Blood Group A Active Substances in Embryonated Chicken Eggs and Their Relation to Egg-Grown Virus

Abstract. Nondialyzable substances similar to blood group A antigens occur in White Leghorn embryos, and in partially purified egg-grown influenza virus preparations. The presence of material with group A activity in the virus itself is not proved. Commercial influenza virus vaccines which were tested contained nondialyzable substances with group A activity. Injections of this nondialyzable material into human volunteers caused an increase in anti-human blood group A_1 and A_2 antibodies.

Chickens possess serologically specific structures similar to blood group A substance (A-like) (*1*). In agreement with these observations, chickens are much better producers of anti-human blood group B than anti-human blood group A agglutinins (see also *2*).

A study of A-like substances in the egg is timely not only because it may further understanding of blood group development during embryonic life, but also because some of the viruses frequently grown in chicken eggs are thought to consist in part of host cytoplasmatic substances. Viruses may thus acquire antigenic specificities peculiar to the host in which they grow. It must be remembered, however, that the laboratory host may bear no relation to the natural environment of the virus.

Embryonated chicken eggs, 10 to 15 days old, from White Leghorn hens were used. Inoculation was carried out with two strains of type A influenza virus, S_{15} and Melbourne, and consisted of 0.5 ml of 10^6 to 10^7 ID_{50} of virus diluted and suspended in buffered saline (0.85 percent NaCl, 0.025*M* phosphate, *p*H 7.2) which contained 200 units per milliliter each of blood group A inactive penicillin and dihydrostreptomycin. Buffered saline was chosen as diluent (except as indicated in Table 1) rather than the conventional broths which contain meat or peptone in order to avoid introduction of large amounts of group A-active substances which are present in these broths. For the same reason casein media treated with blood group A enzyme preparations, such as trypsin, pepsin, or rennin (*3*) was not used. The incubation period was 48 hours.

Table 1 shows the blood group activities of chick allantoic fluid and membrane, and of inoculating fluids. As can be readily seen, dialyzed broth has very high group A activity even when compared to the best source of human group A substance, pseudomucinous ovarian cyst fluid. It is not surprising therefore, to find high blood group A activity in chick allantoic fluid which has been inoculated with broth or with virus suspended in such a broth. Although a very large part of the blood group activity found in this fluid is of extraneous origin, even allantoic fluids and membranes from uninoculated eggs and, better still, from eggs inoculated with virus suspended in saline, contain some material with group A activity which becomes clearly demonstrable on dialysis.

Chorioallantoic membranes from chicken eggs were suspended in water and ground in a Waring blender. These membrane extracts and the allantoic fluids were boiled and the nondialyzable part was fractionated with ethanol containing 0.1 percent sodium acetate (final concentration). The results for the chorioallantoic membranes and fluids were similar. The most active material in both fluids and membranes regularly occurred in those nondialyzable fractions which were not precipitated by ethanol concentrations of up to 90 percent (see Table 1). Preliminary experiments indicate that material so isolated contains more than 25 percent chloroform-methanol extractable lipid-like substances. Blood group active substances also were precipitated at an ethanol concentration from 40 to 75 percent, the same range in which most of the water soluble blood group substances of mammalian origin are found. It is noteworthy that neither group B nor group H(O) activity was observed in any of these preparations.

To aid in determining whether or not egg material with group A activity was associated with influenza virus, virus was purified by adsorption onto, and elution from, red cells by standard procedures. Table 2 shows clearly that group A active material was carried over in the eluate. However, over 85 percent of the virus hemagglutinin was transferred, while less than half of the materials with group A_1 and A_2 activity were found in the eluate. It is impossible to say from these experiments whether or not the active material is an intrinsic part of the influenza virus or merely adsorbed and carried over, especially since some elutable A-active material was adsorbed by O erythrocytes from uninoculated allantoic fluid. From the results of the elution experi-

Table 1. Sources of substances with blood group A activity. The unit is milligrams per milliliter of material which completely inhibits four doses of human anti-A hemagglutinins. The concentration of the inhibitor is its concentration before dilution with the serum in the erythrocyte suspension. Final concentration would be one-third of the values given (see also *2*).*

Source†	Not dialyzed				Dialyzed			
	A_1	A_2	B	H(O)	A_1	A_2	B	H(O)
1) Chick allantoic fluid inoculated (48 hr incubation) with:								
a. Influenza virus suspended in saline	>10	±10	>10	>10	5–10	2.5–5	>10	>10
b. Influenza virus suspended in broth	1.2	0.6–1.2	>10	>10	0.6	0.2–0.3	>10	>10
c. Broth	1.2	0.6	>10	>10	0.6–1.2	0.2–0.3	>10	>10
d. Saline	>10	±10	>10	>10	5–>10	2.5–5	>10	>10
e. Nothing	>10	>10	>10	>10	>10	5	>10	>10
2) Chick chorioallantoic membrane not inoculated or inoculated with influenza virus suspended in saline	±10	5	>10	>10	5–>10	2.5–5	>10	>10
3) Allantoic fluid >90% ETOH fraction					1.2–2.5	0.6	>10	>10
4) Chorioallantoic membranes >90% ETOH fraction					0.6	0.3–0.6	>10	>10
5) Tryptose phosphate broth (Difco)	0.3	0.1–0.2	>10	0.6–1.2	0.05	0.05	>10	0.6
6) Brain heart infusion (Difco)	0.3	0.2			0.1	0.03	>10	0.6
7) Casein hydrolysate (commercial pooled)	±10	5–>10		>10	2.5–5	1.2		>10
8) Horse serum	10	2.5–5	>10	>10	5–10	1.2	>10	±10
9) Human ovarian cyst E.J.	0.1	0.02	>10	>10	0.05	0.01	>10	5–10
10) Influenza virus vaccine (commercial pooled)	5–>10	2.5–±10			1.2	0.3–0.6	>10	>10

* Average of at least three experiments. † All materials boiled and subsequently kept frozen; chloroform and toluene or ethanol added during work up.

Table 2. Distribution of material with blood group A activity in influenza virus* inoculated allantoic fluid. The virus hemagglutinin titer was measured against chick erythrocytes. The blood group activity is expressed as in Table 1. Boiled aqueous suspensions of the nondialyzable part were fractionated with ethanol.

Ethanol fraction (%)	Yield (%)	Mg/ml of material which inhibits	
		Anti A_1	Anti A_2
a. Not absorbed, virus titer 1:16†			
0		5–10	2.5–5
40	57	±5	2.5
75	14	>5	1.2
90	3.5	5–10	2.5–5
>90	12	1.2	0.6
b. Absorbed with 1/3 vol. human group O erythrocytes, virus titer 1:2‡			
0		>10	5
40	67	±10	2.5–5
75	11	5	2.5
90	4	>10	5
>90	9	1.2–2.5	1.2
c. Eluate from human group O erythrocytes of b; virus titer 1:128§			
0		5	1.2–2.5
40	67	5	2.5
75	6.5	5	0.2
90	5.5	>5	±5
>90	~20	2.5–5	1.2–2.5

* Saline suspended. † Average of two experiments, 25 eggs total. ‡ Average of two experiments, 29 eggs total. § About 25 percent of nondialyzable material of part *a* was absorbed and eluted.

ments, however, it can be stated that there is no direct correlation between hemagglutinin titer of the virus and group A activity. These observations call to mind chemical findings that both influenza viruses and embryonated chicken eggs possess similar mucopolysaccharides which contain all sugars of blood group A, B and H(O) substances (*4*).

Crude in vitro active fractions of uninoculated membranes and allantoic fluid, 20 mg total for each rabbit, were given intravenously over a period of 8 days. Two of five rabbits given membrane fractions, and one of four given fluid fractions showed a significant rise in preexisting A_1 and A_2 agglutinins in sera which were obtained 1 week after the last injection. Whether the rise was true immunization or anamnestic response cannot be decided, as all responding animals had preexisting anti-A antibodies.

Of potential practical importance in relation to immunization of humans may be our finding, in the non-dialyzable portion (12 percent or less of total before the 48 hours' dialysis) of commercial influenza virus vaccines from six manufacturers, of material which inhibited anti-human blood group A agglutinins. This may be part of the virus or it may originate from embryonic structures or the diluting fluid or from all of these. The rather high activity of the unfractionated nondialyzable material (Table 1) suggests that much of the A activity may originate from the virus-diluting fluid.

Approximately 5 mg of the nondialyzable autoclaved part of commercial influenza virus vaccine was injected twice subcutaneously on two succeeding days into each of five healthy human volunteers of blood group B or H(O). These had received no injection of tissue-grown vaccines or other group A active materials during the previous 2 years. After 10 days, all subjects showed a four- to eightfold rise in anti-human blood group A_1 and A_2 titers; pre- and postimmunization sera were titrated simultaneously and a new pipette was used for each titration step dilution (*2*). The obvious potential hazards (*5*) inherent in a tissue-grown vaccine which contains even minute amounts (see also *6*) of substances similar to blood group antigens of extraneous and intrinsic origin may be significantly reduced if inoculations were performed with virus which had been suspended in fluid devoid of blood group activity (*7*), and if vaccines were made from more highly purified virus preparations. Complete elimination of blood group-like material from some tissue-grown vaccines, however, may be contingent on the antigenic composition of these tissues.

Note added in proof: Three additional volunteers of blood group O gave a similar increase of anti-A agglutinins when injected subcutaneously with one-fifth of the amount previously used.

GEORG F. SPRINGER*
HARVEY TRITEL
Immunochemistry Section, William Pepper Laboratory, and *Department of Medical Microbiology, University of Pennsylvania, Philadelphia*

References and Notes

1. F. Schiff and L. Adelsberger, *Zentr. Bakteriol. Parisitenk. Abt. I Orig.* **93**, 172 (1924); L. Adelsberger, personal communication, 1962; H. G. Bray, H. Henry and M. Stacey, *Biochem. J.* **40**, 124 (1946).
2. F. V. Dungern and L. Hirschfeld, *Z. Immunitaetsforsch.* **8**, 526 (1911); R. Uyeyama, *Japan. J. Med. Sci. (Social Med. Hyg.)* **3**, 13 (1940); G. F. Springer, R. E. Horton, M. Forbes, *J. Exptl. Med.* **110**, 221 (1959).
3. W. F. Goebel, *J. Exptl. Med.* **68**, 221 (1938); G. F. Springr, C. S. Rose, P. Gyorgy, *J. Lab. Clin. Med.* **43**, 543 (1954).
4. G. L. Ada and A. Gottschalk, *Biochem. J.* **62**, 686 (1956); C. A. Knight in *The Nature of Viruses*, G. E. W. Wolstenholm and E. C. P. Millar, Eds. (Little, Brown, Boston, 1957), p. 222.
5. J. Dausset and G. Vidal, *Sang* **22**, 478 (1951); R. André, B. Dreyfus, C. Salmon, *Bull. Soc. Med. Paris* **67**, 1315 (1951); E. A. Kabat, *Blood Group Substances* (Academic Press, New York, 1956), p. 68; A. S. Wiener, V. J. Freda, I. B. Wexler, G. J. Brancato, *Am. J. Obstet. Gynecol.* **79**, 567 (1960); C. McNeil, *Proceedings of the Ninth International Congress on Blood Transfusion* (S. Karger, New York), in press.
6. E. Witebsky, N. C. Klendshoj, C. McNeil, *Proc. Soc. Exptl. Biol. Med.* **55**, 167 (1944); E. A. Kabat, *Blood Group Substances* (Academic Press, New York, 1956), p. 68.
7. Supported by G-10906, National Science Foundation.
* Established Investigator of the American Heart Association.

9 September 1962; revised 3 October

Partitioning of Body Fluids in the Lake Nicaragua Shark and Three Marine Sharks

Abstract. The relative volumes of major body fluids of freshwater and marine sharks are remarkably similar in spite of the differences in external medium and in osmotic pressure of body fluids. The small differences detected are in agreement with differences reported in comparisons of freshwater and marine teleosts: a slightly higher total water content and a smaller ratio of extracellular to intracellular fluids in freshwater forms.

Whereas there is a somewhat limited literature on the physiology of marine elasmobranchs, very little has been reported on the physiology of forms adapted to fresh water. Since the work of Homer Smith on freshwater elasmobranchs in the 1920's and early 1930's (*1*), the physiology of these animals has apparently been completely neglected. The ready availability of the Lake Nicaragua shark, *Carcharhinus nicaraguensis* (*2*), makes it a good subject for studies on the peculiar osmoregulatory phenomena encountered in elasmobranchs, yet, not a single reference is available on the physiology of this widely known freshwater selachian (*2a*). Most of the reports on the osmoregulation of elasmobranchs have concerned the urea retained in high concentrations, trimethylamine oxide, and the ionic concentration of body fluids. There are appreciable differences between marine and freshwater species in the concentrations of these solutes (*1*). It was thought that further clarification of the osmoregulation of elasmobranchs might be attained by comparing the body-water content and its apportionment among the various ma-