STUDIES OF THE HEMOLYSIS OF RED BLOOD CELLS BY MUMPS VIRUS

III. Alterations in Lipoproteins of the Red Blood Cell Wall*

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Hemolysis of chicken red blood cells by mumps virus (1) has been shown to exhibit many of the characteristics of an enzymatic reaction (2, 3). In the course of further study of the mumps hemolysin (2–5), it was found to possess certain properties in common with lecithinase A, such as similar pH and temperature optima, inhibition by urethane, and inhibition by high concentrations of calcium and magnesium. Furthermore, the hemolytic activity of mumps virus was inhibited by the presence of lecithin (6) which suggests its similarity to a lecithinase, although the viral hemolysin is heat-labile while lecithinase A is heat-stable (5).

The presence of large amounts of lipoproteins in the chicken red blood cell wall, including significant amounts of lecithin, suggests that hemolysis produced by a lecithinase might occur as the result of the splitting off of a fatty acid from lecithin to produce lysolecithin which is known to be highly hemolytic. In order to detect alterations in the phosphatides of the chicken erythrocyte wall following hemolysis by mumps virus, a chromatographic analysis of the products of the reaction was undertaken by methods which would permit the detection of lysolecithin and other phosphatides. The morphological alterations that might occur during hemolysis by mumps virus were studied by comparing normal chicken erythrocytes with those hemolyzed with distilled water and with mumps virus.

These experiments indicate that hemolysis of erythrocytes by mumps virus is of a chemical nature involving a change in the cell wall of a constituent which is sphingomyelin or closely related to it.

Materials and Methods

Virus.—The source of virus for these experiments was amniotic fluid from embryonated hens' eggs infected with mumps virus. Eggs previously incubated for 8 days were inoculated

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via the amniotic route with mumps virus (1–5), and the fluids were harvested after 4 to 5 days' incubation at 36°C. when the eggs began to die.

Ten aliquots of amniotic fluid were centrifuged for 20 minutes at 3,000 R.P.M. to remove any large particles in the fluids. The supernatant fluid was then centrifuged for 20 minutes at 30,000 R.P.M. in the Spinco ultracentrifuge (model L). The pellet was gently washed once with isotonic phosphate-buffered saline (pH 7.2) and then resuspended in 1.0 ml. of the same solution by repeated mixing to give a concentrated virus preparation.

Red Blood Cells.—Red blood cells were obtained from hens by cardiac puncture and were stored in Alsever's solution (7) at 4°C. for not more than 7 days. These cells were washed 3 times with phosphate-buffered saline and then suspended in this solution at a concentration of 4 per cent by volume.

Hemolytic Test.—One ml. of concentrated virus preparation was added to 2.0 ml. of fresh 4 per cent chicken red blood cells in phosphate-buffered saline. As a control, the virus preparation was heated at 60°C. for 20 minutes before addition to the red blood cells. This temperature inactivates the hemolysin (1–3). The tubes were incubated at 37°C. for 2 to 4 hours with mixing every 30 minutes by gentle rotation.

Microscopic Methods.—Samples for microscopic examination were obtained with a micropipette from tubes of non-hemolyzed cells, cells hemolyzed with mumps virus, and cells hemolyzed with distilled water. These were placed on clean glass slides, covered with a coverslip, and viewed with a regular light microscope and a phase microscope. Smears of these same cells were made on glass slides and stained with Wright's stain.

Chromatographic Studies.—At the end of the incubation period, total lipide extracts were made of each suspension of erythrocytes in the hemolytic tests by slowly adding to the tube 5 volumes of methanol and then 5 volumes of chloroform. This mixture was extracted for 15 minutes at 50°C. and then centrifuged for 20 minutes at 3,000 R.P.M. The precipitate was re-extracted with 10 ml. of methanol-chloroform (1:1 by volume). The combined methanol-chloroform extracts were evaporated to dryness under reduced pressure in an atmosphere of nitrogen at 45°C. Methanol was added to the residue and the solution again was evaporated to dryness *in vacuo* in the presence of nitrogen in order to remove the last trace of water.[1] The dry residue was extracted several times with chloroform. The combined chloroform extracts were then evaporated to dryness under nitrogen in a water bath at 45°C. For chromatography this chloroform-soluble material representing the total lipides was taken up in isoamyl alcohol–benzene (1:1 by volume). Ten μl. aliquots containing 100 to 200 μg. of total lipides were analyzed by chromatography on silicic acid–impregnated paper according to the method of Marinetti and Stotz (8), using diisobutyl ketone–acetic acid water and/or chloroform-methanol-water as solvents. Rhodamine-6-G dye was used for color development of the spots under ultraviolet light. Further identification of the phosphatides was carried out by using the ninhydrin reagent for the detection of free amino groups, and the method of Levine and Chargaff (9) for the identification of choline groups. In addition, identification of the phosphatides was confirmed by comparison of their mobilities to those of known compounds. The relative amount of phosphatide present in each spot was estimated by comparing the areas of corresponding spots in control non-hemolyzed samples and in hemolyzed samples when equal quantities (100–200 μg.) of extracts of the samples were applied to the chromatographic paper.

P^{32} Labelling of the Red Blood Cell Phosphatides.—In two experiments the phosphatides of the chicken red blood cells were labelled with P^{32} by the intravenous injection of the chicken with 2 millicuries of P^{32}-labelled orthophosphate 16 hours prior to the collection of the erythro-

[1] The removal of the water was necessary in order to prevent the extraction of hemins in the subsequent chloroform treatment. Hemins catalyzed a rapid formation of peroxidized lipides which streaked extensively on chromatography.

cytes by cardiac puncture. The red blood cells were hemolyzed immediately and total lipide extracts were made. Autoradiograms were prepared of the chromatograms of the total lipide extracts by exposing the chromatograms to Kodak no-screen x-ray film for 1 month. The amount of radioactivity in each phosphatide spot was estimated by the degree of darkening of the developed x-ray film. By superimposition of the autoradiograms on the original chromatograms which were stained with rhodamine-6-G, the radioactivity in each of the phosphatides was ascertained. This method was capable of detecting less than 1 μg. of P^{32}-labelled lysolecithin.

EXPERIMENTAL

Microscopic Observations of Hemolyzed Cells.—Microscopic studies of the morphology of normal chicken red blood cells, those hemolyzed with mumps virus, and those hemolyzed with distilled water, revealed striking differences. The normal cells showed thick, rigid, cell walls and the hemoglobin gave a reddish-brown color to the cytoplasm. Cells hemolyzed with mumps virus demonstrated a thin, very flexible cell wall (usually visible only on careful focusing) and no longer maintained the characteristic shape of chicken erythrocytes. The cytoplasm was completely colorless and transparent. Cells hemolyzed with distilled water exhibited a complete loss of morphological pattern and a pronounced tendency to adhere to one another. Large amorphous clumps of these cells formed rapidly on standing after the addition of the distilled water. Hemoglobin was no longer visible in these cells.

After centrifugation, there was considerable difference in the gross appearance of the residue obtained from cells hemolyzed with mumps virus and those hemolyzed with distilled water. The cells hemolyzed with virus gave a residue which was white and flowed easily down the wall of the tube when it was tipped. Stained smears indicated an increase in the mechanical fragility of these cells such that the nuclei were easily separable from the rest of the cell during the process of making the smear. The residue from cells hemolyzed with distilled water was red, had an extremely gelatinous character, and failed to flow from the tube even when it was inverted. An increase in mechanical fragility similar to that found in the cells hemolyzed with virus was noted.

Hemolytic Activity of Lipide Extracts from Erythrocytes Hemolyzed by Mumps Virus.—To test the possibility that hemolysis by mumps virus might result from the action of lysolecithin formed by the direct action of the virus on the red blood cell phosphatides, an assay system was set up consisting of 0.1 per cent red blood cell suspension in phosphate-buffered saline in which it was possible to detect from 1 to 5 μg. of lysolecithin. This assay system was dependent on the immediate lysis of the red blood cells by lysolecithin with concurrent clearing of the suspension. Lipide extracts of red blood cell suspensions hemolyzed by mumps virus failed to produce any clearing of such an erythrocyte suspension, even after incubation at 37° C. for 3 hours, thus giving no evidence for the presence of lysolecithin in the solution.

To rule out the possibility that lysolecithin might be formed during hemol-

ysis of the red blood cells by mumps virus but subsequently destroyed in the process of hemolysis, the experiments outlined in Table I were carried out by mixing virus and erythrocytes, incubating to permit hemolysis to occur, then adding lysolecithin, and incubating again. After extraction of the lipides as described above, chromatographic analyses were done to detect lysolecithin. No lysolecithin was detected following hemolysis by mumps virus and the lysolecithin added was not destroyed by the processes involved in hemolysis

TABLE I

Detection of Lysolecithin Added to the Hemolytic System

Tube	Red blood cells	Mumps virus	Hemolysis	Lyso-lecithin	Chromatograms
				μg.	
1	2 ml. 4 per cent in PO_4-buffered saline	1 ml. of 12 × conc. active virus	Complete in 1.5 hrs.	None	No lysolecithin detected
2	2 ml. 4 per cent in PO_4-buffered saline	1 ml. of 12 × conc. active virus	Complete in 1.5 hrs.	200	Lysolecithin easily detected
3	2 ml. 4 per cent in PO_4-buffered saline	1 ml. of 12 × conc. virus, heated at 60°C. for 10 min.	No hemolysis until lysolecithin added—then complete hemolysis	200	Lysolecithin easily detected
4	2 ml. 4 per cent in PO_4-buffered saline	1 ml. of 12 × conc. virus, heated at 60° C. for 10 min.	No hemolysis until lysolecithin added—then about 50 per cent hemolysis	100	Lsyolecithin easily detected

by mumps virus, though the amount of lysolecithin necessary to cause hemolysis in this system was easily detectable by paper chromatography.

Tests for Phosphatides in Virus Preparations.—Experiments were designed to determine whether the mumps virus preparations used in these studies contained any phosphatides. Paper chromatographic analysis of a total lipide extract of amniotic fluids containing mumps virus demonstrated that phosphatides were absent. This finding ruled out the possibility that either the virus or the egg amniotic fluids contained detectable phosphatides which might be involved in the hemolytic reaction.

Chromatographic Detection of Alterations in Phosphatides of Erythrocytes Following Viral Hemolysis.—A photographic reproduction of a typical chromatogram illustrating the effect of the mumps virus hemolysin on the red blood cell phosphatides is shown in Fig. 1. The chromatographic analysis of the hemolyzed red blood cells gave no indication of the presence of

lysolecithin in the hemolyzed samples (*i.e.*, no lipide was observed in systems A, B, and C corresponding to the lysolecithin spot (1)). To increase the sensitivity of the method, autoradiograms were made of the chromatograms of lipide extracts from P^{32}-labelled chicken red blood cells hemolyzed by mumps virus. This technique also failed to show the presence of lysolecithin in the reaction mixture, although labelling of the lecithin was observed. Furthermore, no change in the amount of lecithin present was observed by these

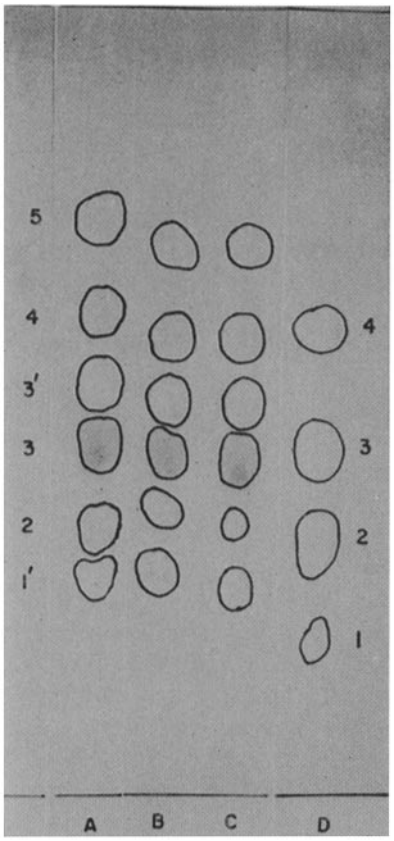

FIG. 1. Chromatograms and superimposed autoradiogram of lipide extracts of P^{32}-labelled chicken erythrocyte phosphatides run in chloroform-methanol (3:1 by volume with 2 per cent water). A = control cells plus heated virus; B = cells hemolyzed for 2 hours with mumps virus; C = cells hemolyzed for 4 hours with mumps virus; D = a standard mixture of authentic non-P^{32}-labelled phosphatides. D-1 is lysolecithin; D-2 is sphingomyelin; D-3 is lecithin; and D-4 is phosphatidyl ethanolamine.

In systems A, B, and C, spots 2, 3, and 4 correspond to sphingomyelin, lecithin, and phosphatidyl ethanolamine, respectively. Spot 1′ corresponds to inositol phosphatide; spot 3′ corresponds to phosphatidyl serine; and spot 5 has not been identified.

techniques. However, a marked decrease in the amount of a phosphatide which gave a positive choline test and had a chromatographic mobility similar to sphingomyelin (Fig. 1, spot 2) was noted in the reaction mixture when compared with the amount found in extracts from control chicken red blood cells which were mixed with heated mumps virus and incubated under identical conditions as the test mixtures of active virus and red blood cells.

A positive test for choline was given by spots 1 (lysolecithin in the reference mixture), 2 (sphingomyelin), and 3 (lecithin). In addition, a weak but non-specific test for choline was given by spot 4 (phosphatidyl ethanolamine). Unsaturated cephalins have been shown by Lea and Rhodes (10) to give such a test. A positive ninhydrin test was given by spots 3′ (phosphatidyl serine) and 4 (phosphatidyl ethanolamine). In the original autoradiograms P^{32} labelling was distinctly visible in spot 4, although this is not apparent in the photographic reproduction.

Under the conditions of these experiments, sphingomyelin was shown to be a minor but definite constitutent of the chicken red blood cell phosphatides and was easily detected chromatographically if sufficient amounts of total lipides were used (100–200 μg.). Rhodamine-6-G dye was found to be more sensitive than the phosphomolybdate reagent for detecting sphingomyelin.[2] Subsequent experiments (11) employing column chromatography permitted the fractionation and isolation of sphingomyelin from the other phosphatides in the chicken erythrocyte. The failure of Turner (12) to detect sphingomyelin in chicken red blood cells may be due in part to the lack of sensitivity of the phosphomolybdate test which he used.

CONCLUSIONS

Hemolysis caused by mumps virus does not appear to be due to osmotic disruption of the chicken red blood cell. The mumps virus hemolysin causes a decrease in the thickness, rigidity, and strength of the red blood cell wall as evidenced by microscopic studies, indicating a marked alteration of the cell membrane, but hemoglobin is released into the solution without a visible rupture of the cell wall. However, an alteration must be occurring in some component of the red blood cell wall which is essential to maintaining its structural integrity. Since the erythrocyte stroma consists mainly of lipoprotein constituents, it is possible that an alteration in one of these components might bring about hemolysis of the cells. The hemolytic agent could conceivably be acting on the protein alone, on the phospholipide, or on a linkage between the protein and the lipide. During hemolysis any one of these reactions might occur as the primary event with one or both of the other occurring secondarily.

[2] In our hands, 2 to 5 μg. of sphingomyelin could be detected with rhodamine-6-G, whereas 30 to 50 μg. were required for detection with the phosphomolybdate reagent.

Since no lysolecithin could be detected following lysis of red blood cells by mumps virus, it seems quite likely that this phenomenon is not due to a lecithinase A action of the virus upon the lecithin in the lipoprotein of the erythrocyte stroma. The inability of the already hemolyzed preparation to inactivate or alter the lysolecithin added to such a system indicated that lysolecithin is not formed and then destroyed during or following hemolysis. An alteration in a component with chromatographic properties similar to sphingomyelin indicated that a change in a portion of the erythrocyte wall of which it is a unit may be responsible, at least in part, for the hemolysis. It is of interest that sphingomyelin is regarded as a structural lipide and is far less active metabolically than is lecithin. However, this alteration in phosphatide concentration might be a secondary manifestation of hemolysis rather than the cause.

SUMMARY

Evidence from microscopic studies indicates that hemolysis caused by the mumps virus hemolysin is a chemical type of hemolysis. Chromatographic analyses of the reaction mixture of erythrocytes and mumps virus following hemolysis indicate that hemolysis is not due to the action of a lecithinase A and that lysolecithin does not play a part in this process. The alteration of a component of the erythrocyte similar to sphingomyelin suggests that some of the phosphatides other than lecithin may be either directly or indirectly affected in the process of hemolysis of red blood cells by the mumps virus.

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