ANTI-GLYCOSYL ANTIBODIES: RESOLUTION OF ANTI-GAL ISOANTIBODIES INTO INDIVIDUAL COMPONENTS BY ELECTROFOCUSING

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SUMMARY: Rabbit anti-galactose isoantibodies which are specific for the terminal galactose units of a streptococcal antigenic diheteroglycan of glucose and galactose have been separated into individual components by a preparative electrofocusing technique. Ultracentrifugation and gel electrophoretic patterns indicate that each component consists of a single protein type of uniform molecular structure and size. Such antibody preparations will be of value for studies on the molecular structure of a natural antibody, on the nature of the bonds in an antigen-antibody complex and on the biological mechanism for the synthesis of antibodies.

Antibodies with molecular uniformity and with specificity for a single structural unit of a natural antigen would be useful for a variety of purposes including studies on the molecular structure of antibodies, on the nature of the bonds in an antigen-antibody complex and on factors involved in the initiation and regulation of antibody synthesis. In a preliminary note (1) we have described the isolation of two sets of anti-glycosyl antibodies from the sera of rabbits immunized with a vaccine of a Group D Streptococcus containing a carbohydrate antigen of glucose and galactose in its cell wall. One set of antibodies, anti-gal antibodies, is specific for terminal galactose units of the antigen, while the other, anti-lac antibodies, is specific for terminal lactose units of the same antigen.

We have now succeeded in separating the anti-gal antibodies into individual components by the electrofocusing method (2,3). On gel electrophoresis, the individual components yielded a single protein band while a reconstituted sample yielded a multiple pattern similar to that of the unfractioned anti-gal antibodies. On ultracentrifugation in an analytical and a preparative centrifuge (1), the anti-gal antibodies

yielded symmetrical patterns indicating homogeneity in molecular size. Although preparations of antibodies of restricted heterogeneity directed against microbial carbohydrate antigens (4-9) and of electrophoretically homogeneous antibodies directed at aromatic haptens (3,8) have been obtained, the preparation of an antibody of molecular uniformity directed against a natural antigen has not previously been achieved.

METHODS AND RESULTS

Isolation of the Anti-gal Antibodies - The anti-gal antibodies were isolated from the sera of rabbits immunized with a vaccine of non-viable cells of Streptococcus faecalis, strain N (9). The isolation procedure involved affinity chromatography (10) on lactosyl Sepharose (11) and elution of the antibodies with 0.5 M galactose solution (1). The antibodies were precipitated from the solution with ammonium sulfate at 50% saturation at 4°. The precipitate was collected by centrifugation, redissolved in saline-phosphate buffer of pH 7 and dialyzed against the saline-buffer solution at 4° for 72 hrs. From 15 ml of antiserum 45 mg of anti-gal antibodies were obtained. The sedimentation rate in a density gradient column, the electrophoretic behavior on polyacrylamide gels, hapten inhibition data and the identification of the carbohydrate and amino acid constituents of the anti-gal antibodies have been presented earlier (1). Further a sample of 0.3 ml of a 0.5% solution of the anti-gal antibodies centrifuged in a Model E centrifuge at 60,000 RPM yielded the patterns shown in Fig. 1.

Separation of Anti-gal Antibodies - Electrofocusing of the anti-gal antibodies was performed with a LKB instrument (Rockville, Maryland) using a LKB 8100 (110 ml) column. The liquid column consisted of a sucrose gradient (5% to 50%) and a 2% carrier ampholyte gradient of pH 6 to 8. A sample of 6 mg of the antibody preparation was introduced into the column along with the gradient solution and electrofocusing was performed at a power of 1.5 to 2 watts for 60 hours at

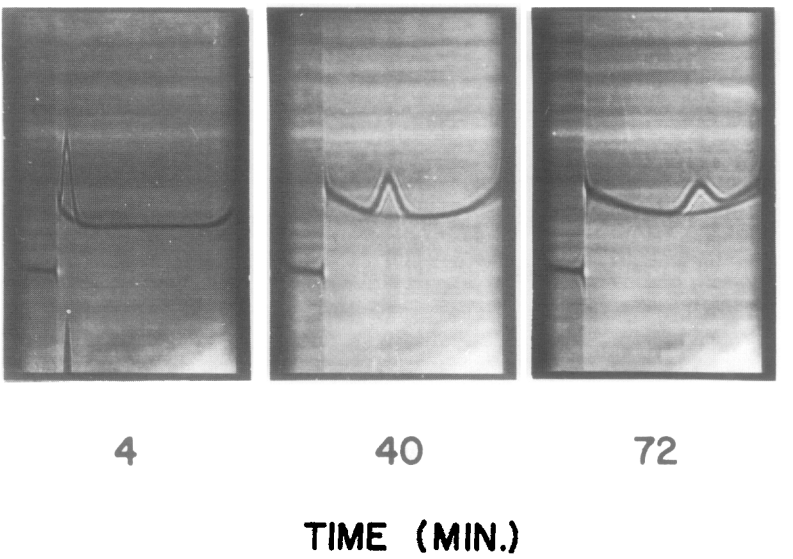

Fig. 1 Sedimentation velocity patterns of the anti-gal antibodies in the analytical ultracentrifuge.

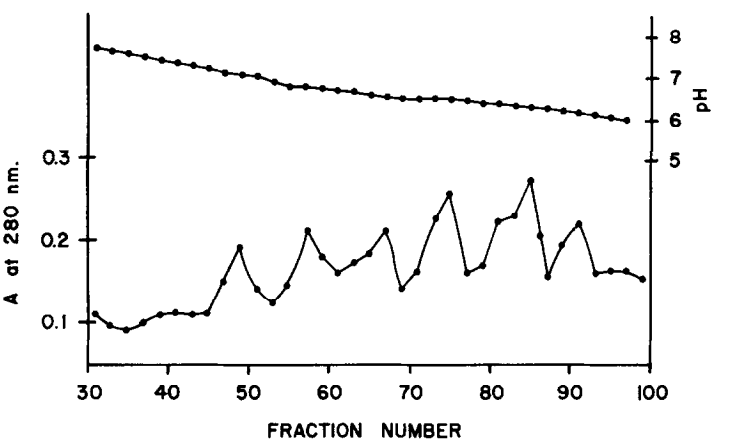

Fig. 2 Electrofocusing pattern and plot of pH values versus effluent volume for the anti-gal isoantibodies.

4°. The column was then emptied at a flow rate of 60 ml/hr and fractions of 1 ml were collected. The absorbance at 280 mu of all fractions and the pH were measured in a Beckman DU spectrophotometer and a Corning pH meter. A plot of the results is shown in Fig. 2.

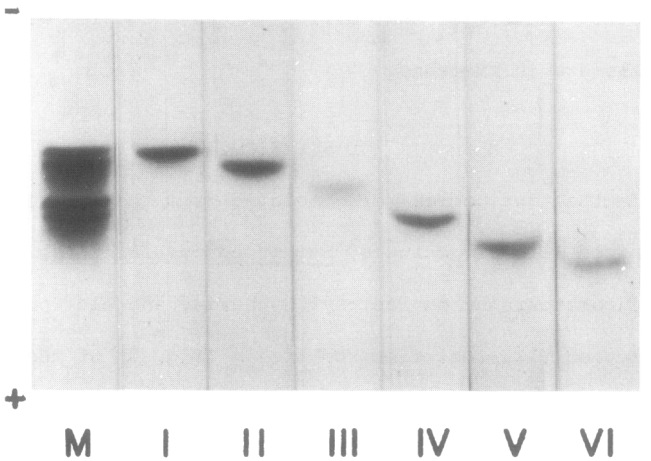

Fig. 3 Gel electrophoretic patterns for the mixture of anti-gal isoantibodies (M) and the individual components (I to VI).

Samples of 50 µl and 200 µl of all fractions and the unfractionated anti-gal preparation were used for gel electrophoresis in 5% polyacrylamide at a constant current of 30 milliamps at pH 8.5 for 6 hours. The gels containing 50 µl samples were stained with Coomassie blue G-250 for locating protein components. A photograph of 6 of the gels (fractions 49, 56, 66, 73, 85 and 90) and the original anti-galactose preparation is reproduced in Fig. 3. The duplicate unstained gels containing 200 µl samples were placed in separate petri dishes and covered with 1% agarose solution. After the agarose had solidified a trough was cut along the polyacrylamide gel and a 0.15% solution of the diheteroglycan was placed in the trough. Diffusion was allowed to proceed in a moist chamber for several days at room temperature. The plates were examined periodically for the appearance of antigen-antibody precipitin bands. A broad, elongated precipitin band appeared with the anti-gal preparation in 48 hrs corresponding to the protein components in the preparation. Bands were not obtained with the individual components because the ampholene interfered with the formation of the antigen-antibody complex as revealed in separate

diffusion tests. Additional agar diffusion tests coupled with immuno-electrophoresis are in progress.

DISCUSSION

Anti-galactose antibodies were isolated from the antisera of rabbits immunized with a vaccine of Streptococcus faecalis, strain N by affinity chromatography on lactosyl Sepharose and elution with galactose solution (1). Gel electrophoresis (Fig. 3) of the preparation showed that the antibody preparation consisted of 6 distinct proteins, each with a characteristic electrophoretic mobility. This antibody preparation reacted with the diheteroglycan of glucose and galactose in the capillary precipitin and the double diffusion in agar test. The antigen-antibody reaction was inhibited by galactose and compounds with terminal β-galactosyl units (1). Since the various proteins react with the same structural unit of the antigen, namely the terminal β-galactosyl unit, the term isoantibody is suggested for the individual components.

The mixture of anti-gal isoantibodies sedimented on density gradient centrifugation as a single band yielding a symmetrical UV scan (1). The sedimentation behavior of the isoantibodies in the analytical ultracentrifuge (Fig. 1) also indicates uniformity in molecular size. The molecular weight for the anti-gal antibodies calculated from the ultracentrifugation data and appropriate formulae (12,13) is 1.5×10^5.

A separation of the anti-gal isoantibodies into individual components has been achieved by an electrofocusing method. The UV absorbance and pH gradient of the fractions from the electrofocusing apparatus are shown in Fig. 2. Forty-five fractions of 1 ml contained the UV absorbing materials. Polyacrylamide gel electrophoresis on 50 µl samples of these fractions showed that a resolution of the isoantibodies into individual components had occurred. Many fractions were composed of a single protein component as shown in Fig. 3 for fractions 49, 56, 66, 73, 85

and 90. However, some overlapping of components did occur and a few fractions consisted of 2 or 3 components. The individual isoantibodies have been numbered from I to VI with the slowest moving component being I and the fastest moving component being VI.

A second set of isoantibodies specific for the lactose moiety of the diheteroglycan was obtained by elution of the lactosyl Sepharose column on which the antibodies were absorbed with lactose solution (1). Experiments on the resolution of the latter isoantibodies into individual components are in progress. The occurrence of antibody populations specific for different structural moieties of the same antigen has also been observed with carbohydrate antigens of the dextran type (14). Studies on the genetic control of synthesis of such sets of isoantibodies are highly desirable and have been initiated in our laboratory. Experiments are also underway on the determination of the amino acid sequence of the individual isoantibodies. Such information will show whether the individual isoantibodies are indeed homogenous in structure and molecular size and the relationship which exists among the individual components of an isoantibody set.

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REFERENCES

1. Pazur, J. H., Miller, K. B., Dreher, K. L. and Forsberg, L. S. (1976) Biochem. Biophys. Res. Commun., 70, 545-550.
2. Valmet, E. (1968) Science Tools, 15, 8-11.
3. Freedman, M. H. and Painter, R. H. (1971) J. Biol. Chem., 246, 4340-4349.
4. Krause, R. M. (1970) Fed. Proceed., 29, 59-65.
5. Kim, Y. D. and Karush, F. (1973) Immunochem., 10, 365-371.
6. Chen, F. W., Strosberg, A. D. and Haber, E. (1973) J. Immunol., 110, 98-106.
7. Freedman, M., Yeger, H., Milandre, J. and Slaughter, M. (1975) Immunochem., 12, 137-147.
8. Freedman, M. H., Guyer, R. B. and Terry, W. D. (1972) J. Biol. Chem., 247, 7051-7061.

9. Pazur, J. H., Anderson, J. S. and Karakawa, W. W. (1971) J. Biol. Chem., 246, 1793-1798.
10. Cuatrecasas, P., Wilchek, M. and Anfinsen, C. B. (1968) Proc. N. A. S. (U.S.A.), 61, 636-643.
11. Wofsy, L. and Barr, B. (1969) J. Immunol., 103, 380-382.
12. Martin, R. G. and Ames, B. N. (1961) J. Biol. Chem., 236, 1372-1379.
13. Schachman, H. K., in S. P. Colowick and N. O. Kaplan (Editors) (1957) Methods in Enzymology, Vol. IV, pp. 32-103, Academic Press, New York.
14. Cisar, J., Kabat, E. A., Dorner, M. M. and Liao, J. (1975) J. Exp. Med., 142, 435-459.