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ANTIGENIC AND SIZE DIFFERENCES BETWEEN SOMATIC AND TESTICULAR CYTOCHROMES C*

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Enzyme-linked immunosorbent assays (ELISA) of somatic (c_s) and testicular (c_t) cytochromes of rat, mouse, rabbit, and beef with rabbit anti-rat cyt c_t antibody exhibited two different antigenic profiles, indicating the presence of two different antigenic structure between cyt c_t and c_s. SDS-polyacrylamide gel electrophoresis of cyt c showed that the molecular size of rat, rabbit and beef cyt c_t is slightly smaller than that of their cyt c_s. However,the electrophoretic mobility of mouse cyt c_t is almost identical to that of mouse cyt c_s, but slightly slower than that of rat cyt c_t. These results indicate that mouse and rat cyt c_t are different despite the identical amino acid sequences for both rat and mouse cyt c_s.

There are two forms of cytochrome (cyt) c in mammalian tissues; somatic (c_s) and testicular (c_t) cyt c (1,2,3). From studies with mouse cyt c, Hennig has reported that 13 amino acid residues are different between mouse cyt c_s and c_t (1). Since then, the presence of cyt c_s and c_t have been reported in the testes of rat (2), rabbit (3), and beef (3), but their primary sequences have not been determined.

Our recent antigenic analysis of these cyt c_t with mouse monoclonal antibodies (McAb) to rat cyt c_t has indicated that rat cyt c_t is probably different from mouse cyt c_t (3). However, it has been reported that the amino acid sequences are identical between rat and mouse cyt c_s (4). Therefore, we have further investigated whether or not rat cyt c_t is identical to mouse cyt c_t. In this communication, we report the antigenic and electrophoretic differences between cyt c_s and c_t of mouse, rat, rabbit, and beef.

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MATERIALS AND METHODS

Purification of cyt c_t- Cyt c_s and c_t were purified from testes and heart of rat, mouse, rabbit, and bull, as described previously (2,3).

Preparation of horse apo-cyt c- Apo-cyt c was prepared from horse heart cyt c (Sigma) according to the procedure of Zimmermann et al. (6).

SDS-PAGE (Polyacrylamide Gel Electrophoresis)- 15% SDS-PAGE was performed according to the procedure of Laemmli (7).

Preparation of phosphorylated BSA (pBSA)- pBSA was prepared from BSA and POCl according to the procedure of Vunakis et al. (8).

Production of Polyclonal Rabbit Antiserum to Rat Cyt c_t- Because of the weak immunogenicity of mammalian cyt c (9), rabbits were injected subcutaneously with a mixture of rat cyt c_t (250 μg) and pBSA (250 μg) in complete Freund's adjuvant once a week for three weeks. Subsequently, each rabbit was injected subcutaneously with the same amount of the cyt c_t and pBSA mixture in incomplete Freund's adjuvant 2 more times, every other week. The animals were bled once every two weeks and boosted intermittently when the titer started to decline. For the booster injections, cyt c_t (250 μg) was used without pBSA.

Preparation of Rabbit Anti-Rat Cyt c_t IgG Fraction- For preparation of anti-rat cyt c_t IgG fraction, immunoaffinity chromatography was performed as follows to remove anti-pBSA and anti-rat cyt c_s antibodies which are contained in the antiserum: pBSA (184 mg) and rat cyt c_s (60 mg) were separately coupled to 6 g and 4 g of CNBr-activated Sepharose gel, respectively, according to the procedure provided by the supplier (Pharmacia). The pooled antiserum was subjected to 33% ammonium sulfate saturation. After the centrifugation, the pellet was suspended in phosphate-buffered saline (PBS). After centrifugation, the clarified supernatant was applied to a pBSA-immobilized Sepharose affinity column (10 ml bed volume), and the column was extensively washed with PBS. The void volume was again applied to the regenerated pBSA-Sepharose column. The affinity chromatography was repeated 3 times.

The void volume obtained from the above pBSA-Sepharose affinity chromatography was applied to rat c_s-immobilized Sepharose column (10 ml bed volume). The column chromatography was performed as described above for the pBSA-Sepharose chromatography. The affinity chromatography with the void volume was repeated 4 times to remove any antibodies which cross-react with rat cyt c_s. The void volume was then precipitated at 50% ammonium sulfate saturation. After the centrifugation, the pellet was dissolved in PBS and dialyzed against PBS. This was used as the "anti-rat cyt c_t IgG fraction".

Enzyme-Linked Immunosorbent Assays (ELISA)- ELISA was performed as described by Kim and Nolla (3). Briefly, polystyrene 96-well plates were coated with 100 ng of cyt c_t, and incubated overnight at 4°C. The coated immunoplates were washed, and blocked with 1% gelatin in PBS for one hour at room temperature. The plates were washed, after which each well was filled with 0.1 ml (1 to 100 μg/ml) of a series of diluted rabbit anti-rat cyt c_t IgG fraction and incubated for one hour. After washing, 0.1 ml of 1,000-fold diluted goat anti-mouse immunoglobulin peroxidase conjugate was added to each well. The plates were incubated for one hour and were washed. Then, 0.15 ml of 1 mM ABTS (2,2'azino-di-(3-ethylbenzthiazoline sulfonic acid)) in 0.1 M citrate-phosphate buffer, pH 4.0, was added to each well. After 30 min, 0.05 ml of 0.2 M citric acid, pH 2.2, was added to stop the peroxidase reaction. Absorbance in each well was recorded at 405 nm using a Dynatech Microelisa Auto-Reader (MR 580). For blanks, a parallel procedure was performed with 0.1 ml of 1% gelatin in PBS. The absorbance units were computed from the average values of the triplicate wells for each antigen.

RESULTS AND DISCUSSION

The testes of rat, mouse, rabbit and beef contains 2 forms of cyt c: cyt c_s and c_t (1,2,3). It has been reported by Hennig (1) that 13 amino acid residues

are different between mouse cyt c_s and c_t. Therefore, it was of interest to determine whether such a considerable amino acid residue difference also occurs between cyt c_t and c_s of other animals.

Since antigenic determinants represent surface protein structure whose conformation is determined by the primary amino acid sequence, a cross-reactivity study of cyt c_s and c_t with specific anti-cyt c antibodies should provide useful information on the differences in local amino acid sequence which constitutes the surface antigenic determinants. For such studies, either McAb or PcAb can be utilized as structural probes. A previous study with McAb detected the presence of both common and species specific antigenic determinants in rat cyt c_t (3). However, the production of a library of McAb for an antigen is a very tedius and difficult process, though not impossible. On the other hand, utilization of a polyclonal antibody (PcAb) is more convenient and useful because production of PcAb is easier than that of McAb, and also because PcAb is a mixture of heterogeneous antibodies with different specificities for various antigenic determinants in an antigen. Therefore, antigenic analysis with PcAb would reveal "overall" antigenic differences rather than a single antigenic difference for an antigen. For these reasons, the antigenicity of cyt c_t and c_s was investigated using polyclonal rabbit anti-rat cyt c_t antibodies.

Because of the considerable homology of the amino acid sequences among mammalian cyt c, rabbit anti-rat cyt c_t antiserum was repeatedly applied to a rat cyt c_s-immobilized Sepharose column in order to remove antibodies which cross-react with rat cyt c_s. The anti-rat cyt c_t IgG fraction was obtained from the void volume and then used as a reagent for the cross-reactivity study of cyt c_s and c_t. As shown in Fig. 1, ELISA tests with polyclonal anti-rat cyt c_t showed two binding profiles between cyt c_t (solid lines) and c_s (broken lines) of rat, mouse, rabbit, and beef. The anti-rat cyt c_t showed a higher binding to all cyt c_t studied, compared to cyt c_s. The results also suggest that the antigenicity of cyt c_t of mouse, rabbit, and beef is to some degree similar to that of rat cyt c_t. The binding of the anti-rat cyt c_t antibodies to cyt c_s at high concentrations (above 50 μg/ml) is probably due to antibodies which leaked

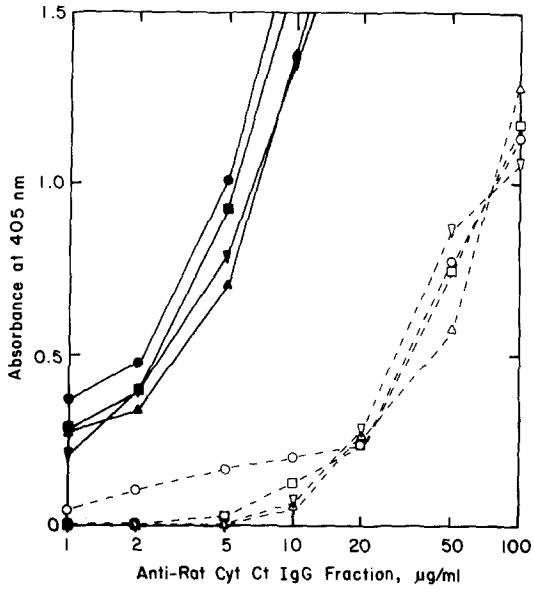

Fig. 1. Cross-reactivity profiles of cyt c_t (solid lines) and c_s (broken lines) of mouse, rat, rabbit, beef with rabbit anti-rat cyt c_t IgG fraction. The absorbance at 405nm was measured from ELISA. ● and ○, rat cyt; ▲ and △, mouse cyt; ■ and □, rabbit cyt; ▼ and ▽, beef cyt.

into the void volume during the repeated affinity chromatography procedures. The leakage was probably caused by antibodies which have weak binding affinities to the rat cyt c_s-Sepharose affinity gel. However, immunoblot assays (10) indicated that the anti-rat cyt c_t (0.245 mg/ml) recognized only rat cyt c_t but not rat heart cyt c_s (results not shown), indicating a high antigenic specificity for cyt c_t. As shown in the Fig. 1, the PcAb which contains various antigenic specificities does not discriminate the antigenic differences among these cyt c_t but clearly does discriminate "overall" differences between cyt c_t and c_s. However, the resolution of the two binding profiles between cyt c_t and c_s suggests two different surface structures between cyt c_t and c_s. This further suggests that cyt c_t of mouse, rabbit, and beef share an "overall" similar antigenic surface structure to rat cyt c_t. However, the cyt c_t are different from their corresponding cyt c_s.

The similar antigenic profile among cyt c_t of rat; mouse, rabbit, and beef suggests that their molecular size could be similar. Therefore, we examined their molecular size using SDS-PAGE technique. Molecular weights of cyt c_s of rat

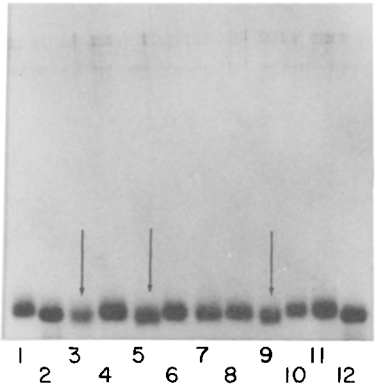

Fig. 2. 15% SDS-polyacrylamide gel electrophoretic patterns of cyt c_s and c_t. The gel was stained in Coomassie Brilliant Blue dye solution. Lanes 3, 5, 7, and 9 contain cyt c_t of beef, rat, mouse, and rabbit. Lanes 4, 6, 8, and 10 contain cyt c_s of beef, rat, mouse, and rabbit. Lanes 1 and 11 contain horse holo-cyt c. Lanes 2 and 12 contain horse apo-cyt c. The arrows indicate cyt c_t of beef (Lane 3), rat (Lane 5), and rabbit (Lane 9), which have a slightly faster electrophoretic mobility than other cyt c. Mouse cyt c_t (Lane 7) shows almost identical mobility with mouse cyt c_s (Lane 8) and rat cyt c_s (Lane 6). Horse apo-cyt c (11,800) and holo-cyt c (12,400) are used as molecular markers. Each lane contains about 2 μg of cyt c.

and mouse (12,132), rabbit (12,220), and beef (12,327) are very similar to that of horse holo-cyt c (12,384). As shown in Fig. 2, horse apo-cyt c (11,770; Lanes 2 and 12) and horse holo- cyt c (12,384; 1 and 11) showed a slight difference in electrophoretic mobilities, indicating that 15% SDS-PAGE resolves proteins with molecular weight differences of 600 daltons. The electrophoretic mobilities of all 4 cyt c_s as well as mouse cyt c_t are very similar to horse holo-cyt c_s (12,384). This confirms that rat and mouse cyt c_s are probably identical. Although mouse cyt c_t and c_s have a similar molecular size, their antigenicity is distinct as shown in Fig. 1. On the other hand, 3 cyt c_t (rat, rabbit, beef: marked with arrows in Fig. 2) are similar in molecular size to horse apo-cyt c (11,770), but are slightly smaller than all 4 cyt c_s (average mol. wt. 12,200). However, the electrophoretic mobility of the mouse cyt c_t (Lane 7 in Fig. 2) is almost identical to the 4 cyt c_s, including cyt c_s of mouse (Lane 8, Fig. 2) and rat (Lane 6), but slightly slower than rat cyt c_t (Lane 5 in Fig. 2). Therefore, it is clear that the molecular weight of rat cyt c_t is slightly smaller than that of mouse cyt c_t, by at least 600 daltons. Furthermore, 15% SDS-PAGE with a mixture of rat cyt c_t and c_s showed a blurred, fused double band (results not

shown), indicating a small difference in their molecular size. In contrast to the report that the amino acid sequences are identical between mouse and rat cyt c_s (4), this study shows that mouse and rat cyt c_t are not identical, as previously suggested (3).

REFERENCES

1. Hennig, B. (1974) Eur. J. Biochem. 55, 167-183.
2. Kim, I.C. (1980) Arch. Biochem. Biophys. 203, 519-528.
3. Kim, I.C., and Nolla, H. (1987) Canad. J. Biochem. Cell Biol. In Press.
4. Scarpulla, R.C., and Wu, R. (1983) Cell 32, 473-482.
5. Hantgan, R.T., and Taniuchi, H. (1977) J. Biol. Chem. 252, 1367-1374.
6. Zimmermann, R., Hennig, B., and Neupert, N. (1981) Eur. J. Biochem. 116, 455-460.
7. Laemmli, U.K. (1970) Nature 227, 680.
8. Vunakis, H.V., Kaplan, J., Lehrer, H.I., and Levine, L. (1966) Immunochemistry 3, 393-402.
9. Reichlin, M., Nisonoff, A., and Margoliash, E. (1970) J. Biol. Chem. 245, 947-954.
10. The Bio-Rad Immunoblot Assay. Bio-Rad Laboratories.