MONOCLONAL ANTIBODIES TO HUMAN ERYTHROCYTE GLUCOSE 6-PHOSPHATE DEHYDROGENASE

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Received 12 August 1980

1. Introduction

Several antisera against human erythrocyte glucose 6-phosphate dehydrogenase (G6PD, type B) have been raised in rabbits and used for the characterization of genetic variants of this enzyme protein [1–4]. Use of the anti-G6PD antisera for estimating the specific activity of G6PD variants has recently been criticized and shown to be inadequate to this purpose [5]. Moreover, both the specificity and strength of conventional antisera are not satisfactory enough to allow a precise characterization of G6PD variants associated with severe deficiency of activity [6].

In an attempt to overcome these limitations, we used cell fusion techniques to obtain anti-G6PD antibody-secreting hybridomas [7]. By this approach, a number of antibodies directed to human G6PD were isolated after fusion of an 8-azaguanine-resistant mouse myeloma line with splenocytes from a mouse hyperimmunized with G6PD.

2. Materials and methods

2.1. *Purification of G6PD from pooled human erythrocytes*

Homogeneous G6PD (type B) was obtained as in [8,9]. Assays of G6PD activity and protein determination were done as in [9].

Abbreviations: G6PD, glucose 6-phosphate dehydrogenase; HAT, hypoxanthine-aminopterine-thymidine; PBS, phosphate-buffered saline (pH 7.2) [21]; BSA, bovine serum albumin; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis

2.2. *Immunization and cell hybridization*

A BALB/c mouse was immunized at 20 days intervals with 4 subcutaneous injections of pure G6PD (50 μg each) in Freund's complete adjuvant. Four days prior to removal of the spleen, the mouse was given an intravenous injection of G6PD (50 μg) in 0.1 ml 0.9% NaCl. Spleen cells (2.5×10^7) and P3 × 63 Ag8U1 myeloma cells (a generous gift of Dr M. D. Scharff) (0.5×10^7) were fused with polyethylene glycol 1000 as in [10]. After fusion the cells were resuspended in selective growth medium (HAT), in Dulbecco's modified Eagle's medium with 20% fetal calf serum, seeded in 72 wells of Costar plates (cat. no. 3524) in 0.5 ml aliquots and cultured at 37°C in a humidified 5% CO_2 atmosphere. Fourteen days after fusion vigorously growing cell colonies were seen in all wells.

2.3. *Screening procedures, cloning and expansion of hybridomas*

Supernatants of the cultures were tested for anti-G6PD activity by means of two different procedures:

(a) Direct hemagglutination, using $CrCl_3$-treated, G6PD-coated sheep erythrocytes, as in [11];

(b) Solid phase binding assay as in [12], using 100 μl aliquots of pure G6PD (100 μg/ml PBS) in each of the 96 wells of a flexible polyvinyl chloride microtitration plate (Microtiter Plates, cat. no. 1-220-29), which was kept overnight at 25°C. After extensive washings with PBS, 100 μl of the various culture supernatants were added and left 4 h at 25°C. The wells were washed 5 times, then 100 μl ^{125}I-labelled rabbit anti-mouse Ig antiserum (50 000 cpm) were added to each well (see below for radioiodination). After 5 washings, the

wells were cut with a hot steel wire and counted for radioactivity. A supernatant was considered positive when the bound radioactivity exceeded by at least 4 times the one recorded in a control assay in which the supernatant from a P3X63 culture was used.

Cells from wells showing anti-G6PD antibody activity were cloned in soft agar as in [13]. Clones were selected on the basis of strong positivity, grown to mass culture and injected i.p. into BALB/c mice pretreated with Pristane (2,6,10,14-tetramethylpentadecane). The monoclonal proteins were then purified from ascitic fluid as in [14] and used as such or, in the case of the protein produced by clone G 12.3 (see section 3), after coupling to Sepharose 4B (Pharmacia) [6,15].

2.4. *Radioiodination*

Purified monoclonal proteins and the IgG fraction from a rabbit anti-mouse Ig antiserum (prepared as in [16]) were radioiodinated using the chloramine T procedure [17].

2.5. *Class of monoclonal antibodies*

To determine the class of the G 12.3 monoclonal antibody, Ouchterlony analysis was performed using anti-class, anti-K and anti-λ rabbit anti-mouse specific antisera (Bionetics).

2.6. *Solid phase binding assay for competition experiments*

This was started in triplicate as above for the screening of culture supernatants. Various dilutions of ascitic fluids (50 μl) in 5% (w/v) BSA were added to each well together with 50 μl ^{125}I-labelled G 12.3 purified antibody (100 000 cpm) diluted in 5% BSA. After 4 h at 25°C, the wells were washed 5 times with PBS, cut and counted for radioactivity.

2.7. *Catalytic activity of G6PD in the presence of monoclonal antibodies*

Homogeneous G6PD (100 ng) was preincubated in 1 ml spectrophotometric cuvettes for 2 h at 25°C with varying amounts (10 μg, 1 μg, 100 ng and 25 ng total protein) of each of the 7 ascitic fluids in section 3. Controls were run in parallel in which the anti-G6PD monoclonal antibodies were replaced by identical amounts of an ascitic fluid containing mouse IgG or IgM myeloma proteins. Reactions were started by addition of 0.3 mM NADP and of 2 mM G6P (final conc.) and recorded at 340 nm with a Gilford mod. 2400 spectrophotometer.

2.8. *Immunoabsorption and analysis of G6PD by polyacrylamide slab gel electrophoresis (SDS–PAGE)*

Human erythrocytes were washed twice with 0.15 M KCl and hemolysates were obtained by hypotonic lysis with 9 vol. water, followed by centrifugation. Lysates were concentrated to 280 mg Hb/ml by dialysis under vacuum against PBS. Part of the lysates (1.5 ml) was absorbed on 100 μl Sepharose-bound mouse IgG in order to avoid non-specific retention. After incubation for 4 h, both the pre-absorbed lysate and a fraction of native, unabsorbed lysate (1 ml) were stirred with G 12.3–Sepharose (50 μl) for 4 h at 4°C. After extensive washings with PBS, bound G6PD was eluted by boiling in 5% (w/v) SDS and the eluates were analyzed by SDS–PAGE on gel slabs using the Tris system [18]. A sample of homogeneous G6PD which had been stirred sequentially with both immunoabsorbents was run in parallel.

3. Results

3.1. *Screening of positive hybridomas*

After hybridization, 10 of 72 wells were found positive for anti-G6PD activity at the solid phase binding assay. Out of these 10 supernatants, two (G 12 and G 32) showed marked direct hemagglutination of sheep erythrocytes coated with homogeneous human G6PD. Cells from the 10 positive wells were cloned in soft agar and the resulting clones tested by binding assay. Seven clones showing high anti-G6PD antibody activity (G 1.6, G 12.3, G 14.5, G 20.1, G 27.5, G 32.4 and G 43.6) were selected, expanded in suspension culture and grown as ascites in BALB/c mice for mass production of antibodies.

3.2. *Characteristics and specificity of G 12.3 antibody*

The antibody secreted by clone G 12.3 appeared immediately to be the most interesting on the basis of both hemagglutination titer and affinity of G6PD binding. It was therefore chosen for further characterization. Table 1 summarizes the properties of this antibody, classified as IgM K, in comparison with those of a conventional rabbit antiserum to G6PD obtained and characterized in [4].

The Sepharose-bound G 12.3 antibody precipitates from erythrocyte lysates of normal subjects a single

Table 1
Comparative reactivity of monoclonal G 12.3 antibody and rabbit antiserum with human G6PD

Assay system	Monoclonal G 12.3 anti-G6PD (ascites)	Rabbit anti-G6PD (serum)
Hemagglutination[a]	$1:4 \times 10^6$ [b]	$1:8 \times 10^3$ [b]
Binding assay[a]	$1:10^6$ [b]	$1:10^3$ [b]
Radial immunodiffusion[a]	+	+
Ring test[c]	+	+

[a] See section 2; [b] absolute dilution; [c] performed as in [19]

protein of M_r 52 000 which is identical to pure G6PD (fig.1). Moreover, this immunoabsorbent failed to bind any protein from erythrocyte lysate of a subject with complete deficiency of G6PD activity and classified as having the Mediterranean variant of G6PD (not shown). Both results prove that the G 12.3 antibody is monospecific for human G6PD, while our anti-G6PD rabbit antiserum, even after proper absorption steps, has been found to interact with several erythrocyte proteins.

3.3. *Probing of antigenic determinants of G6PD by way of monoclonal antibodies*

The catalytic activity of purified G6PD is completely unaffected by preincubation with each of the various ascitic fluids, this suggesting that none of the monoclonal antibodies interacts with the catalytic sites of the enzyme protein.

In an attempt to start the immunochemical characterization of native G6PD, competition experiments were carried out which aimed at clarifying whether or not the same determinants on the G6PD molecule are recognized by the different monoclonal antibodies. Purified G 12.3 antibody was radioiodinated and tested in a competitive binding assay. The results in table 2 indicate that binding of radioiodinated G 12.3 protein to G6PD is strongly inhibited by the antibody secreted by G 32 hybridoma, even at those high dilutions which obscure the competition afforded by unlabelled G 12.3 protein. These patterns suggest binding to the same region of G6PD molecule of both 12.3 and G 32 antibodies, the latter being characterized by a consistently higher affinity. Only limited competition is afforded by the G 43.6 ascites at the various dilutions, while the other monoclonal antibodies appear to interact at distinct antigenic sites.

4. Discussion

Monoclonal antibodies produced by hybridomas provide a number of advantages over conventional antisera: they are obtainable in large amounts and are characterized by high titers, by a single defined specificity and by remarkable reproducibility of interaction with the corresponding antigen. Monoclonal antibodies to human G6PD provide no exception and may represent a tool of considerable analytical potential for the following reasons. They may profitably be used to develop immunohistochemical techniques

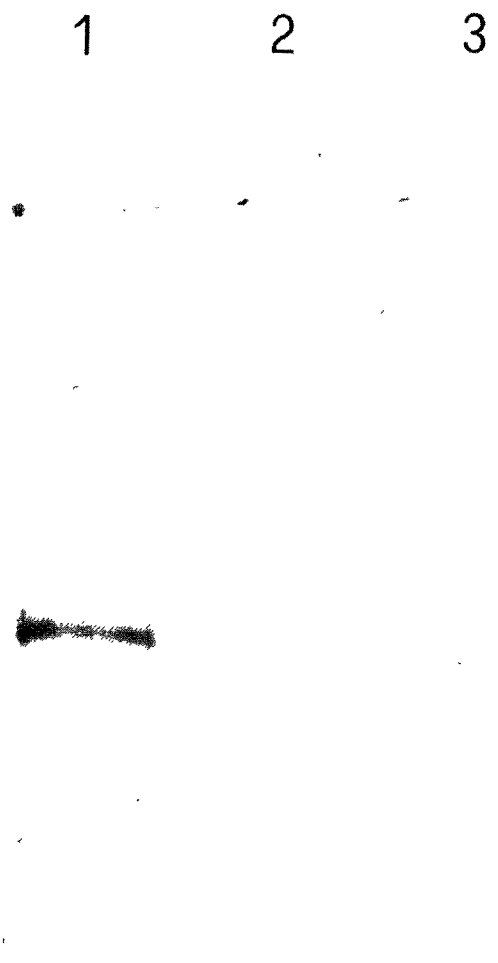

Fig.1. Immunoabsorption of G6PD from a human erythrocyte lysate by Sepharose-coupled G 12.3 purified antibody. For experimental details, see section 2. (1) Purified pre-absorbed G6PD; (2) pre-absorbed hemolysate; (3) non pre-absorbed hemolysate.

Table 2
Inhibition of binding of radioiodinated G 12.3 antibody to human G6PD by ascitic fluids[a]

Ascites	Dilution of ascites in 5% (w/v) BSA			
	1:10	1:100	1:1000	1:2000
Ig G_{2b} (MPC-11)[b]	0	0	0	0
G 1.6	0	0	0	0
G 12.3	94.6	75.4	36.9	7.3
G 14.5	19.4	0	2.1	9.1
G 20.1	28.9	6.3	0	0
G 27.5	37.8	6.1	0	0
G 32.4[c]	9.2	2.2	2.6	3.6
G 43.6	22.3	15.2	19.7	22.0
G 32	97	91.5	61.8	100

[a] Experiments were performed in triplicate as in section 3. Results were expressed as % of inhibition and equated to:

$$100 - \frac{\text{cpm bound in the presence of ascites from hybridomas}}{\text{cpm bound in the presence of control ascites } (IgG_{2b})} \times 100$$

[b] MPC-11 mouse myeloma cell line [13]; [c] The difference between inhibitions by uncloned G 32 and clone G 32.4, respectively, may be due to substantial changes undergone by the protein (identified as IgM) upon culturing, e.g., the disappearance of J chain: this view is supported by loss of hemagglutination and retention of binding capacity in the clone (see text)

aimed at localizing this commonly used marker of the X chromosome in a number of human cell types and of somatic cell hybrids between humans and other species. They may be also used for discriminating among G6PD-positive and G6PD-negative cells, a situtation which is typical of the heterozygous condition of G6PD deficiency and raises difficult diagnostic problems [20]. The product of clone G 12.3 and probably the other monoclonal antibodies described here because of their specificity toward different determinants on the G6PD molecule (table 2), appear to be useful probes for topological studies: these can be performed on the native G6PD molecule, on eventual discrete species arising from post-translational changes (e.g., by intracellular proteolysis) and on structural variants of this enzyme protein. Another application which can be envisioned for monospecific anti-G6PD antibodies is their use for selective immunoprecipitation of ribosome-bound nascent G6PD and for subsequent investigations on the molecular organization of the G6PD gene in the human X chromosome by means of specific mRNA and cDNA probes.

Acknowledgements

This work was supported by grants from the Italian CNR, P. F. Tecnologie Biomediche and P. F. Medicina Preventiva.

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