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Removal by Heparin–$MnCl_2$ of Nonspecific Rubella Hemagglutinin Serum Inhibitor* (32743)

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The serology of rubella virus infections was simplified, enormously, by the description of methods for demonstrating viral hemagglutination (HA) and antibodies by hemagglutination-inhibition (HI) (1). The hemagglutinin is produced in BHK-21 cells and agglutinates erythrocytes from day-old chicks. The HI tests require that a nonspecific inhibitor of rubella virus hemagglutinin be removed from serum by absorption with kaolin. This report describes another, more effective, system for removing the interfering inhibitor. Antibody titers determined with the new system are compared with those of the kaolin-absorbed sera of 100 elderly persons.

Materials and Methods. Antigen. High titered seed virus (M-33 strain, originally supplied by Dr. Paul Parkman) maintained in RK-13 cells was inoculated into BHK-21 cells (provided by Mr. Monroe Vincent, Microbiological Associates, Inc.) which were fed with Eagle's basal medium without serum. The BHK supernate was tested for HA daily and harvested when the titer was satisfactory, generally 1:16-1:32. Following its harvest, the supernate was treated with ether and Tween 80 (2) and stored frozen until used.[1]

Reference test. This test was performed as described by Stewart, *et al.* (1) except that room temperature incubation was used throughout. Erythrocytes were obtained from pools of day-old chick blood mixed with an equal volume of modified Alsever's solution.[2] Dextrose–gelatin–barbital buffered saline (DGV) was the diluent employed. All sera were inactivated for 30 min at 56°C and absorbed with 25% kaolin for 20 min at room temperature. Before centrifugation, a drop of 25–50% chick erythrocytes was added. After 1 hour at 4°C, the tubes were centrifuged and their supernate removed. These were assumed to represent 1:4 dilutions of the sera.

Heparin–manganous chloride treatment. Aliquots of each serum were treated with heparin-$MnCl_2$ as suggested by Mann *et al.* (4) except that the two reagents were mixed

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[1] Antigen was prepared by Dr. Alvin Novack, formerly of this Department.

[2] Among other erythrocytes tested for reactivity with rubella virus hemagglutinin, it was subsequently found that cells from Muscovy ducks yield titers at least as good as those obtained with day-old chick cells. Adult duck cells are employed routinely in this laboratory.

TABLE I. Study Population by Age and Sex.

Age group (years)	Sex		Totals
	Male	Female	
50–59	1	3	4
60–69	6	2	8
70–79	7	25	32
80–89	13	26	39
90–99	6	11	17
Totals	33	67	100

before their addition to the sera in a single solution. Sodium heparin, injection, U.S.P. (Riker), containing 5,000 USP units/ml with benzyl alcohol (0.9%) was the heparin source. No attempt was made to remove antichick erythrocyte hemagglutinins from these sera, which will be discussed later. Since the amount of heparin–$MnCl_2$ added to the serum represented only one tenth of its volume, the latter was considered to have been undiluted.

Sera. These were obtained in November 1963 as preimmunization samples for an influenza vaccine study which antedated the local epidemic of rubella which began in the spring of 1964. The sera were stored at −20°C until thawed for use. Aliquots of 0.5 ml were removed for each test system.

A unit of antigen was the highest dilution in which 4+ hemagglutination was noted. The antibody titer of a serum was the highest dilution in which there was total (0) inhibition of agglutination. All tests were performed by the microtiter method (3). Erythrocytes were added in 0.2% concentration in DGV.

Results. The age and sex distributions of the study population are summarized in Table I. Females accounted for 67 of the 100 and 88% were 70 or more years of age.

The HI titers observed with the two test systems were surprisingly similar (Table II). Sera treated by the two methods were titrated in parallel rows. Except for the differences in their initial wells, they could be read almost interchangeably. A positive HI test was not demonstrated in only one serum (a 90 year old female). Despite repeated testing, her serum was consistently read as <1:4 following kaolin treatment and as negative, undiluted, following heparin–$MnCl_2$. Thus, 99% of this population of elderly persons had measurable HI antibodies for rubella virus.

Since most of the sera contained high titers of HI antibodies, interference by chick erythrocyte agglutinins was not a significant problem. Such agglutination is coarse and visibly different from that induced by rubella virus.

TABLE II. Comparative Rubella HI Titers, by Sex, Observed after Serum Treatment with Kaolin or Heparin–$MnCl_2$.

HI titer (reciprocal)	Male (no.)		Female (no.)		Both	
	Kaolin	Hep.–$MnCl_2$	Kaolin	Hep.–$MnCl_2$	Kaolin	Hep.–$MnCl_2$
<undil.	—[a]	0	—	1	—	1
undil.	—	0	—	0	—	0
2	—	0	—	0	—	0
<4	0	0	1	0	1	0
4	0	0	0	0	0	0
8	1	0	0	0	1	0
16	2	1	3	4	5	5
32	2	2	10	6	12	8
64	9	5	16	17	25	22
128	5	11	17	19	22	30
256	9	7	12	11	21	18
512	4	3	5	5	9	8
1024	1	4	3	4	4	8
Totals	33	33	67	67	100	100

[a] — = Not tested.

TABLE III. Chick Erythrocyte Agglutinins Remaining after Treatment of Sera[a] with Heparin–$MnCl_2$.

Reciprocal serum dilution	Male	Female	Both
Undil.	0	1	1
2	3	9	12
4	12	17	29
8	11	22	33
16	5	15	20
32	1	3	4
64	0	0	0
128	1	0	1
Totals	33	67	100

[a] These sera were *not* absorbed with chick erythrocytes.

In sera which have high enough rubella HI titers, chick cell agglutination is followed by one or more wells in which inhibition of agglutination is complete. Removal of red cell agglutinins is obviously desirable, since in negative or low titered sera one cannot be confident that virus antibody was not missed, but the effectiveness of absorption, as practiced, is not complete. Such absorption was not incorporated into the heparin–$MnCl_2$ method, although it was demonstrated to be entirely feasible in other experiments. The determinations summarized in Table III reveal that while antichick red cell titers as high as 1:128 were detected, 95% of the sera had titers of $\leq$1:16. By comparison (Table IV), 73% of the kaolin-treated sera which had been absorbed with chick cell had antichick erythrocyte agglutinins in titers $<$1:4; 26% had titers of 1:4; and 1% were positive at 1:8. Among the unabsorbed sera, 75% had titers $\leq$1:8. Were they diluted fourfold, these results would be equivalent to the 73% that were negative at $<$1:4 in the kaolin-treated, absorbed sera. Thus, absorption with chick red cells increased the number of sera reacting with these cells at dilutions of $<$1:4 about five times, but still left 27% reacting at $\geq$1:4. The latter is approximately one-third the number that was observed in the unabsorbed sera. The effects of chick red cell absorption were unchanged when the sera were so treated before or after kaolin or heparin–$MnCl_2$.

TABLE IV. Chick Erythrocyte Agglutinins Detected in Sera Treated with Kaolin.[a]

Reciprocal serum dilution	Male	Female	Both
$<$4	27	46	73
4	6	20	26
8	0	1	1
Totals	33	67	100

[a] These sera *were* absorbed with chick erythrocytes.

On occasion and unpredictably, kaolin fails to fully remove nonspecific rubella virus hemagglutinin inhibitor, resulting in inconsistencies in the titers observed in repeated tests with the same serum. Such has not been the case following heparin–$MnCl_2$. This difference may be explained by the results observed in another group of experiments.[3] Six randomly selected, adult sera which had been obtained for other purposes were examined before and after aliquots had been treated in the usual fashions with kaolin or heparin–$MnCl_2$. Each was then examined with appropriate antisera by the micro-Ouchterlony method. While their IgA and IgM levels remained essentially unaffected by either treatment, IgG concentrations, however, were reduced an average of 43% by kaolin and 25% by heparin–$MnCl_2$. The significance of this difference, at best, is marginal ($p<0.10$). Individual decreases following the kaolin were 38, 39, 39, 40, 47, and 56%, while those after heparin–$MnCl_2$ were much more irregular, 0, 0, 27, 29, 44, and 52%.

When changes in their beta-lipoprotein contents were measured, the two methods proved to be quite dissimilar. Four of the six untreated sera had titers of beta-lipoprotein[4] of $>$1:32; the other two were 1:32. Following treatment with heparin–$MnCl_2$, no beta-lipoprotein was detected in any of the six while kaolin removed it completely from only three, reduced it to 1:4 in two, and to 1:16, in one.

[3] Conducted in Dr. Joseph Lunn's laboratory with the aid of Miss Suzanne Milicich. The author is indebted to both of them for these results.

[4] Determined with goat, anti-human beta-lipoprotein serum, Hyland No. GP 10-64F.

Heparin–$MnCl_2$ failed, as expected, to remove the heat-labile hemagglutinin inhibitor of *M. pneumoniae* (5).

Discussion. The description of an HI test for rubella virus antibodies by Stewart *et al.* (1) has served to catalyze epidemiological and clinical studies of rubella. Improvements and simplifications of the original technique are to be expected. Halmer *et al.* (6) already have confirmed the finding by Schmidt and Lennette (7) that the alkaline extraction of BHK-21 infected rubella virus cells produces superior yields of complement-fixing antigen. They have shown, also, that such treatment increases hemagglutinin content as well. The demonstration that heparin–$MnCl_2$ removes inhibitor more effectively than does kaolin, represents another improvement since it is simple, rapid, consistently effective and does not reduce the immunoglobulin content significantly.

The data reported here confirm those published by Mann *et al.* (4) to the effect that heparin–$MnCl_2$ consistently removes nonspecific viral (in this case rubella) serum HA inhibitor with only a minor effect upon immunoglobulins. The results with the latter were somewhat different, thus probably reflecting differences in techniques and, perhaps, sampling. In regard to the latter, two of the six sera which we studied had unchanged IgG levels. There was essentially no disagreement on the comparative efficiency of the two methods in removing beta-lipoprotein.

The requirement for erythrocytes from day-old chicks represents something of a problem in that they are not readily available, quite expensive and difficult to collect aseptically. When examined properly, almost every lot has been found to contain bacteria, which may be the most significant factor in their storability. As was mentioned earlier, we now use red cells from both male and female, adult Muscovy ducks. These are at least as sensitive as those from day-old chicks, cheaper, can be collected aseptically and can be stored for at least 3 weeks.

The persistence of antibodies in this elderly population is of some interest. The sera had been obtained at least several months before a major outbreak of rubella occurred in this area. Since all of the donors were residents of two institutions for elderly persons, their exposures to children and young adults had been limited for some time. It seems reasonable to assume that HI antibodies for rubella are persistent enough to indicate long-term protection and to provide useful data from epidemiological surveys.

Field *et al.* (8) in a comparative study of rubella HI and neutralizing antibody tests in Great Britain found that 92% of the sample 20–45 years of age (the oldest category listed) had HI antibodies for rubella. Similarly, 97% of children 15–19 years old were positive. These results are quite like those obtained in the present study except for the marked differences between the ages of the sampled populations.

Summary. Rubella HI nonspecific inhibitor is removed from serum more effectively by heparin–$MnCl_2$ than by kaolin. Heparin–$MnCl_2$ treatment removes beta-lipoprotein from serum; kaolin does this less efficiently. Among 100 elderly persons, 99 were found to have rubella HI antibodies.

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