This assay required neither pure antigen nor monospecific antiserum. Due to considerable difficulty encountered in achieving final purification of β-glucuronidase and in acquiring monospecific antiserum, we followed their general approach, with some modifications, to develop an enzyme immunoassay for this enzyme. The results obtained with this immunological approach to human β-glucuronidase deficiency are reported here. A preliminary report of this work was presented previously (16).

METHODS

Normal human β-glucuronidase. Partially purified placenta enzyme was prepared by suspending frozen placenta pieces (5-cm cubes) in 0.05 M Tris-HCl, 0.10 M NaCl buffer, pH 8.0, and homogenizing them in a Waring Blendor (Waring Products Div., Dynamics Corp. of America, New Hartford, Conn.). After centrifugation at 2,500 *g*, the supernatant was batch-absorbed with the IgG fraction of goat anti-rat β-glucuronidase coupled to Sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) (17). After mixing at 4°C overnight, the immune IgG-Sepharose was washed successively with 0.15 M NaCl, 1 M NaCl, and water. The enzyme was then eluted with 6 M urea. This eluate was made 3 M in urea and 0.02 M in potassium phosphate, pH 5.8, and centrifuged at 1,000 *g* for 10 min. The supernatant was applied to a carboxymethyl-Sephadex column suspended in the phosphate buffer and gradient-eluted with 0.13 M potassium phosphate, pH 7.2. The eluate contained partially purified β-glucuronidase. After addition of 1 g glycerol/9 ml enzyme solution to afford cryoprotection, the preparation was stored in aliquots at −70°C. This preparation was used for immunization of animals and as standard blocking enzyme and standard indicator enzyme (see below).

Crude placenta, liver, and platelet enzyme were prepared by suspending each tissue in 0.02 M Tris-HCl, 0.075 M NaCl, pH 7.5, and homogenizing the suspension in a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, N.Y.). Triton X-100 was added to the homogenate at a ratio of 0.2 g/100 ml, and the homogenate was centrifuged at 100,000 *g* for 30 min. The supernatant was recovered. After addition of 0.1 vol of 50% glycerol, the enzyme was stored in aliquots at −70°C for use in immunoassay.

Fibroblast enzyme was obtained from extracts of cultured fibroblasts. These cells were established in culture from skin biopsies from normal control patients. They were grown to confluence in 1,330-cm² glass roller bottles for 14 days after a 1:5 split, with Eagle's minimal essential medium containing 15% heat-inactivated (60°C for 60 min) fetal calf serum, 100 u/ml penicillin, 100 μg/ml streptomycin, and 15 mM Hepes buffer, pH 7.6. Fibroblasts were washed twice with 50 ml of phosphate-buffered saline, removed from the bottles by scraping, sedimented, resuspended in an equal volume of 0.01 M Tris-HCl, pH 7.5, containing 0.2% Triton X-100, and broken by sonication. The high-speed supernatant (100,000 *g* for 1 h) was assayed for protein and lysosomal enzyme concentration as previously described (13). After addition of 0.1 vol of 50% glycerol, the enzyme was stored in aliquots at −70°C for use in immunoassay.

Patient and heterozygote β-glucuronidase. Fibroblasts were obtained from four β-glucuronidase-deficient patients and two obligate heterozygotes (the parents of patient 3). Growth of fibroblasts and preparation of extracts was as described for normal enzyme. Patient 1 (T.Y.) was a 13-yr-old male with mild skeletal involvement, hypertension, and obstructive vascular disease (8). Fibroblasts were provided by Dr. A. Beaudet (Baylor College of Medicine, Houston, Tex.). Patient 2 (J.E.) was a 6-yr-old white male with moderately severe manifestations like those reported for patient 3 (7). Dr. Laird Jackson (Jefferson Medical College, Phildelphia, Pa.) provided a skin biopsy for fibroblasts. Patient 3 (C.R.) was a 3½-yr-old Negro male with a moderately severe form of the disease (7). He had mental retardation, moderately severe skeletal involvement, and hepatosplenomegaly. The mother (D.R.) and father (C.S.) of patient 3 were clinically normal. Patient 4 (K.B.) was the most severely affected patient (8). Skin fibroblasts were provided by Dr. A. Beaudet. Clinical features of this patient included severe growth and psychomotor retardation, macrocephaly, corneal clouding, severe skeletal deformities, hepatosplenomegaly, hernias, and numerous episodes of bronchopneumonia. Death occurred at 2¾ yr of age. Fibroblasts from patients 2 (J.E.) and 3 (C.R.) are available from the Human Mutant Cell Repository, Institute for Medical Research, Camden, N. J., as strains GM-151 and GM-121, respectively.

Protein and enzyme levels of the fibroblast extracts used in these studies are shown in Table I. Note that all four homozygous deficient patients had some residual β-glucuronidase activity.

Animal β-glucuronidase. Fresh liver tissue was obtained from various animals, including sheep, rabbit, rat, and calf. Liver enzyme was prepared as described above for human liver enzyme.

Preparation of antiserum. Antiserum was prepared in a goat by the subcutaneous injection of 40,000 U (166 μg) of the partially purified normal human placenta β-glucuronidase emulsified in complete Freund's adjuvant. The goat was boosted with the same dose of antigen emulsified in incomplete Freund's adjuvant on day 32, with 9,000 U on days 60 and 104, and with 4,500 U on day 132. The antiserum used in this study was harvested on day 141. Immunodiffusion in agarose gel of this antiserum against the partially purified antigen gave two precipitin lines. One precipitin line stained specifically for β-glucuronidase activity by the simultaneous diazo coupling method of Hayashi et al. (18).

Preparation of antiserum-Sepharose (AS-Sepharose) and

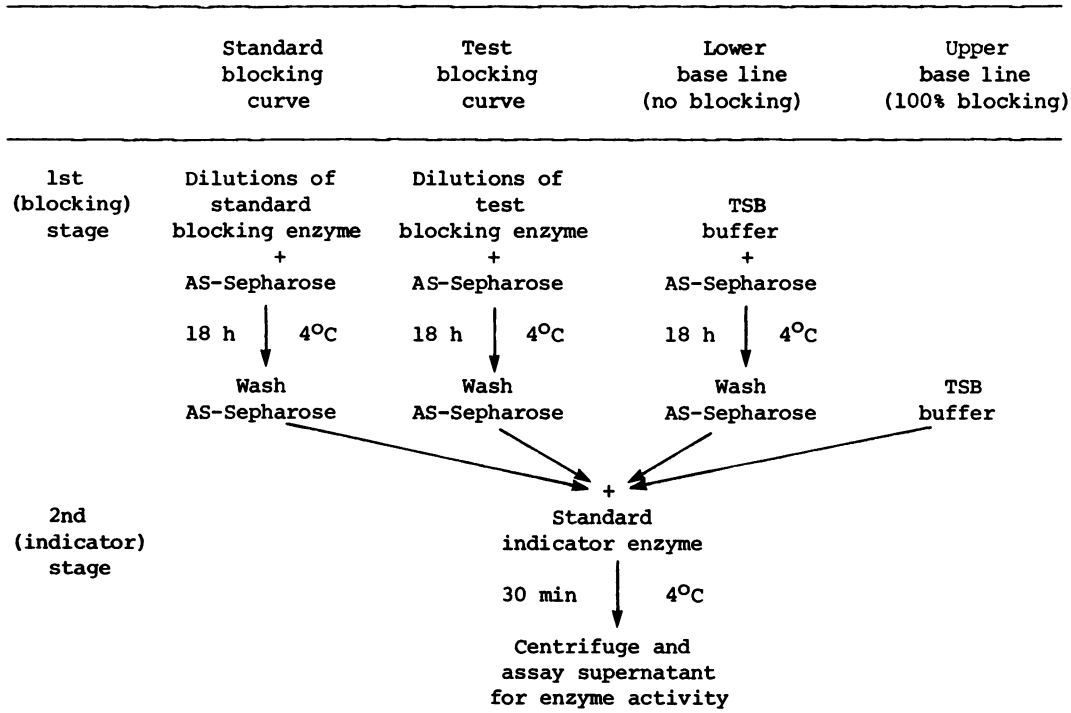

FIGURE 1 Procedure for blocking EIA. Controls are not shown.

normal serum-Sepharose (NS-Sepharose). Goat antiserum and normal goat serum were separately coupled to Sepharose 4B (17). Sepharose 4B was activated with cyanogen bromide at a ratio of 300 mg/g Sepharose. Sera were coupled to the activated Sepharose in 0.2 M citrate buffer, pH 6.0, at a ratio of 1 mg protein/ml packed Sepharose. For some experiments, sera were coupled at a ratio of 10 mg protein/ml of packed Sepharose, or the IgG fraction obtained by DEAE cellulose chromatography was coupled at a ratio of 1 mg protein/ml packed Sepharose. After mixing by rotation overnight at 4°C, coupled Sepharose preparations were blocked with 1 M ethanolamine and washed extensively as described (15). The final wash consisted of 0.02 M Tris-HCl, pH 7.2. Both AS- and NS-Sepharose were stored at 4°C in the last buffer containing 0.1% sodium azide.

β-Glucuronidase activity assay. β-Glucuronidase activity was assayed fluorometrically with 10 mM 4-methylumbelliferyl-β-D-glucuronide (Koch-Light, Elk Grove Village, Ill.) as substrate (13). 1 U of β-glucuronidase activity is defined as the activity which liberates 1 nmol of 4-methylumbelliferone/h.

The blocking enzyme immunoassay (EIA) for β-glucuronidase. The blocking EIA was modified from that previously described by Neuwelt et al. (15) for the detection of CRM for arylsulfatase-A. In this assay, aliquots of AS-Sepharose were exposed to various amounts of standard or test blocking enzyme during a first incubation. The AS-Sepharose was then washed, and its ability to bind a constant amount of standard indicator enzyme activity was determined in a second incubation. An impaired ability to bind standard indicator enzyme indicated blocking of antigen-binding sites of the AS-Sepharose by β-glucuronidase antigen. Blocking activity was plotted against the amount of β-glucuronidase catalytic activity added, rather than against protein. The reason for this modification deserves emphasis. Since the specific activity of β-glucuronidase in cultured fibroblasts increases several-fold with increasing cell density (13), there is no simple relationship between β-glucuronidase activity (and presumably CRM) and protein in extracts from normal cultured fibroblasts. Plotting blocking activity against the amount of β-glucuronidase activity in the extracts was possible in all of the studies presented, because even the extracts from homozygous deficient patients had some residual β-glucuronidase activity. However, if this assay was used for extracts with no measurable β-glucuronidase activity, the blocking activity could be plotted against protein (bearing the above reservation in mind) or against catalytic activity of another lysosomal enzyme, such as α-galactosidase or α-mannosidase (see Table I).

Partially purified normal human placenta β-glucuronidase, prepared as described above, was used as the standard blocking enzyme and as the standard indicator enzyme. The buffer used to dilute Sepharose preparations, standard or test blocking enzyme solutions, and standard indicator enzyme solutions was 0.02 M Tris-HCl, 75 mM NaCl, pH 7.2, containing 1.0% bovine serum albumin (TSB buffer). The dilution of AS-Sepharose used in the blocking EIA was determined as follows: 0.4 ml of TSB buffer, 0.5 ml of a 1:400 dilution of standard indicator enzyme, and 0.1 ml of serial twofold dilutions of AS-Sepharose, NS-Sepharose, or TSB buffer were added to multiple screw-cap glass tubes. The tubes were mixed and incubated on a rotator at 4°C for 30 min. After centrifugation at 1,000 *g* for 3 min at 4°C, 0.5 ml of supernatant was removed and assayed for β-glucuronidase activity. The percent enzyme bound was determined and plotted against the dilution of AS- or NS-Sepharose. The dilution of AS-Sepharose that bound approximately 33% of the enzyme under the above conditions was chosen. This was a 1:1.5 dilution for most Sepharose preparations. NS-Sepharose was used as a control for nonspecific binding at this same dilu-

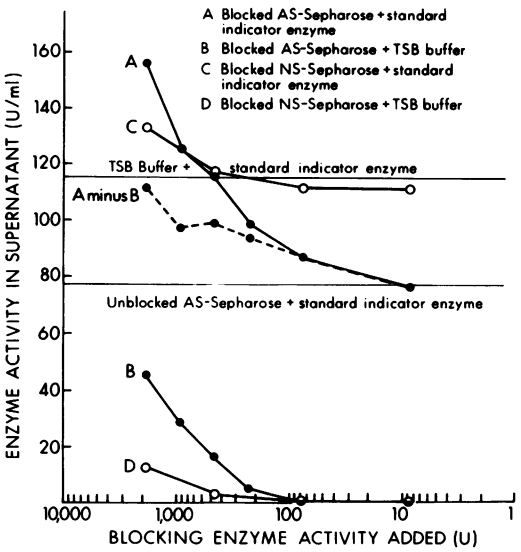

FIGURE 2 A standard blocking curve and its controls. The ability of the blocked and washed AS-Sepharose or NS-Sepharose to bind a constant amount of standard indicator enzyme (curves A and C) or to release blocking enzyme in the presence of buffer (curves B and D) are indicated. Curve A minus B is the blocking curve corrected for released enzyme. The blocking curves expected for test enzymes with altered ratios of immunoreactivity to enzymatic activity are discussed in the text. The specific activity of the standard blocking (and indicator) enzyme was 241,000 U/mg protein.

tion; it occasionally bound a small amount of standard indicator enzyme, ranging from 0 to 6%.

The blocking EIA was performed as follows: for the first (blocking) stage of the assay, 0.4 ml of TSB buffer, 0.5 ml of serial dilutions of standard or test blocking enzyme, and 0.1 ml of appropriately diluted AS-Sepharose were added to multiple screw-cap glass tubes. Tubes were mixed and incubated on a rotator at 4°C for 18 h. The AS-Sepharose was washed by centrifuging the tubes at 1,000 *g* for 3 min at 4°C and removing all but 0.1 ml (which contained the Sepharose) by aspiration through a pipette with a short U-tip. 7.4 ml of ice-cold TSB buffer was added, the tubes were mixed and recentrifuged, and all but 0.1 ml was again aspirated. For the second (indicator) stage of the assay, 0.4 ml of TSB buffer and 0.5 ml of a 1:400 dilution of standard indicator enzyme were added to the tubes containing blocked and washed Sepharose. The tubes were mixed, incubated on a rotator at 4°C for 30 min, and again centrifuged. 0.5 ml of supernatant was removed and assayed for β-glucuronidase activity. Standard and test blocking enzyme were run simultaneously on every assay. The level of enzyme activity remaining in the final supernatant from the second stage of the assay was plotted against the amount of standard or test blocking enzyme activity added in the first stage of the assay.

The lower base line (no blocking in first stage) was determined by adding 0.9 ml of TSB buffer to 0.1 ml of AS-Sepharose for the first incubation (i.e., omitting blocking enzyme) and proceeding as in the first and second stages of the assay. The theoretical upper base line (100% blocking in first stage) was determined by adding 0.5 ml of TSB buffer to 0.5 ml of a 1:400 dilution of standard indicator enzyme in tubes containing no AS-Sepharose and proceeding as in the second stage of the assay with the incubation at 4°C. Complete blocking in the assay would displace the curve from the lower base line to the theoretical upper base line. Percent blocking was calculated by dividing the observed displacement (units per milliliter observed − units per milliliter at lower base line) by complete displacement (units per milliliter at upper base line − units per milliliter at lower baseline), and multiplying by 100%. A diagram of the assay is shown in Fig. 1.

Two controls were included. A control for the maximum amount of blocking enzyme bound by the AS-Sepharose in the first stage of the assay and subsequently released in the second stage (release control) was performed by substituting 0.5 ml TSB buffer for the standard indicator enzyme. A control for nonspecific binding by the AS-Sepharose was performed by substituting NS-Sepharose for AS-Sepharose in various tubes.

Due to the limited amounts of patient and heterozygote blocking enzymes available and also to the limited number of tubes that could be included in one assay because of technical considerations, each point on the blocking curves represented a single sample determination. To assess reproducibility, every test blocking enzyme was assayed at least twice. While the exact level of enzyme activity in the final supernatant for each dose of blocking enzyme varied slightly from one assay to another, the difference (or similarity) observed between test blocking curve and standard blocking curve was always consistent. The standard deviation of the enzyme assay at an enzyme level of 100 U/ml (in the working range of the EIA) was 1.8 U/ml, and coefficient of variation was 1.8%.

RESULTS

Characteristics of the blocking EIA for β-glucuronidase. The experimental and control curves for the standard blocking enzyme are shown in Fig. 2. Experimental curve A rises above the upper base line, indicating that some of the enzyme bound by AS-Sepharose in the first stage of the assay is released in the second stage of the assay. Control curve B indicates that released enzyme contributes appreciably to curve A at high doses of blocking enzyme but not at low doses. While the displacement of the lower portion of curve A from the lower base line represents true blocking, the displacement of the middle to upper portions of curve A represents a combination of true blocking and a gradually increasing component of release of blocking enzyme. One can approximate a true blocking curve by correcting for the "release phenomenon." If for each dose of blocking enzyme, the points on curve B are subtracted from curve A, a "minimum blocking curve" representing only blocking can be constructed. Since curves corrected for released enzyme (A minus B) were felt to be a considerably more accurate representation of true blocking than uncorrected curves, corrected curves were used in the data to be presented. Portions of curves not requiring correction are indicated by solid lines; portions requiring corrections are indicated by dashed lines.

Control curve C of Fig. 2 is below the upper base line at low doses of blocking enzyme, indicating a small amount of nonspecific binding of standard indicator enzyme by NS-Sepharose. At high doses of block-

ing enzyme this curve rises above the upper base line, indicating that some enzyme was bound in the first stage of the assay and released in the second stage. Control curve D indicates the extent of this enzyme release. Curve C thus represents the release of nonspecifically bound blocking enzyme. Since the corrective factor of curve D is included in the corrective factor of curve B, curve D was not run routinely.

Many efforts were made to maximize the avidity of the AS-Sepharose for enzyme and thus minimize the release phenomenon. The goat was injected with low doses of immunogen on multiple occasions in an effort to enhance the avidity of the antiserum. AS-Sepharose bound enzyme activity more avidly when coupled at a ratio of 1 mg protein/ml packed Sepharose than at 10 mg/ml. Consequently, the former ratio was used. Use of Sepharose to which the purified IgG fraction of the goat antiserum was coupled at a ratio of 1 mg protein/ml packed Sepharose did not improve avidity or reduce the release phenomenon. Consequently, whole AS-Sepharose was used. The release phenomenon was reduced by carrying out the incubations of both the first and second stages of the assay at 4°C rather than 37°C. Additionally, the binding of enzyme activity to the AS-Sepharose was as rapid at 4°C as at 37°C. Accordingly, incubation temperatures were kept at 4°C. When the incubation during the first stage of the assay lasted 1 h, the standard blocking curve failed to reach the upper base line. Accordingly, this incubation was prolonged to 18 h. While this allowed more complete blocking of the lower affinity antigen-binding sites of the AS-Sepharose, it also enhanced the release phenomenon. Nevertheless, this latter incubation time was chosen as the best compromise methodology.

In the studies below, the assay presented in Fig. 2 was used to compare the blocking curves of test enzymes with that of standard enzyme. A blocking curve for a test enzyme with no CRM would be expected to fall along the lower base line. For a test enzyme with greater reduction in immunoreactivity than enzymatic activity, the blocking curve would be expected to shift to the left of the standard blocking curve. A test enzyme with greater loss of enzymatic activity than immunoreactivity would have a blocking curve shifted to the right of the standard curve. A test enzyme with proportional reduction of immunoreactivity and enzymatic activity (e.g. in a mutant fibroblast extract with low levels of normal enzyme) would be expected to give a blocking curve that fell along the standard blocking curve.

Comparison of β-glucuronidase from different human sources. The ability of human β-glucuronidase from placenta, liver, fibroblast, and platelet sources to block in the EIA is shown in Fig. 3. Crude placenta, liver, fibroblast, and platelet enzymes were each assayed simultaneously with the partially purified placenta enzyme used as standard blocking enzyme. Enzyme from each of the four sources generated blocking curves indistinguishable from those generated by the standard blocking enzyme. Thus no antigenic differences could be detected between placenta, liver, fibroblast, and platelet enzymes with goat antiserum to the placenta enzyme.

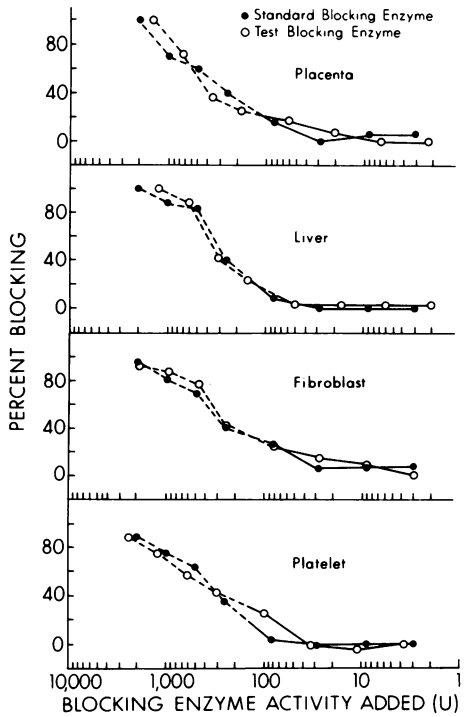

FIGURE 3 Blocking curves of β-glucuronidase from different human organ sources. The percent blocking of the binding capacity of AS-Sepharose for standard indicator enzyme is shown. Crude placenta, liver, fibroblast, and platelet enzymes were used as test blocking enzymes, and partially purified placenta enzyme as standard blocking enzyme. Curves are corrected for the release of blocking enzyme where this control was positive (dashed lines). The specific activities of the blocking enzymes in units per milligram protein were: standard, 241,000; placenta, 169; liver, 956; fibroblast, 471; and platelet, 227.

Comparison of β-glucuronidase from cultured fibroblasts of patients with β-glucuronidase deficiency mucopolysaccharidosis. The ability of extracts from cultured fibroblasts of patients with β-glucuronidase deficiency mucopolysaccharidosis to block in the EIA is shown in Fig. 4. Release controls performed with the patients' fibroblast extracts indicated that no β-glucuronidase activity was released from the blocked and washed AS-Sepharose during the second stage of the assay for any of the blocking doses used. Thus the blocking curves attained with patients' fibroblast extracts did not require correction. Fibroblast extracts from all four patients with β-glucuronidase deficiency blocked in the EIA, indicating the presence

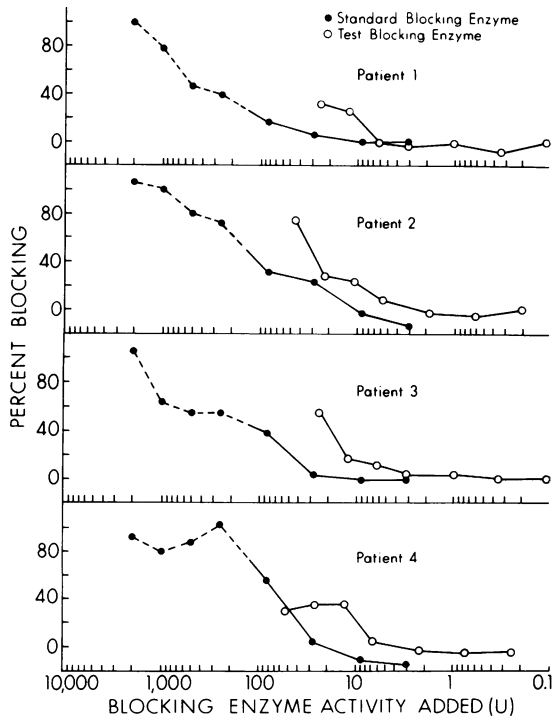

FIGURE 4 Blocking curves of extracts from cultured fibroblasts of patients with β-glucuronidase deficiency mucopolysaccharidosis. Patient fibroblast extracts were used as test blocking enzymes. The specific activities of the blocking enzymes in units per milligram protein were: standard 241,000; patient 1, 8; patient 2, 11; patient 3, 16; and patient 4, 18.

of CRM. The patient fibroblast extracts contained at least as much blocking activity per amount of enzyme activity as did normal enzyme (Fig. 4). The extract of patient 4, the most severely affected patient clinically, generated a severely compromised blocking curve. While this enzyme antigenically cross-reacted with normal enzyme, it was also deficient in the antigenic determinants present on normal enzyme. This is demonstrated by the fact that the two highest doses of blocking enzyme failed to block more than the third highest dose, indicating that the maximum blocking capacity of the CRM (approximately 36%) had been attained. The extracts of patients 2 and 3, less severely affected clinically, generated less severely compromised blocking curves. These curves demonstrated a minimum of 76% and 56% blocking, respectively, and did not reach an upper plateau at the highest available doses of blocking enzyme. The correlation between degree of blocking activity in fibroblast extracts and clinical severity is not complete. The extract of patient 1, the least affected patient clinically, generated a blocking curve demonstrating only 32% blocking. In this case, however, the curve had not yet definitely reached an upper plateau at the highest available dose of blocking enzyme, and the maximum blocking capacity of the mutant protein is uncertain. It should be noted that there is no a priori reason to expect a perfect correlation between the degree of antigenic loss of mutant protein and clinical severity of disease. Antigenic activity and catalytic activity might reside in different portions of the β-glucuronidase molecule and be affected independently by at least some mutations.

Comparison of β-glucuronidase from cultured fibroblasts of two obligate heterozygotes for β-glucuronidase deficiency mucopolysaccharidosis. The ability of enzyme from cultured fibroblasts of two obligate heterozygotes for β-glucuronidase deficiency (mother and father of patient 3) to block in the EIA is shown in Fig. 5. Release controls performed with these extracts indicated that no enzyme activity was released from the blocked and washed AS-Sepharose during the second stage, except for the two highest doses of the father's extract. Thus the blocking curves obtained with heterozygotes' fibroblast extracts did not require correction except at those two points. Fibroblast extracts from both heterozygotes blocked in the EIA. Both fibroblast enzymes contained at least as much blocking activity per amount of enzyme activity as did normal enzyme (Fig. 5). This result would be expected if the heterozygote fibroblasts contained both normal and mutant enzyme. Since β-glucuronidase is a tetramer (19–21), the heterozygote enzyme most likely represents varying combinations of normal and abnormal monomers in a tetrameric enzyme (22).

Comparison of β-glucuronidase from livers of dif-

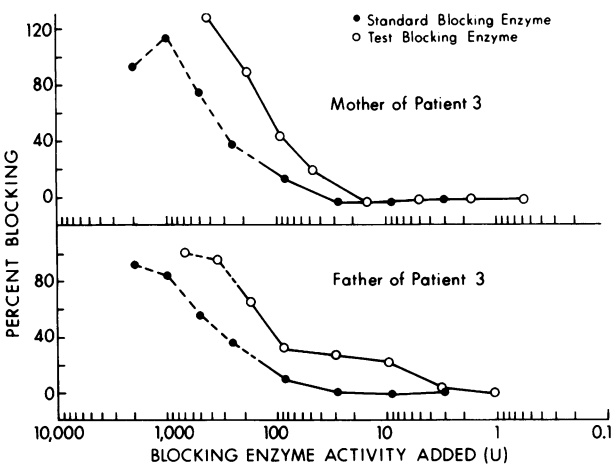

FIGURE 5 Blocking curves of β-glucuronidase from cultured fibroblasts of two obligate heterozygotes for β-glucuronidase deficiency mucopolysaccharidosis. Fibroblast extracts of the mother and father of patient 3 were used as test blocking enzymes. The specific activities of the blocking enzymes in units per milligram protein were: standard, 241,000; mother, 130; and father, 206.

ferent mammals. The ability of liver enzyme from sheep, rabbits, rats, and calves to block in the EIA is shown in Fig. 6. Release controls indicated that no enzyme activity was released from the blocked and washed AS-Sepharose during the second stage of the assay for the lower blocking doses used; however, gradually increasing amounts were released for the intermediate and higher blocking doses. Thus the mid and upper portions of the curves are corrected for release. Enzyme preparations from liver of all animals studied possessed material that antigenically cross-reacted with the human enzyme. Human liver enzyme generated a blocking curve similar to that of the standard blocking enzyme (Fig. 3). The blocking curves for the sheep, rat, and calf enzymes were shifted to the left of the standard blocking curve. This shift indicated that the animal enzymes possessed less blocking activity for the antibody to human enzyme per unit of catalytic activity than normal human enzyme. The rabbit enzyme appeared quite similar to the human in the EIA. However, the blocking curve for rabbit enzyme stored for several months at −70°C approached that of the other animals. The blocking and catalytic activity of human enzyme stored for several months at −70°C was stable.

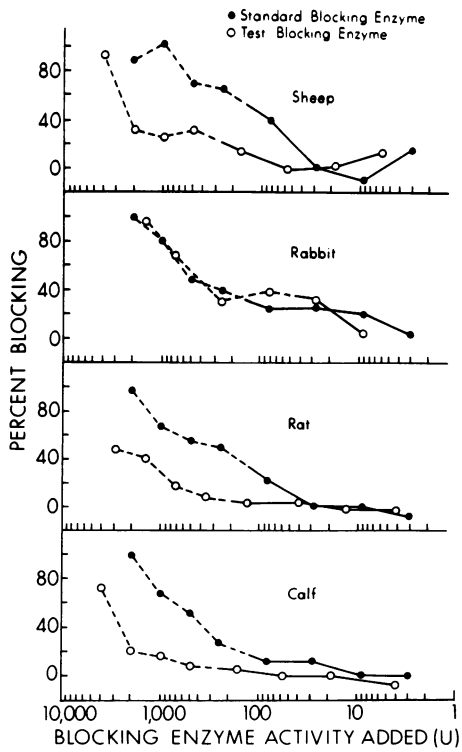

FIGURE 6 Blocking curves of β-glucuronidase from liver of different mammalian species. Crude sheep, rabbit, rat, and calf enzymes were used as test blocking enzymes. The specific activities of the blocking enzymes in units per milligram protein were: standard, 241,000; sheep, 598; rabbit, 745; rat, 464; and calf, 366.

DISCUSSION

The presence of CRM in extracts of fibroblasts from deficient patients indicates that, in each of the mutant fibroblast cell lines, at least one of the two mutant alleles specifies a cross-reactive antigen or that two possibly different mutant alleles complement one another in producing a cross-reactive antigen. The findings in fibroblast extracts from the one pair of heterozygotes tested are compatible with the interpretation that both produce normal and abnormal enzyme.

One might question the importance of demonstrating CRM in mutant extracts containing some residual β-glucuronidase activity, since the presence of CRM might be assumed from the residual activity. However, the low level of β-glucuronidase activity measured with the artificial substrate could be due to lack of specificity of another hydrolase. The presence of CRM cannot be assumed from low levels of residual activity when considering enzyme replacement in a deficient patient.

Another important question concerning the interpretation of these studies is whether the low level of "residual" β-glucuronidase activity could represent bovine β-glucuronidase taken up from the fetal calf serum in which the fibroblasts were grown (13). This possibility was greatly reduced in these studies by using fetal calf serum heated to temperatures that inactivate bovine β-glucuronidase. The blocking curves observed with extracts of mutant fibroblasts cannot be attributed to bovine enzyme taken up from fetal calf serum for two reasons. For bovine enzyme to produce the degree of blocking observed would require the addition of 100 times the amount of enzymatic activity present in the mutant extracts. Furthermore, the uptake of CRM from the heat-inactivated fetal calf serum is unlikely because the blocking activity of bovine enzyme was completely eliminated by the thermal inactivation used to destroy the β-glucuronidase activity in fetal calf serum.

β-Glucuronidase from different human tissue sources is known to be heterogeneous electrophoretically and to vary in the proportion of high and low in vitro "uptake" forms (12, 23). However, no antigenic differences could be detected by the EIA when studied with an antiserum against the placental enzyme. The placental enzyme is predominantly the low-uptake form. It is possible that antibody to the high-uptake form of the enzyme, which has characteristic isoelectric properties (23), might distinguish antigenic determinants not shared by other forms of the enzyme.

These studies extend experience with the blocking EIA described by Neuwelt et al. (15) for arylsulfatase

A to the assay of mutant and other forms of β-glucuronidase. The chief advantage of the EIA is that it requires neither pure antigen nor monospecific antibody. However, the assay has some limitations. The most troublesome feature of the assay described was the release phenomenon. Whether or not this phenomenon was a problem in the initial description of the method (15) is uncertain. In that report, two of the blocking curves with normal enzymes appeared to reach an upper plateau at the upper base line, which suggests that there was little, if any, release. However, many of the other blocking curves did not reach an upper plateau and failed to reach the upper base line. The control, in which blocked and washed AS-Sepharose was incubated in buffer alone, was not reported (15). The release phenomenon may have been prominent in our assay because the initial stage of incubation of AS-Sepharose with blocking enzyme was prolonged to 18 h. This allowed enzyme to be bound to a higher proportion of lower affinity sites. While this improved the sensitivity of the assay, it also enhanced the release phenomenon.

The explanation for the absence of the release phenomenon with the patients' fibroblast extracts (Fig. 3) is uncertain. This could be related at least in part to the dose of blocking enzyme activity added. Release was not observed with standard blocking enzyme until a dose of at least 100 U had been added (Fig. 4). A dose this high was not reached with the patients' extracts.

It would appear that a high-avidity antiserum is of utmost importance to the success of this assay. The sensitivity of our assay was greater and the release phenomenon less with the goat antiserum used than with a rabbit antiserum we tried initially. A possible explanation may be inferred from Fig. 6. The close antigenic similarity between the rabbit and human liver enzymes suggests that rabbits might have recognized fewer antigenic determinants in the human enzyme as foreign than did the goat, which produced an antiserum of greater avidity. Even with the more avid goat antiserum, the release phenomenon could not be completely eliminated. In spite of the indicated limitations of the EIA, it is clear from these studies that this assay provided useful information in a system where neither pure antigen nor monospecific antiserum was available.

The CRM in extracts of fibroblasts from β-glucuronidase-deficient patients is a favorable finding from the standpoint of immunological acceptance of enzyme replacement therapy in such patients. An immune response directed against infused enzyme could lead to treatment failure or to iatrogenic disease (14). However, whether or not these patients possess sufficient CRM to obviate an immune response to infused enzyme cannot be determined from this study.

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