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Adsorption Characteristics of Influenza Viruses to Cholesterol and Fatty Acids. (24697)

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A new and interesting phenomenon, viral combination with cholesterol(1) and other lipids(2) in chromatographic columns was recently discovered by Youngner and Noll. This reaction might be applicable to preparation of improved influenza vaccines since virus was reported to be adsorbed to cholesterol from infected allantoic fluids, whereas nonviral material was not. Thus, passage of infected chorioallantoic fluid through suitable columns of cholesterol resulted in 15-fold purification of virus. Addition of cholesterol to the biologic might be desirable also since Youngner and Noll presented evidence that inoculation of animals with the cholesterol-influenza complex induced elevated antibody responses by adjuvant action. Our work amplifies these basic studies and in particular describes marked differences in combining capacity with cholesterol which exist for several strains of influenza virus.

Methods. Chromatographic columns were prepared by grinding cholesterol to fine powder with mortar and pestle. Weighed amounts of cholesterol (5 g) were then made into a paste-like consistency with phosphate buffered saline, pH 7.2 (PBS); quantitatively transferred to glass columns and packed with slight positive pressure and repeated washings with PBS. Care was exercised to prevent drying or cracking of columns. Virus suspensions of known concentration were then introduced into the columns and effluent fractions assayed for hemagglutinin (HA) titer with standard pattern tests using 0.5% chicken erythrocytes(3). Cholesterol-virus slurries were prepared using weighed amounts of powdered cholesterol to which virus suspensions of known concentration and quantity were added with grinding in mortar and pestle. The mixture was transferred to Erlenmeyer flasks and incubated at room temperature 30 minutes; spun in refrigerated centrifuge at 1500 rpm 30 minutes and supernate assayed for HA titer. Adsorption efficiency was compared on the basis of either total HA units/g cholesterol or % retained.

Results. Early experiments with crude infected allantoic fluid made it clear that the virus-cholesterol system contained complex variables since the affinity of adsorption to cholesterol differed markedly among strains of influenza virus (Table I). Among type A strains, a gradient was found with Asian isolate, "Formosa," readily combining with cholesterol, whereas other A strains showed decreased affinity. Type B strain, Great Lakes (B/GL), did not combine with cholesterol to any measurable degree. When this type of experiment was repeated with strains partially purified by centrifugation and resuspension in PBS, the virus preparations behaved quite differently In particular, a significant increase was observed in retention value with A/PR 301 and B/GL strains

Additional investigations were carried out with these 2 strains which had demonstrated a marked difference in adsorption rate when partially purified Formosa strain allantoic fluid was separated from virus by high-speed centrifugation and used as suspending medium for sedimented B/GL strain virus. Similarly, sedimented B/GL virus was resuspended in its own allantoic fluid and in PBS. The same rate of retention was found for B/GL virus when suspended in any of above

TABLE I. Cholesterol Retention of Influenza Viruses from Allantoic Fluid or Buffered Saline.

Virus strains	HA units/g		% virus	
	Allantoic fluid	PBS*	Allantoic fluid	PBS
A/Asian/Formosa/57	32000†	3976	100	96
A/PR8/34	7600	10816	59	65
A/PR301/54	1380	4096	43	100
B/GL/54	0	2850	0	68

* Phosphate-buffered saline, pH 7.2.
† Hemagglutinating units with 0.5% chicken erythrocytes.

menstrua (Table II). In contrast, A PR 301 strain, subjected to similar treatment, was most readily adsorbed when it had been resuspended in PBS. Suspensions prepared with centrifugally sedimented pellets of this virus in either its own original allantoic fluids, or fluid which once contained Formosa virus, were retained by cholesterol columns to the same degree.

When virus suspensions were recycled through fresh cholesterol columns, percentage adsorbed remained constant with PR 8 as shown by Youngner and Noll(1), while with B/GL suspensions, a decrease in adsorption was noted with secondary percolates (Table III). Lack of adsorption with B GL effluents during second passage suggested that results obtained during the first passage were due to mechanical trapping of aggregates present in original suspensions. To confirm this conclusion, a column of cholesterol was saturated with B GL strain virus and subjected to usual saline washing procedures. This was continued until wash fractions were free of demonstrable virus by the HA test. Ten ml of saline were then introduced and the column mechanically agitated by stirring.

TABLE II. Absorption of B/GL and A/PR 301 Strains by Cholesterol from Allantoic Fluids or Saline Suspensions.

Virus strain	Suspending medium		% HA units retained
B/GL	As harvested		0
	Resuspended in PBS		42
	Idem	Formosa harvest fluid	40
	"	B/GL harvest fluid	49
PR 301	As harvested		43
	Resuspended in PBS		100
	Idem	Formosa fluid	53
	"	PR 301 "	54

This procedure resulted in complete release of virus from the column. Data derived from such experiments with B/GL and other viruses are illustrated in Fig. 1. It was evidently desirable to conduct further experiments by mixing the lipid with virus suspensions in a slurry. These were then clarified by centrifugation and supernatant fluids assayed for virus. Comparison of results with the cholesterol slurry and column methods is provided in Table IV. The most striking differences were seen with allantoic fluid virus suspensions of PR 301 and PR 8 strains. Significant amounts of each virus were retained by cholesterol columns, while no adsorption was noted when viruses were mixed with cholesterol in slurries. In contrast, PR 301 and PR 8 viruses suspended in saline readily combined with cholesterol either in slurry or column. These results suggest that inhibitors in allantoic fluid were most apparent using the slurry method.

TABLE III. Per Cent Retention of Influenza Virus by Cholesterol Columns in Series.

Cycle	Virus strain	
	PR8	B/GL
1	59	39
2	50	6

Youngner and Noll(2) found that other lipids such as aliphatic fatty acids will also absorb viruses, and we have corroborated their observations with additional influenza strains. Table V presents summary of observations with palmitic and stearic acids. In general, higher retention values for all strains were obtained with palmitic acid than with cholesterol, while stearic acid bound a greater amount of virus than did palmitic acid under identical conditions.

Experiments were performed in mice and

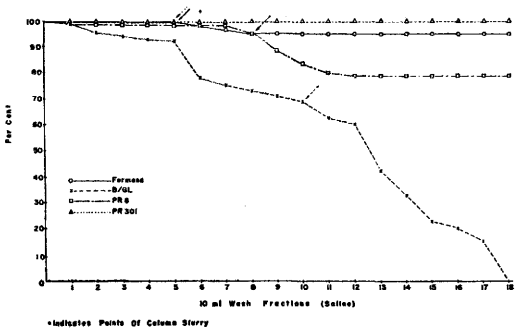

FIG. 1. Per Cent Retention of concentrated influenza viruses on cholesterol columns during successive washings.

guinea pigs to establish degree of antibody enhancement in animals which had been injected with influenza virus suspensions to which varying amounts of cholesterol or stearic acid had been added. With such mixtures, not all virus antigens in a polyvalent

TABLE IV. Adsorption of Influenza Strains to Cholesterol by Slurry and Column Method.

Method	Virus strain	% virus retention Allantoic fluid	PBS
Slurry	PR301	0	100
Supernate assay	Formosa	93	100
	PR8	0	75
	B/GL	0	0
Column	PR301	43	100
Effluent assay	Formosa	100	96
	PR8	59	78
	B/GL	0	0

influenza vaccine would be bound to the lipid. It was of interest to learn whether a cellular response at site of injection was of basic importance or whether virus-cholesterol

TABLE V. Absorption of Influenza Strains by Lipids Using the Slurry Method.

Adsorbent	Virus strain	% virus retention Allantoic fluid	PBS
Cholesterol	PR8	0	75
($C_{27}H_{46}O$)	PR301	0	100
	Formosa	93	"
	B/GL	0	0
Palmitic acid	PR8	61	81
($C_{16}H_{32}O_2$)	PR301	81	100
	Formosa	97	94
	B/GL	0	25
Stearic acid	PR8	50	100
($C_{18}H_{36}O_2$)	PR301	93	"
	Formosa	98	"
	B/GL	50	75

complexes were necessary to obtain an adjuvanted response. Data from guinea pig experiments where hemagglutinin-inhibiting (HI) antibody was measured with PR8 and B/GL strains are shown in Table VI. These animals were injected with 1 ml of standard 4 strain commercial 1958 polyvalent vaccine diluted 1:5 and, therefore, received only 20

TABLE VI. Antibody Responses in Guinea Pigs to Polyvalent Influenza Vaccine Mixed with Cholesterol or Stearic Acid.

Route of inj.	Adsorbent (5 mg/ml)	Mean titers* with test strains: PR8 3 wk	PR8 6 wk	GL 3 wk	GL 6 wk
Subcut.	None	5	8	3	0
	Cholesterol	10	0	0	0
	Stearic	13	10	5	0
Intramusc.	None	6	4	2	0
	Cholesterol	16	4	8	0
	Stearic	13	3	5	0

* Hemagglutination-inhibiting antibody titers in sera from 5 or 6 animals/group.

CCA (chick cell agglutinating units) each of PR8 and B/GL components. Average antibody titers obtained were low. However, groups which received 1 ml of vaccine mixed with 5 mg cholesterol or stearic acid developed 2-fold greater antibody titers as measured with PR8 while responses against B/GL generally were very low. Adjuvant effects observed with PR8 at 3 weeks were not evident after 6 weeks. Youngner and Noll(1) reported striking enhancement of antibody responses but a greater proportion of virus in their preparation had been adsorbed to cholesterol.

Discussion. It has long been known that allantoic fluid contains inhibitors for hemagglutination and that certain strains of influenza virus possess enzymes capable of destroying such inhibitors(4). Therefore, differences in adsorption to cholesterol suggested that materials contained in allantoic fluid might differentially affect this adsorption. When virus strains were partially purified by resuspension in PBS, there was a significant increase in adsorption with A/PR 301 and B/GL strains, with the former going from 43 to 100% and the latter from 0 to 68% retention. Further studies indicated 2 factors

were operating. One was clumping which resulted from centrifugation, the other was inhibitory activities in allantoic fluid. B/GL virus will not combine with cholesterol. The apparent retention was due to mechanical filtration of clumps from saline suspensions by the cholesterol columns. Composition of type B strain is clearly different since it does not combine with cholesterol as do type A viruses. In contrast, adsorption of A/PR 301 strain to cholesterol is affected by inhibitory materials in allantoic fluids. Adsorption increased when this virus was resuspended in PBS as shown by results from either column or slurry experiments. It is also apparent that Formosa allantoic fluids are not free from inhibitory substances because PR 301 strain resuspended in those fluids did not readily combine with cholesterol. An analogous set of reactions occurs with these 2 strains in HI tests with serum which contains non-specific inhibitory substances(5). Here again, the Formosa strain is completely resistant and readily agglutinates erythrocytes, whereas with the A/PR 301 strain, the hemagglutination pattern will not form in presence of serum inhibitors.

With fatty acids tested the inhibitor content of allantoic fluid observed in cholesterol reactions did not appear to play as significant a role. In contrast to its behavior with cholesterol, type B/GL strain readily combined with stearic acid.

Summary. Marked differences were noted among strains of influenza with respect to adsorption to powdered cholesterol, palmitic acid and stearic acid. It has been shown that some strains suspended in allantoic fluids adsorb to lipids less efficiently than when suspended in buffered saline. Behavior of type B/GL strain was significantly different from type A strains, especially with cholesterol or palmitic acid as adsorbent. Mixtures of minimal amounts of influenza virus with cholesterol or stearic acid elicited an enhanced antibody response in animals when tested at 3 weeks.

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Serum Proteins, Lipoproteins and Glycoproteins. II. Muscular Dystrophy and Related Diseases in Patients.* (24698)

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Biochemical changes in various forms of muscular dystrophy have been summarized(1, 2). They include changes in serum proteins but in such investigations in clinical muscular dystrophy, classification of patients on the basis of commonly recognized forms of the disease was not always made uniformly or stated clearly. These uncertainties may account for some discrepancies in results. In this report, various forms of muscle disease were strictly differentiated from each other in tabulation of results. The largest group comprised children with pseudohypertrophic muscular dystrophy (PMD). Other muscle diseases investigated were myositis, myotonia dystrophica and amyotrophic lateral sclerosis. Some patients with scoliosis and with Perthes disease also were studied, since sometimes muscular wasting was present which was of secondary nature. The purpose of this investigation was to find significant changes of serum proteins, lipoproteins and glycoproteins

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