Further Observations on Cold Hemagglutinin Activity in Encephalomyocarditis, M.M., Mengo Encephalomyelitis and Columbia-SK Immune Sera. (18847)

BENJAMIN V. SIEGEL.* (Introduced by Sidney Raffel.)

From the Department of Bacteriology and Experimental Pathology, Stanford University School of Medicine, Calif.

Cold hemagglutinins are serum entities which possess the property of aggregating human erythrocytes, in particular erythrocytes of the O group, at low temperatures. Stats and Wasserman(1) have reviewed the subject quite extensively, and the introductory descriptive material below derives in the main from that source.

Cold hemagglutination was first recognized by Landsteiner(2) who showed it to depend upon a definite antigen-antibody reaction. The reaction can be distinguished from the usual iso- or hetroagglutination by its high sensitivity to thermal change; as a rule it occurs to only a slight extent or not at all above 22°C. The phenomenon of agglutination in the cold can be reversed by warming to temperatures above 20°C and caused to recur by cooling to below 10°C. Cold hemagglutinins do not lose their activity after heating to 56°C for 30 minutes, and, like other agglutinins, they can be stored in the cold with only slight diminution in potency. Cold hemagglutinins have been described(1) as gamma globulins. Antibody nitrogen studies have also revealed that a titer of cold agglutinins of 1:2560 is equivalent to 1.473 mg nitrogen per ml.

Positive cold hemagglutination reactions have thus far been demonstrated chiefly with sera from cases of primary atypical pneumonia. The only other sera which elicit this phenomenon with any regularity are those from individuals with African trypanosomiasis. Cold hemagglutinins at high titers have also been reported in sera from cases of acquired hemolytic anemia and in some patients with Raynaud's disease(1).

A preliminary serological study(3) showed an increased activity of cold agglutinins in the sera of rabbits immunized with a mouse brain suspension of encephalomyocarditis virus. This suggested that perhaps other viruses, particularly those of murine poliomyelitis, or as Warren *et al.*(4) suggest, the encephalomyocarditis group, might also induce this response. The experiments described below were carried out to determine the extent to which these viruses may elicit cold hemagglutinin production, and to investigate the possibilities of such a technic in providing an additional differential criterion for the encephalomyocarditis group.

Preparation of antisera. Ten per cent suspensions of infected mouse brain in isotonic saline were used as immunizing antigens. The rabbits employed were not of an inbred stock, but stemmed from several different families. They were 10 weeks of age or older at the start of the experiment; this in light of past studies by Wheeler(5) who found that the normal cold-agglutinin titer of newborn rabbits diminished considerably during the first weeks following birth. Two or 3 rabbits were used for each virus. Prior to immunization all rabbits were bled for normal specimens of serum. At weekly intervals, over a period of 3 weeks, 1 cc amounts of the antigen suspensions were injected into the animals by the intramuscular route. During the fourth and fifth weeks 2 cc and 3 cc amounts respectively, were introduced intraperitoneally. Following a 10-day rest period the animals were bled from the heart. The blood samples were al-

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1. Stats, D., and Wasserman, R. L., *Medicine*, 1943, v22, 363.

2. Landsteiner, K., *München. med. Wschuschr.*, 1903, v50, 1812.

3. Siegel, B. V., *Bact. Proc.*, 1951, 96 abstract.

4. Warren, J., Smadel, J. E., and Russ, S. B., *J. Immunol.*, 1949, v62, 387.

5. Wheeler, K. M., *J. Immunol.*, 1938, v34, 409.

lowed to clot for 2 to 3 hours at room temperature, and the expressed sera were removed, spun, and then stored at —20°C until ready for testing. The titers of the inocula employed, expressed as the reciprocals of the logarithms of the LD_{50}, were 8.1, 8.5, 7.9 and 7.0 for EMC, M.M., Mengo, and Columbia-SK, respectively.

Cold hemagglutination test. Although all human erythrocytes can be agglutinated in the cold by hemagglutinating sera, group O cells were used because they were found generally to yield highest titers. The human O blood employed was stored in an equal volume of Alsever's solution at refrigerator temperature; prior to testing the cells were washed 3 times in isotonic saline and then brought up to 2% by volume with saline.

Progressive 2-fold dilutions of serum in saline, from undiluted to 1:512, were pipetted in 0.05 cc quantities into serological tubes (10 x 75 mm), and 0.05 cc of the 2% red cell suspension was added to each tube. The tubes were shaken and then placed in the refrigerator (4°C) for 30 minutes. At the end of this time the tube mixtures were centrifuged for 1 minute at 1500 rpm and returned to the refrigerator for another 30 minutes, following which they were read for agglutination under the low power of the microscope. To gain some idea about the thermal range of the reaction for the several sera, similar tests were carried out at 22°C and at 37°C. In addition, reversals of the reaction were studied, those sera initially tested at 4°C being later studied at 37°C and those tested at 22°C and 37°C later studied at 4°C. All readings were carried out rapidly at room temperature, the end point being determined by the last tube showing distinct clumping under low power magnification.

Results. The findings are condensed in Tables I and II. The former delineates the results in terms of average titers, while the latter pictures the outcome of tests with pooled sera. Titers, in both cases, are expressed as the reciprocal of the highest dilution of serum which gave agglutination.

Since there was the possibility that normal mouse brain tissue itself might be the inciter of increased cold hemagglutinin production, a control was run with two rabbits which, following the regular cardiac bleeding, were subjected to inoculation with a 10% mouse brain suspension in isotonic saline. In this connection it might be mentioned that Nakamura(6) had found that lipids in raw milk, when injected into normal rabbits, caused increases in the titer of cold hemagglutinins. From the tables it appears that the mouse brain tissue actuated an average 2-fold increase in the titer and some alteration in the thermal amplitude; while the results obtaining from titrations with the same sera pooled manifested no change in titer. For practical purposes then it seems reasonable to assume that any marked changes evinced in the virus-immunized rabbits could be ascribed to the activity of the

TABLE I. Cold Hemagglutinins in Rabbit Sera for Human O Erythrocytes both Before and After Immunization with Viruses of the Encephalomyocarditis Group. Titers based on avg values of individual sera.

Virus	No. of sera	Avg titer at 37°C	22°C	4°C
Normal mouse brain	2 N*	2	2	24
	2 I †	4	6	48
Encephalomyocarditis	3 N	1	2	18
	3 I	2	10	138
M.M.	2 N	1	1	9
	2 I	5	17	80
Mengo encephalomyelitis	3 N	1	6	37
	3 I	2	20	64
Columbia-SK	3 N	3	8	17
	3 I	3	12	69

* Normal. † Immune.

TABLE II. Cold Hemagglutinins in Rabbit Sera for Human O Erythrocytes both Before and After Immunization with Viruses of the Encephalomyocarditis Group. Titers based on pooled serum specimens.

Virus	No. of sera pooled	Pooled titer at 37°C	22°C	4°C
Normal mouse brain	2 N*	Undil.	4	32
	2 I†	2	4	32
Encephalomyocarditis	3 N	Undil.	Undil.	8
	3 I	8	8	128
M.M.	2 N	Undil.	2	2
	2 I	16	32	128
Mengo encephalomyelitis	3 N	Undil.	Undil.	8
	3 I	2	8	8
Columbia-SK	3 N	Undil.	2	8
	3 I	2	8	64

* Normal. † Immune.

6. Nakamura, I., *Keijo. J. Med.*, 1931, v2, 425.

virus itself.

Further perusal of Table I reveals that EMC virus occasioned approximately an 8-fold increase in titer, M.M. virus a 9-fold increase, and Columbia-SK a 4-fold increase; these changes were accompanied always by augmented thermal amplitudes. In the case of Mengo virus, the increase in titer was of a similar order of magnitude as that following vaccination with the normal mouse brain suspension. In the experiments with pooled sera (Table II) the results were much more striking. A study of the table indicates that EMC virus instigated a marked production of cold hemagglutinins, the immune titer being 16-fold that of the normal, while M.M. virus initiated a 64-fold increase in titer and Columbia-SK an 8-fold increase. Again Mengo virus acted like normal mouse brain, in this case evoking no change.

Discussion. It appears on the basis of these preliminary findings that of these 4 related viruses, M.M., EMC, Columbia-SK, and Mengo encephalomyelitis, the last alone manifests no propensity for provoking the production of cold hemagglutinin.

It appears also from these results that there is a direct relationship between titer and thermal amplitude, a finding in line with those made earlier by Bialosuknia and Hirszfeld(7), and Kettel(8). Hirszfeld(9) was of the opinion, too, that the thermal amplitude reflects also the combining affinity of the antibodies, the agglutinins with greater affinity being less dependent upon conditions of temperature.

The observations of Warren, Smadel, and Russ(4), Dick(10), Olitsky and Yager(11), and Schultz(12) have all pointed to the existence of a generic relationship and common antigenicity among the viruses of the encephalomyocarditis group. While no evidence is presented to the contrary, the findings here might suggest that these viruses may differ in their antigenic make-up insofar as they relate to the production of antibodies against human red blood cells.

The number of variables entering into experiments of this kind suggest, in addition to the use of a larger number of rabbits, employment of inbred animals of the same families and of approximately the same ages. As a further measure to insure against adventitious results, the use of rabbits with the same normal cold hemagglutinin titers would be desirable.

Summary. Cold hemagglutination studies were undertaken to explore the possibilities of this method for neurotropic viral analysis. Preliminary experiments had indicated that such an approach might provide additional differential criteria for the viruses of the encephalomyocarditis family. The observations reported here suggest that while M.M., EMC, and Columbia-SK viruses instigate increased cold hemagglutinin production in rabbits, Mengo encephalomyelitis virus is without such effect. Further work is indicated using larger numbers of animals of more uniform age and genetic constitution.

7. Bialosuknia, W. and Hirszfeld, L., *Compt. Rend. Soc. Biol.*, 1924, v90, 1198.

8. Kettel, K., *Compt. Rend. Soc. Biol.*, 1929, v100, 371.

9. Hirszfeld, L., *Klin. Wchnschr.*, 1924, v3, 1180

10. Dick, G. W. A., *J. Immunol.*, 1949, v52, 375.

11. Olitsky, P. K., and Yager, R. H., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 719.

12. Schultz, E. W., personal communication.

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