Comparative Antigenicity of Native and "Desialized" Orosomucoid in Rabbits.* (27790)

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Several investigators have noted that pure or partially purified preparations of the serum glycoprotein orosomucoid are very poor antigens in rabbits(1,2). However, precipitating antibodies to this protein have been observed in the serum of rabbits or horses immunized with whole human serum(3,4,5). Precipitating antibodies at a relatively low titer have been formed against pure orosomucoid in chickens(6,7).

Orosomucoid contains large amounts of galactose, mannose, glucosamine, and sialic acid(8). The sialic acid is entirely limited to terminal non-reducing positions(9), and thus might be expected to be an important determinant in the immunochemical specificity of this protein. However, this does not appear to be the case, since precipitation arcs are found when orosomucoid, from which sialic acid is removed by treatment with neuraminidase, is studied by immunoelectrophoretic methods(3,10). In this laboratory it has been observed that chicken antibodies to orosomucoid react equally well with native and desialized orosomucoid.

As a further extension to this problem it seemed desirable to prepare antibodies to orosomucoid denuded of its sialic acid component. This report demonstrates a significant enhancement of the antigenicity in rabbits of purified orosomucoid preparations when the sialic acid is largely removed by treatment with the neuraminidase present in influenza virus.

Materials and methods. Antigen preparation. Orosomucoid was prepared from Fraction VI of pooled normal human plasma† by the method of Bezkorovainy and Winzler (11), using DEAE- and CM-cellulose columns. This protein fraction, homogenous by paper electrophoretic and ultracentrifugal data, contained 11.8% N-acetylneuraminic acid, assayed by the method of Warren(12); 12.4% hexosamine by the procedure of Elson and Morgan(8); 17.0% hexose by an orcinol method(8); and 60.5% protein by the method of Lowry, *et al.*(13).

Removal of sialic acid from 600 mg of orosomucoid was effected by formalin-inactivated influenza virus.‡ Each of six 100-mg portions of orosomucoid was dissolved in 5.0 ml phosphate buffer-saline (pH 6.8) and 0.21 neuraminidase units§ (in the form of liquid influenza vaccine) were added. The mixtures were incubated at 37°C, with occasional shaking, for 18 hours. One hour centrifugation at 35,000 rpm rendered the supernatants free of vaccine particles. After exhaustive dialysis and lyophilization of the pooled mixtures, approximately 520 mg of "desialized" orosomucoid were recovered. This material contained 3.7% sialic acid, indicating that 68.6% of this component had been removed from the native glycoprotein. As previously shown(9,14), no amino acids or other sugars were liberated during the incubation.

Immunization. Two groups of 9 white male rabbits weighing about 1.5 kg were immunized with native and partially desialized orosomucoid respectively. Ten milligrams of antigen in 1 ml Freund's adjuvant were injected intramuscularly (thigh) on the 1st, 8th and 17th days. The animals were bled by cardiac puncture on the 27th day and the antisera obtained were stored at 5°C.

Examination of antisera. Qualitative tests were carried out by layering serial dilutions of the antiserum over a constant concentra-

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§ A unit of enzyme activity is defined as that amount which cleaves 1 micromole of N-acetylneuraminic acid from an excess of neuraminil lactose in 1 minute at 37°C at pH 7.0.

tion of the antigen (100 μg/ml) in 2 × 20 mm tubes, and observing the appearance of a ring at the interface within the next 2 hours. The reverse procedure, employing constant antiserum concentration and increasing dilutions of the antigen, was also used.

Quantitative precipitin determinations were carried out by mixing 0.25 ml of antiserum with increasing amounts of the antigen (0-150 μg) and diluting to a final volume of 1.5 ml with normal saline. The reactants were incubated at 37°C for 2 hours and then placed at 5°C for 18 hours. At the end of the incubation period the tubes were centrifuged in the cold at 3000 rpm for 30 minutes. Supernatant solutions were decanted and used for antibody or antigen excess tests. The precipitates were then washed twice by suspension in saline and finally obtained free of soluble protein by centrifugation as above. The amount of protein in the precipitates, suppended in 0.5 ml saline, was determined essentially by the method described by Lowry, *et al.*(13).

Double-diffusion analysis was carried out in plates of 0.75% Ion-agar‖ in 0.05 M, pH 8.6, veronal buffer. Rabbit antisera and pooled normal human serum were used undiluted; concentrations of the other antigens are specified in the figure. The plates were incubated at 37°C and were observed twice daily for 4½ days.

All antisera were tested qualitatively and quantitatively with native orosomucoid as the test antigen. In addition, 5 antisera from each group were examined with both native and desialized orosomucoid, as well as by the double-diffusion technic.

Results. In spite of marked variations in responses of individual rabbits to either form of the antigen, the "modified" orosomucoid generally elicited more potent antisera than the native glycoprotein.

Fig. 1 shows a typical pair of quantitative precipitin curves obtained when native orosomucoid reacts with the 2 types of antisera. Similar curves were obtained when the modified orosomucoid was employed as the test antigen.

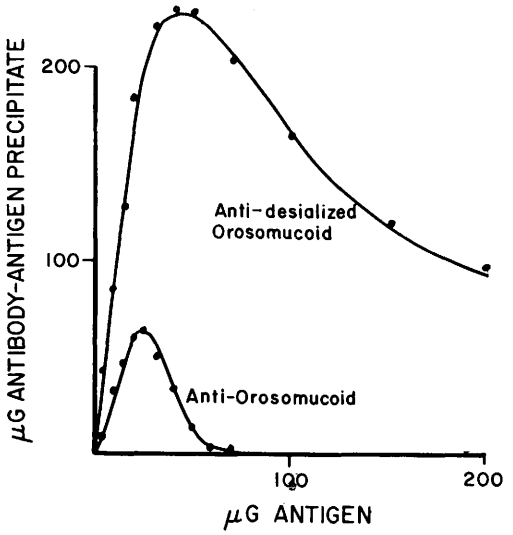

FIG. 1. Representative pair of quantitative precipitin curves of the two types of antisera examined with native orosomucoid as the test antigen.

Qualitative examination showed that antisera produced against desialized orosomucoid formed interface rings with both antigens at much greater dilutions (1:32) than did antisera prepared against the native protein (1:1); some of the latter showed no reaction at all.

The results of the quantitative precipitin determinations are summarized in Table I. The antibody response of individual rabbits within each group varied widely. However, there is a significant difference between the 2 groups, $P<0.05$, with the antibody-protein measured at the equivalence peak being considerably higher in the antisera of rabbits immunized with desialized orosomucoid. The average ratio of antibody to antigen protein at the equivalence zone peak ranged from 1.7 with the anti-native orosomucoid to 5.9 with the anti-desialized orosomucoid antisera. Differences in reactivity of the antisera with the 2 forms of the antigen were detectable but were neither consistent nor of great magnitude.

When 5 undiluted antisera from each group were examined by the double diffusion technic, 4 antidesialized orosomucoid and only

‖ Consolidated Laboratories, Inc., Chicago Heights, Ill.

one anti-native orosomucoid antisera showed the expected precipitation arcs with both antigens and human serum (Fig. 2). Concentration of the antigens was varied from 25 to 100 μg/ml; human serum was undiluted.

Discussion. Two points are emphasized by these results. It is evident that sialic acid is not an important determinant group in the interaction of orosomucoid with its specific antibody, since the native and desialized orosomucoid react similarly with antibodies to either preparation.

The antigenicity of desialized orosomucoid is significantly greater than native orosomucoid. The basic chemical reason for this is not known. The physical properties of desialized orosomucoid are significantly different from those of the native glycoprotein. It is more readily digested by proteolytic enzymes (15). It is appreciably less soluble and is more easily precipitated with ammonium sulfate than is native orosomucoid. The desialized glycoprotein also is a less acidic protein, having the mobility of a β-globulin at pH 8.6 rather than that of an α_1-globulin characteristic of the native glycoprotein(9).

TABLE I. Quantitative Precipitation of Orosomucoid at Equivalence Point by Antibodies to Native and Desialized Orosomucoid.

	μg antibody protein/ml antiserum*	
	Test antigen:	
Antisera	Native OM†	Desialized OM†
Anti-native OM		
R-29	390	NT‡
R-30	32	NT
R-31	28	NT
R-32	0	NT
R-39	0	0
R-40	344	312
R-41	248	152
R-42	156	108
R-43	0	0
Mean and stand. dev.	133 ± 158	114 ± 116
Anti-desialized OM		
R-15	748	NT
R-20	214	NT
R-21	496	NT
R-28	462	NT
R-44	208	120
R-45	384	922
R-46	360	220
R-47	1392	1324
R-48	1416	1516
Mean and stand. dev.	631 ± 464	820 ± 632
p-value	<.05	<.10

* Values given refer to highest point of equivalence zone in the quantitative precipitin tests.
† OM = Orosomucoid.
‡ NT = Not tested.

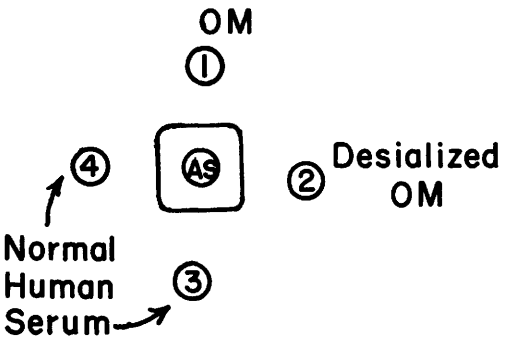

FIG. 2. Schematic representation of results obtained by the double-diffusion technic. Lines of identity of OM, desialized OM and antigens in human serum.

The increase in antigenicity of desialized orosomucoid is very practical, since it permits more consistent preparation of rabbit sera of reasonably high anti-orosomucoid titer. It will be interesting to determine whether the antigenicity of other sialic acid-containing glycoproteins is increased by treatment with neuraminidase.

Summary. Neuraminidase treatment of orosomucoid from human plasma results in an increase in the antigenicity of the glycoprotein in rabbits. Native and desialized orosomucoid react in a qualitatively and quantitatively similar manner with antibodies to the normal or desialized antigen. Thus the sialic acid, though a terminal sugar, is not an important determinant antigenic group.

1. Jager, B. V., *Fed. Proc.,* 1953, v12, 423.
2. Kornberg, L., Lipari, R., *ibid.,* 1955, v14, 469.
3. Schultze, H. E., Schwick, G., *Behringwerk-Mitteilg.,* 1957, Heft 33, 1, N. G. Elwert, Marburg.
4. Grabar, P., *Adv. Protein Chem.,* 1958, v13, 1.
5. Biserte, G., Havez, R., Hayem-Levy, A., *Clin. Chim. Acta,* 1960, v5, 272.
6. Silberberg, S., Goodman, M., Kefalides, N. A., Winzler, R. J., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 641.
7. Goodman, M., Ramsey, D. S., Simpson, W. L., Brennan, M. J., *J. Lab. Clin. Med.,* 1957, v50, 758.
8. Winzler, R. J., *Methods of Biochem. Analysis,* 1955, v2, 279.
9. Popenoe, E. A., *Biochim. Biophys. Acta,* 1959, v32, 584.

10. Vaux St. Cyr, C. de, *7th Colloquium Sint Jans Hospital-Brügge,* 1959.
11. Bezkorovainy, A., Winzler, R. J., *Biochim. Biophys. Acta,* 1961, v49, 559.
12. Warren, L., *J. Biol. Chem.,* 1959, v235, 1971.
13. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., Randall, R. J., *ibid.,* 1951, v193, 265.
14. Mayron, L. W., Robert, B., Winzler, R. J., Rafelson, M. E., Jr., *Arch. Biochem. Biophys.,* 1961, v92, 475.
15. Yamashima, I., *Acta Chem. Scand.,* 1956, v10, 1666.

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Inhibition of Arbor Viruses (Group A) by a Protein-Like Constituent of a Corynebacterium.* (27791)

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During the course of experiments in which Sindbis virus was being cultivated in primary human amnion cell cultures(1) it was observed that the expected cytopathic effects of this agent failed to occur in one preparation. This culture proved to be contaminated with a bacterium which was subsequently classified by Miss Elizabeth King of the U. S. Public Health Service Communicable Disease Center as a corynebacterium. The species, however, could not be identified. It was demonstrated that the bacterium regularly inhibited cytopathogenicity of Sindbis virus and the experiments described here were undertaken to determine the nature of the bacterial factor responsible for the viral inhibition and its mode of action.

Methods. It was noted that the bacterium grew better in bovine amniotic fluid (BAF) tissue culture medium(2) than in bacteriologic media such as beef broth and thioglycolate. BAF medium was therefore used as routine to propagate the organism. The original contaminated fluid from the tissue culture was diluted with 25 ml of BAF medium and stored at 6°C. In subsequent experiments 0.5-ml aliquots of this suspension which contained approximately 20,000 viable bacteria per ml were inoculated into 100-ml aliquots of BAF medium which were then incubated for 3 days at 37°C. Total counts at this time gave 50,000,000 viable organisms per ml of culture. The bacteria were removed by centrifugation at 800 rpm for 10 minutes, the supernate (medium supernate, MS) was passed through a Millipore filter (pore size: 0.45 mμ) and then assayed for viral inhibitory capacity. Bacteria were suspended in 5 ml of BAF medium and then sonically disrupted for 30 minutes. The suspension was frozen and thawed 3 times, and again sonicated for 30 minutes. The preparation was then filtered through a Millipore filter. The filtrate (bacterial concentrate, BC) was assayed for inhibitory capacity.

Except as otherwise noted, assay of bacterial inhibitory factor (BIF) was carried out in 3-4-week-old primary cultures of human amnion cells employing 100 $TCID_{50}$ of Sindbis virus as the test dose. In these assays materials to be tested for inhibitory capacity were diluted serially by a factor of 2 in BAF medium and 1-ml aliquots were added to each of 1-3 amnion cell cultures immediately before inoculation of virus.

Titer of a given preparation of BIF is expressed as the highest dilution which completely suppressed CPE (Cytopathic Effect) in human amnion cells on the day that in virus control cultures approximately 50% of the cells exhibited CPE. Unless specifically stated all preparations of BIF used were BC.

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