Association Between the Erythrocyte Hemagglutination Inhibitor and the M-N Blood Group Substances.* (28594)

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(Introduced by R. J. Winzler)
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A potent glycoprotein inhibitor of viral hemagglutination from human erythrocytes, homogeneous in paper and moving boundary electrophoresis, by ultracentrifugal analysis, column chromatography on various grades of Sephadex, and end-group analysis has been described(1,2). It is believed to be identical with the surface component of human erythrocytes with which influenza viruses interact to cause hemagglutination. This inhibitor also has M-N blood group activity. A recent paper (3) describes some observations on the structure of this glycoprotein. Data are presented here indicating that the viral hemagglutination inhibitor and the M-N blood group activity are contained in the same protein.

Materials and methods. The stromal inhibitor used for these studies was prepared from pooled human red cells containing about equal activities of M and N agglutinogens. The preparative procedure described earlier (2) was modified to include a final passage of the glycoprotein through a G-75 Sephadex column.

Lee and PR8 strains of influenza virus were propagated in eggs as described previously (2). The red cells used were pooled, 3 X washed, human, type O, Rh positive, type M and N in roughly equal quantities and made up in buffered saline to 0.5% for viral hemagglutination or viral hemagglutination inhibition titrations, or to 2% for serological titrations.

Viral hemagglutination and hemagglutination inhibition titrations were carried out by the serial dilution technique described earlier (2).

Rabbit anti-M and anti-N sera were obtained from Ortho Pharmaceutical Corp., Raritan, N. J. Antisera were titered by making serial 2-fold dilutions of the individual serum or of a 1:1 mixture of anti-M and anti-N, in phosphate buffered saline (PBS, 0.9% saline buffered at pH 7.4 with 0.015 M phosphate). To 0.1 ml of each dilution was added 0.1 ml of buffered saline and 0.1 ml of 2% red cells in saline. These were mixed and allowed to stand at room temperature for 20 minutes before being read for agglutination.

M-N activity was titrated by making serial 2-fold dilutions of a solution containing the agglutinogen in buffered saline. To 0.1 ml of each dilution was added 0.1 ml of titrated M-N mixture (diluted just enough to give a definite positive agglutination with a 0.1 ml 2% red cell suspension). This mixture was shaken and incubated 30 minutes at 37°C. To this was added 0.1 ml of 2% suspension of red cells. Again the tubes were shaken, allowed to stand at room temperature for 20 minutes, centrifuged for one minute, and read for agglutination. One unit of M-N activity is defined as the reciprocal of the greatest dilution of test material that will just inhibit the agglutination of type M-N cells under these test conditions.

Results. The first type of experiment was designed to show that the stromal glycoprotein was active as a viral hemagglutination inhibitor and that addition of mixed anti-M and anti-N sera reduced this activity by combining with the glycoprotein.

Incubations of anti M-N sera, of glycoprotein, and of anti M-N sera with glycoprotein at 2 concentrations were carried out for 30 minutes at 37°C and serially diluted for hemagglutination inhibition. The anti M-N sera were prepared by mixing 0.4 ml of rabbit anti-M and 0.4 ml rabbit anti-N and used undiluted. The glycoprotein solution was made with 2 mg dissolved in one ml PBS. Additional details and results are shown in Table I.

The anti M-N serum reacted with the glycoprotein to decrease its inhibitory activity at

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TABLE I. Effect of Anti M-N Serum on Inhibition of Viral Hemagglutination by a Stromal Glycoprotein.

		Hemagglutination inhibition (reciprocal of dilution)
I.	.25 ml glycoprotein (.5 mg)	8192
II.	.25 " glycoprotein (.05 mg)	512
III.	.25 " anti M-N serum*	<8
IV.	.25 " glycoprotein (.5 mg) + .25 " anti M-N serum	2048
V.	.25 " glycoprotein (.05 mg) + .25 " anti M-N serum	<8
VI.	.25 " glycoprotein (.5 mg) + .25 " normal rabbit serum	8192

* The anti M-N serum above had a hemagglutination titer of 16 units.

both concentrations. At the 0.5 mg level only a moderate decrease in titer resulted indicating that the anti M-N serum might be approaching saturation with the inhibitor. The anti M-N serum completely eliminated the activity of 0.05 mg of glycoprotein, suggesting that, at this level, it was totally in combination with antibody.

A second type of experiment was designed to determine whether removal of the viral hemagglutination inhibitor from solution by binding to virus and centrifugation resulted in concurrent removal of the M-N agglutinogen. A parallel decrease in both activities would be strongly suggestive of a single protein having both activities.

For this purpose, the Lee strain of influenza virus was used since it tends to form a more stable complex with red cells in the cold. The virus was first concentrated by centrifugation at 10,000 $\times$ *g* for one hour at 4°C, the supernatant removed, and the pellet reconstituted to one-twelfth its original volume. The final titer was 4096 hemagglutinating units per ml. Since, by definition, one hemagglutination inhibition unit is that quantity of inhibitor which will prevent the agglutination of 0.5 ml of 0.5% human type O Rh positive red cells by 4 hemagglutinating units of virus, the proportions of virus and inhibitor were adjusted so that at one level exact equivalence would be attained and all inhibitor should be combined with virus. This required dilution of the inhibitor to 4096/4 or 1024 hemagglutination units per ml.

To each of 6 test tubes were added one ml of inhibitor and varying volumes of virus including the theoretical equivalent volume, and all tubes brought to 2 ml with PBS. The tubes were shaken well and permitted to stand for one hour at 4°C. All of the mixtures were centrifuged for one hour at 100,000 $\times$ *g* and 4° C. The viral hemagglutination inhibition and serological titrations were performed on the supernatant solutions. The results are shown in Table II.

It is seen that both activities diminish as the amount of virus added is increased. Thus the concomitant removal of both activities is demonstrated as the glycoprotein is removed from solution by binding to the virus and centrifugation.

Discussion. Within a period of less than a year, Romanowska(4), Klenk and Uhlenbruck(5), and Kathan *et al*(2) reported preparation from human erythrocytes of glycoproteins having M and N blood group specificity as well as viral hemagglutination inhibition. Earlier, Springer and Ansell(6) had shown that the M and N blood group agglutinogens of human erythrocytes are inactivated by influenza viruses and by the

TABLE II. Removal of Hemagglutination Inhibitor and M-N Agglutinogens by Influenza Virus.

Inhibitor vol (ml)	Viral suspension vol (ml)	Buffer vol (ml)	Hemagglutination inhibition titer	M-N titer
1.0	0	1.0	256	320
1.0	.6	.4	64	320
1.0	.8	.2	64	80
1.0	.9	.1	16	40
1.0	1.0	0	16	20
0	1.0	1.0	0	0

Varying amounts of virus were added to inhibitor solution, removed by centrifugation, and the supernatants titered.

receptor-destroying-enzyme of *V. cholerae*. This is consonant with our observation(3) that the glycoprotein obtained by the previously described procedure from red cells pre-treated with influenza viruses is devoid of hemagglutination inhibitory activity. The similarity in chemical composition and in sensitivity to neuraminidase between the stromal hemagglutination inhibitor and the M-N blood group mucoids has led us to suspect that these may be the same substance. However, Klenk and Uhlenbruck(5,7,8) have suggested that they are different because M-N blood group active mucoids can be isolated from the supernatant solutions of erythrocytes treated with proteolytic enzymes such as trypsin, papain, ficin, or bromelin at which time the erythrocytes were still agglutinable by influenza viruses. The soluble mucoids were not inhibitors of viral hemagglutination. The latter observation can be explained by the studies of Gottschalk(9) as well as our own observations, which indicate that a certain minimum moleculer weight is essential for a glycopeptide to function as a viral hemagglutination inhibitor irrespective of the structural aspects of the oligosaccharide moiety with which the viruses would interact. Their observation that erythrocytes can be agglutinated even after proteolytic removal of M-N specific mucoids may be due to incomplete removal of sialic acid-containing substances. Mäkelä *et al*(10) showed that only about half of the sialic acid is lost from red cells by trypsin. The remainder of the sialo-mucoids may continue to function as sites for viral attachment.

We are now preparing stromal hemagglutination inhibitory glycoprotein from pure M or pure N human red cells to look for differences between them. No obvious differences have been found in carbohydrate composition.

Summary. The data reported indicate that a stromal glycoprotein, homogeneous by several criteria, is an active inhibitor of viral hemagglutination and has M-N blood group specificity. Both properties decrease in activity when the protein is removed from solution by mixing with influenza virus and centrifuging the mixture or when it is reacted with anti-M-N serum. It has always been observed that the disappearance of the one property of the protein is accompanied by the disappearance of the other. These results are interpreted as an indication that a single protein possesses both of these properties.

1. Kathan, R. H., Johnson, C. A., Winzler, R. J., *Acta Soc. Med. Upsaliensis,* 1959, v64, 290.

2. Kathan, R. H., Winzler, R. J., Johnson, C. A., *J. Exp. Med.,* 1961, v113, 37.

3. Kathan, R. H., Winzler, R. J., *J. Biol. Chem.,* 1963, v238, 21.

4. Romanowska, E., *Naturwiss.,* 1960, v47, 66.

5. Klenk, E., Uhlenbruck, G., *Z. physiol. Chem.,* 1960, v319, 151.

6. Springer, G. F., Ansell, N. J., *Proc. Nat. Acad. Sci.,* 1958, v44, 182.

7. Uhlenbruck, G., *Z. Bakt., Parasit. Infekt. Krankh. u. Hyg. Ref.* 1960-61, v177, 197.

8. ———, *Nature,* 1961, v190, 181.

9. Gottschalk, A., in *The Chemistry and Biology of Sialic Acids and Related Substances,* Cambridge Univ. Press, London, 1960.

10. Mäkelä, O., Miettinen, T., Pesola, R., *Vox Sang.,* 1960, v5, 492.

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Antagonism of Morphine and of Reserpine by Levorotatory and Dextrorotatory Isomers of 2,2-Diphenyl-4-(2-Piperidyl)-1,3-Dioxolane HCl. (28595)

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The compound, 2,2-diphenyl-4-(2-piperidyl)-1,3-dioxolane HCl, has 2 asymmetric carbon atoms and is composed of 2 racemates of different melting points. The lower melting racemate, dioxadrol*, was separated into the *d* isomer, dexoxadrol*, and the *l* isomer, levoxadrol*(1).

* Generic names.