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CROSS-REACTING MATERIAL TO MONOCLONAL ANTI-G6PD[1] IN THE ABSENCE OF CATALYTIC ACTIVITY

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SUMMARY: Monoclonal antibody prepared against highly purified rat liver G6PD was used to probe the mode of regulation of this enzyme in a mammalian model system. Material cross-reacting with antibody against liver G6PD was found in similar amounts in extracts of two genetically related rat hepatoma cell lines, only one of which exhibits detectable enzymatic activity when both are cultured under identical conditions *in vitro*. The data suggest a post-translational event is necessary for the expression of catalytic activity for G6PD in this model system.

G6PD has been the subject of intense study due in part to the great number of variant forms characterized in human populations (1,2). Still the mechanism(s) of its expression remains unclear (2). A cell culture system has been described as a model for studying the regulation of G6PD activity in mammalian cells (3). We report here observations with a monoclonal antibody to highly purified rat liver G6PD (4,5), employed to gain insight into the mechanism of expression of this enzyme in this unique model.

Cells of the U/B 9QD line, derived from the H-35 Reuber hepatoma, fail to exhibit detectable G6PD activity when grown *in vitro* but manifest this activity when grown as tumors *in vivo* (3). Another line, U/B 9QS, arose from U/B 9QD by spontaneous chromosome loss *in vitro*. Concommitant with the reduction in

[1] Abbreviations: G6PD: glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP oxidoreductase, EC 1.1.1.49); 6PGD: 6-phophogluconate dehydrogenase (6-phospho-D-gluconate: NADP oxidoreductase, EC 1.1.1.43); PBS: phosphate-buffered saline; NADP: nicotinamide-adenine dinucleotide phosphate, oxidized form; NADPH: nicotinamide-adenine dinucleotide phosphate, reduced form; ELISA: enzyme-linked immunosorbent assay; and β-GAL: β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23).

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chromosome mode, G6PD activity became readily demonstrable in U/B 9QS cells and has remained so after repeated transfer in vitro (6). The appearance of this enzyme activity in vivo in the parenchyma of tumors formed with cells phenotypically negative for G6PD suggested the presence of an intact structural gene under negative control in vitro. It was of interest to determine whether this gene was functioning under in vitro conditions when catalytic activity was not apparent. Evidence for the presence of inactive structural protein, gained with monoclonal anti-G6PD, affirms the expression of the structural gene and suggests some post-translational event is necessary for the realization of G6PD activity in this mammalian model system.

METHODS AND MATERIALS

Cell Lines and Culture Techniques. The origin and description of the cells and culture techniques used in this study have been published (3,6). G6PD activity is not demonstrable by spectrophotometric or cytochemical methods in U/B 9QD cells grown in vitro. The U/B 9QS cell line, derived from U/B 9QD by chromosome loss, exhibits G6PD by either assay method. Both lines were maintained under identical culture conditions; each was checked periodically by the method of Chanock, et al. (7) and shown to be free of Mycoplasma species.

Preparation of Cell Extracts. Cells from cultures of U/B 9QD and U/B 9QS were washed in PBS and disrupted in a Potter-Elvejhem homogenizer in 0.1 M Tris buffer, pH 7.6, containing 0.1 mM NADP, 35.5 mM $MgCl_2$, and 2 mM β-mercaptoethanol. Homogenate supernatants were separated by centrifugation in an Eppendorf microcentrifuge. Aliquots were taken for protein measurements at 280 nm, for 6PGD and G6PD activity determinations by the spectrophotometric method, and as sources of antigens for immunoassays.

Enzyme Activity Assay. G6PD activity was determined from the difference between 6PGD plus G6PD activity and that of 6PGD alone, by a modification of Dror's spectrophotometric method (4). A 50 µl aliquot of test sample was added to 1 ml of reaction mixture containing 4 µmol of 6-phosphogluconate and 1 µmol of NADP in 0.1 M Tris, pH 7.6, with 35.5 mM $MgCl_2$. Total dehydrogenase activity, 6PGD plus G6PD, was determined separately by including 4 µmol of glucose-6-phosphate to the above mixture. One enzyme unit was defined as the quantity of enzyme which formed 1 µmol of NADPH per minute under the defined assay conditions. The rate of reduction of NADP was determined from the rate of change in absorbance at 340 nm at 30°C.

Preparation of Monoclonal Antibody. Monoclonal antibody was obtained from a hybridoma clone after fusing mouse myeloma cells with spleen cells of hyperimmunized mice as described (5). The antigen used for this purpose was a greater than 6000-fold purified G6PD preparation from rat liver (4).

Immunoassay. Cell extracts were assayed for antigenic activity by the ELISA method of Engvall and Perlmann (8) essentially as described by Douillard and Hoffman (9). Replicate wells of polyvinylchloride microtiter plates were coated with homogenate supernatants from either U/B 9QD or U/B 9QS by incubation overnight at room temperature (100 µl per well). The wells were washed with PBS containing 0.15% bovine serum albumin and 0.02% sodium azide, incubated with 100 µl of 1% bovine serum albumin in PBS for 1 hour at room temperature, then washed again. The wells were next treated overnight at room temperature,

with various dilutions of concentrated culture supernatants from either the parent mouse myeloma cell line or a hybridoma clone producing antibody against rat liver G6PD. The wells were washed as before and treated with a hybridoma screening reagent. The latter was β-galactosidase conjugated to $F(ab')_2$ fragments of sheep anti-mouse IgG (light and heavy chain specific). Excess reagent was washed off, and the substrate, p-nitrophenyl-β-D-galactoside, was added. The reaction was followed according to the manufacturer's protocol, and the results measured at 405 nm with a Bio-Tek ELISA reader. Assay conditions were adjusted so that β-galactosidase activity was directly proportional to the amount of antigen or antibody over a finite dilution range. Cellular extracts were adjusted to the same A_{280}, duplicate aliquots were tested at each level of antibody, and mean response was corrected for blank values obtained with myeloma supernatants. Dose/response results obtained with each antigen, 9QD and 9QS, were fit by the method of least squares and compared by the Student's t-test with the programable Monroe calculator, Model 1860.

Materials. β-mercaptoethanol, glucose-6-phosphate, 6-phosphogluconate, and NADP were purchased from Sigma Chemical Co. (St. Louis, MO). Polyvinylchloride microtiter plates were obtained from Dynatech (Cambridge, MA), and the hybridoma screening reagent from Molecular Diagnostics (Rockville, MD). All other chemicals were reagent grade.

RESULTS

Dehydrogenase Activity. Homogenate supernatants from U/B 9QD and U/B 9QS, adjusted to similar total protein concentrations, showed identical 6PGD activity. This is illustrated in a copy of a typical tracing obtained with the spectrophotometric assay (Fig. 1, Sections A and C). U/B 9QD preparations did

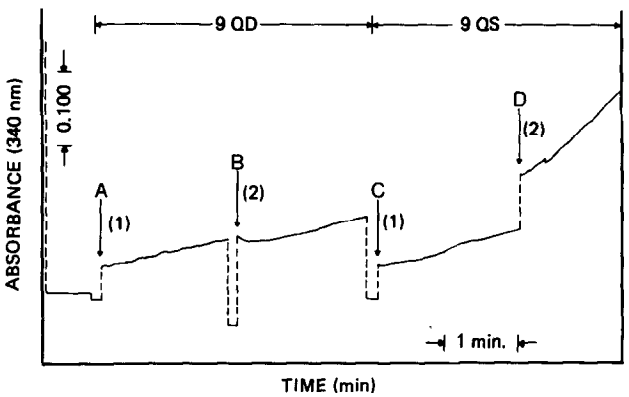

Fig. 1. Spectrophotometric assay for 6PGD and G6PD. Washed cultured cells were homogenized in TRIS/Mg/β-mercaptoethanol and centrifuged in a microcentrifuge. 50 µl of supernatant fluid was added to 1.0 ml reaction mixtures containing: (1) 6PGD assay: 4 µmol 6-phosphogluconate, 1 µmol NADP, 0.1 M TRIS/35.5 mM $MgCl_2$, pH 7.6, (2) 6PGD + G6PD assay: as for (1) plus 4 µmol glucose-6-phosphate

Rate of change in absorbance was followed at 340 nm; 30°C. G6PD activity was determined from the difference in absorbance observed with (1) and (2) above.

Letters indicate separate sections of the overall assay.

Arrows show points of addition of indicated preparations, 9QD or 9QS.

Numbers identify enzyme assay mixtures, for 6PGD (1) or G6PD(2).

TABLE 1. IMMUNOASSAY FOR G6PD

Antigen[a]	Culture Supernatant	β-Galactosidase Activity (A_{405})				
		10µl[b]	20µl	30µl	40µl	50µl
U/B9QD	Mouse Myeloma	.054[c]	.041	.065	.068	.049
(A_{280}: 1.935)	Hybridoma clone GDR1	.639	.847	1.153	1.290	1.442
U/B9QS	Mouse Myeloma	.115	.085	.075	.068	.049
(A_{280}: 2.035)	Hybridoma clone GDR1	.594	.817	1.056	1.629	1.440

Cell-free extracts of G6PD-positive and G6PD-negative hepatoma cell lines were tested for antigenic activity with a monoclonal antibody to purified rat liver G6PD by the ELISA method (9).

[a]Cell extracts (homogenate supernatants)
[b]Volumes of myeloma and hybridoma culture supernatants
[c]All values represent the means of 2 determinations

not exhibit any further dehydrogenase activity when glucose-6-phosphate was included in the assay (Fig. 1, Section B). However, under the same conditions U/B 9QS showed 0.105 units of G6PD activity per ml (Fig. 1, Section D).

Immunoassay. Homogenate supernatants from both U/B 9QD and U/B 9QS cells grown in vitro contained similar amounts of cross-reacting material against the culture supernatant of a hybridoma clone (GDR1) producing antibody specific for highly purified rat liver G6PD. Typical results are shown in Table 1. β-GAL indicator activity was linearly related to the amount of antibody over a finite dilution range, for cell-free preparations of both lines. This is shown in Fig. 2. The slopes of the straight lines fit for each cell extract by the method of least squares were shown to be identical when analyzed by the Student t-test.

DISCUSSION

The use of monoclonal antibodies allows the determination of specific activity in the strictest sense, i.e. that based on immunoreactive structural protein and not on total protein (10). Such antibodies make possible comparative measurements even in the absence of catalytic activity and may serve as sensitive probes into the mechanism of expression of enzyme activity.

The data presented here shows that a catalytically defective protein is present in U/B 9QD (G6PD-negative) cells at apparently the same level as that of the active structure in genetically related G6PD-positive cells. This suggests a post-synthetic event is necessary for the ultimate realization of catalytic

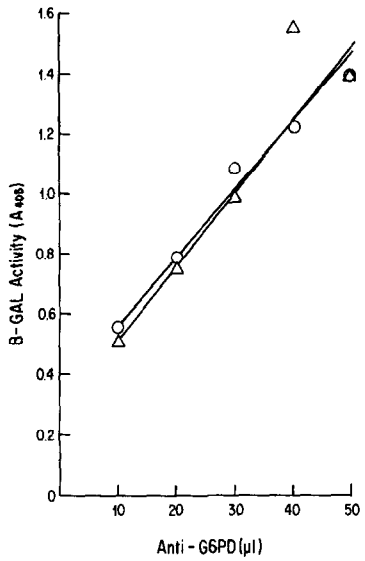

Fig. 2. Comparative Immunoassay (ELISA) for G6PD. One hundred microliters of cell-free extracts of each antigen, 9QD (-0-) and 9QS (-Δ-), were fixed to polyvinylchloride microtiter plates and treated with 100 µl of various dilutions of culture supernatant of a hybridoma secreting a monoclonal antibody against highly-purified rat liver G6PD. A species specific secondary antibody, conjugated with β-GAL, was added; excess was washed away. A chromogenic substrate was then added and galactosidase activity was followed according to the directions of the manufacturer (Molecular Diagnostics). Indicator enzyme (β-GAL) activity is shown on the ordinate after correction for absorbance due to parental mouse myeloma supernatant used as a control for the primary antibody. Each point is the mean of duplicate determinations.

activity for G6PD in the model employed here. The model also illustrates the potential for negative regulation of this event under in vitro conditions (3,6).

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