Detection of Staphylococcal Antibodies in Human Gamma Globulin and Serum by Hemagglutination Tests.* (24990)

E. NETER, E. A. GORZYNSKI, A. M. DRISLANE, A. H. HARRIS, AND E. RAJNOVICH
Statler Research Labs., Children's Hospital, Depts. of Pediatrics and Bacteriology, University of Buffalo Medical School, and N. Y. State Dept. of Health, Albany

Plasma fraction II of Cohn, available as commercial gamma globulin, contains many, but not all, antibodies present in blood plasma of random adult population. Its protective value against such virus diseases as measles and infectious hepatitis has been clearly established and that against certain other viral infections is being explored. Recent studies have revealed that human gamma globulin also protects against certain bacterial infections in animals(1-4) and, when used in conjunction with suitable antibiotics, is said to yield favorable results in severe human infections(5,6). Previous investigations suggested that human gamma globulin contains both "complete" and "incomplete" antibodies against somatic (O) antigens of enteric pathogens, including salmonellae, shigellae, and enteropathogenic *Escherichia coli*(7). "Incomplete" antibodies were demonstrated by means of Coombs' hemagglutination and enzyme hemagglutination tests. In the former, antigen modified human erythrocytes were incubated with human gamma globulin and, after washing, tested with human globulin (Coombs') antiserum. In the latter procedure, red blood cells were treated with certain proteolytic enzymes, including trypsin, pancreatic protease, ficin, or papain, either before or after antigen modification, and then tested with gamma globulin. Both tests yielded considerably higher titers than the conventional agglutination and hemagglutination procedures and resemble those used for demonstration of "incomplete" Rh antibodies. Our study revealed that these methods lend themselves also to detection and titration of certain staphylococcal antibodies in human gamma globulin and serum.

Materials and methods. The antigens were prepared as follows. Several freshly isolated strains of *Staphylococcus aureus, citreus,* and *Bacillus subtilis* were grown on brain veal agar in Kolle flasks for 18 hours at 37°C. The resulting growth was removed with 25 ml of phosphate hemagglutination buffer (pH 7.3) (Difco)/flask. The suspensions were centrifuged in refrigerated centrifuge (International, #PR-2, 24,500 G) for 20 minutes and resulting supernates in appropriate dilution employed as antigen. Uninoculated Kolle flasks were treated in like fashion and the washings used for control purposes. Human erythrocytes (blood group O) (2.5% suspension) were washed 3 times with hemagglutination buffer. To the sediment was added the antigen in appropriate dilution in quantities sufficient to restore a 2.5% erythrocyte suspension. The mixtures were incubated in waterbath at 37°C for 30 minutes. The red blood cells were washed 3 times and then used in following tests. *Hemagglutination test*: The antigen modified erythrocytes (vol. 0.2 ml) were added to equal volumes of human gamma globulin or serum in serial dilutions. The mixtures were incubated at 37°C for 30 minutes and centrifuged at 1000 rpm 1 to 2 minutes. The resulting hemagglutination was read grossly. *Enzyme hemagglutination test*: The antigen modified erythrocytes were centrifuged. Pancreatic protease (Nutritional Biochemicals Corp., Cleveland, O.) in concentration of 250 μg/ml was added to the sediment in amounts to yield a 2.5% erythrocyte suspension. The mixtures were incubated at 37°C 30 minutes; the cells were washed 3 times and then tested with gamma globulin or serum as described above. *Coombs' hemagglutination test*: The antigen modified red blood cells were added to gamma globulin or serum in serial dilutions as above; the mixtures incubated at 37°C for 30 minutes; the cells were then washed 3 times. These suspensions (vol. 0.1 ml) were mixed with equal volume of commercially available

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TABLE I. Hemagglutination by Human Gamma Globulin and Serum of Erythrocytes Modified by Staphylococcal Antigen.

Staphylococcal antigens and antibodies		Staphylococcal antigen dilutions (reciprocal) 2	10	50	250	1250	2	10	50	250	1250
		Hemagglutination					Protease hemagglutination				
Staphylococcus aureus		Hemagglutinin titers (reciprocal)									
Strain D	(a)*	160	160	160	<20	<20	1280	1280	1280	160	<20
	(b)	80	40	20	<10	<10	80	160	80	<10	<10
Strain C	(a)	<20	<20	<20	<20	<20	1280	1280	160	<20	<20
	(b)	<10	<10	<10	<10	<10	160	160	<10	<10	<10
Strain 6048	(a)	<20	<20	<20	<20	<20	320	20	<20	<20	<20
	(b)	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Staphylococcus citreus											
	(a)	<20	<20	<20	<20	<20	20	<20	<20	<20	<20
	(b)	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10

* a = Human gamma globulin. b = Pooled human serum.

Coombs' antiserum, incubated at 37°C 30 minutes, and centrifuged at 1000 rpm for 2 minutes. The resulting hemagglutination was recorded. Several lots of human gamma globulin, including preparations from donors in U.S.A., India (courtesy of Dr. P. R. Dutt, New Delhi), and Japan (courtesy of Dr. I. S. Danielson, Lederle Labs., Pearl River, N. Y.), as well as several pools of inactivated human serum from 20 healthy adults each were employed.

Results. The results of a typical experiment on titers of antibodies in human gamma globulin (U.S.A.) and pooled serum against human red blood cells modified by antigens from 3 strains of *Staphylococcus aureus* and 1 strain of *S. citreus* are summarized in Table I.

Antibody titers in gamma globulin were considerably higher (8 to 128) in the protease hemagglutination than in basic hemagglutination test, when antigens from 3 strains of *Staphylococcus aureus* were used for modification of erythrocytes. It is evident also that the antigen from strain D was active in higher dilution than that from strain C. The antigen from strain 6048 was least active. The degree of increased sensitivity of protease hemagglutination test is related to amount of antigen used for erythrocyte modification and presumably to that present on surface of red blood cells. It is evident that, depending upon source of antigen and amount of antigen used for erythrocyte modification, the protease hemagglutination test yields positive results when the basic test is negative. The Table shows also that protease hemagglutination test is somewhat more sensitive than hemagglutination procedure when human serum is titrated. Repeated experiments yielded essentially similar results. The antigen from *Staphylococcus citreus* was almost completely ineffective. Control experiments, not shown in Table, revealed that sterile fluids prepared from Kolle flasks and used for modification of erythrocytes gave entirely negative results, as did protease treated red blood cells tested with gamma globulin and serum in appropriate dilutions. That merthiolate, used as preservative in commercial gamma globulin, did not contribute to above findings is evident from observations that merthiolate itself in like concentrations failed to agglutinate red blood cells treated with protease and/or staphylococcal antigen, and that merthiolate-free gamma globulin yielded results similar to those obtained with commercial gamma globulin containing this preservative.

Further experiments showed that other coagulase-positive and coagulase-negative strains of staphylococci as well as several strains of *B. subtilis* also yielded antigens suitable for these hemagglutination reactions. Two additional strains of *Staphylococcus citreus* gave negative results. Gamma globulin preparations from India and Japan had antibody titers similar to those present in specimens from U.S.A.

A comparison of Coombs' staphylococcal hemagglutination test with the protease and

basic hemagglutination procedures revealed that the former 2 yielded essentially identical titers of antibodies in several lots of commercial human gamma globulin and were considerably more sensitive than the basic hemagglutination method.

Regarding heat stability of staphylococcal antigen, it remained active for erythrocyte modification after heating in boiling water for 15 minutes at pH 2 to 5, but lost its activity when heated at pH 7.2 to 9. In contrast, lipopolysaccharides from gram-negative enterobacteriaceae are more effective in modifying erythrocytes after being heated at alkaline pH(8).

Absorption experiments revealed that suspensions of *Staphylococcus aureus* (strains C and D) and of *B. subtilis* removed the antibody from human gamma globulin, when the latter was tested with red blood cells modified by either of the 3 antigens. In contrast, suspensions of *E. coli* 0111:B4 and 0127:B8 did not remove these antibodies. It is interesting that *Staphylococcus citreus,* which did not yield a suitable antigen for erythrocyte modification, also failed to remove the antibody from gamma globulin.

Discussion. The present investigation revealed that use of red blood cells treated with pancreatic protease and a staphylococcal antigen made possible the detection of antibodies in considerably higher titers in both human gamma globulin and serum than did that of erythrocytes modified by antigen alone. Enhanced sensitivity was noted also when staphylococcal hemagglutination test was combined with the Coombs' method. These observations extend previous studies on basic staphylococcal hemagglutination test used for detection of antibodies in serum(9-13). At least 2 explanations may be considered for results described here. (1) It is conceivable that the enzyme and Coombs' hemagglutination procedures detect "incomplete" staphylococcal antibodies, for such methods are useful for demonstration of "incomplete" Rh antibodies. This possibility, however, must remain speculative, unless it were possible to demonstrate actual differences in antibody molecules between "complete" and "incomplete" staphylococcal antibodies. (2) Alternatively, it is conceivable that the enzyme and Coombs' hemagglutination tests merely render the basic hemagglutination procedure more sensitive and that all 3 methods detect an identical antibody. Since protease and other enzymes(14) are equally effective when used either before or after antigen modification of red blood cells, it is unlikely that they merely make possible the attachment of larger amount of antigen to the surface of cells. Rather, it is suggested that enzymatic removal of some substrate from the surface of red blood cells allows more antigen molecules to participate in the reaction with antibodies and in hemagglutination, as discussed elsewhere(15,16).

It is likely that the staphylococcal antigen is similar to, or identical with, that described by Rantz and associates(17), who demonstrated an antigen common to most gram-positive bacteria and suggested the name "nonspecies specific (NSS) antigen." Our observations that both *B. subtilis* and *Staphylococcus aureus,* in contrast to *E. coli* and *Staphylococcus citreus,* remove from human gamma globulin the antibodies operative in hemagglutination test, and the fact that the staphylococcal antigen is heat stable at acid, but not at alkaline pH, support this assumption. It is possible also that the antigens common to staphylococcus and streptococcus, described by Pakula and Walczak(10) as well as by Znamirowski *et al.*(11), are of the Rantz type. Further studies are needed to isolate and characterize this antigen. Investigations are in progress to determine the conditions (*e.g.* effect of lysozyme) for antigen release from bacterial cells.

The question arises as to whether the staphylococcal antibodies demonstrated in rather high titers in human gamma globulin by means of the enzyme and Coombs' hemagglutination tests have protective value. Experiments to elucidate this problem are being carried out in collaboration with Dr. Myron Fisher, Detroit, Mich. In particular, the protective effect, if any, of human gamma globulin after absorption with *Staphylococcus aureus, S. citreus, B. subtilis,* and other bacteria is being studied. Should antibodies of the Rantz type be effective *in vivo* in experimental

infections, their role in apparently non-specific resistance should be considered along with properdin and other factors.

Summary. Two modifications of a staphylococcal hemagglutination test, utilizing protease for treatment of erythrocytes or Coombs' antiserum, are described and are more sensitive than the basic hemagglutination method for titration of staphylococcal antibodies in human gamma globulin and serum. These antibodies probably are directed against an antigen of the Rantz type, as the antigen is heat stable at acid and labile at alkaline pH and since both *Staphylococcus aureus* and *B. subtilis,* in contrast to *E. coli* and *Staphylococcus citreus,* remove antibodies from human gamma globulin. The significance of the data is discussed.

1. Fisher, M. W., Manning, M. C., *Antib. Ann.,* 1957-1958, 572.

2. Fisher, M. W., Manning, M. C., *J. Immunol.,* 1958, v81, 29.

3. Millican, R. C., Rust, J., Rosenthal, S. M., *Science,* 1957, v126, 509.

4. Sonea, S., Frappier, A., Borduas, A., *Union Méd.,* Canada, 1956, v85, 1028.

5. Waisbren, B. A., *Antib. Chemoth.,* 1957, v7, 322.

6. Ravina, A., *Presse Méd.,* 1957, v65, 1776.

7. Neter, E., Drislane, A. M., Harris, A. H., Gorsynski, E. A., *Am. J. Pub. Health,* 1959, in press.

8. Neter, E., Westphal, O., Lüderitz, O., Gorzynski, E. A., Eichenberger, E., *J. Immunol.,* 1956, v76, 377.

9. Rountree, P. M., Barbour, R. G. H., *Australasian Ann. Med.,* 1952, v1, 80.

10. Pakula, R., Walczak, W., *Acta Microbiol. Polonica,* 1955, v4, 235.

11. Znamirowski, R., Collins, A. M., Neelin, E., Roy, T. E., *Can. J. Publ. Health,* 1959, v50, 30.

12. Oeding, P., *Acta Pathol. Microb. Scand.,* 1955, v37, 116.

13. ———, *ibid.,* 1957, v41, 435.

14. Neter, E., Gorzynski, E. A., Cohen, E., *Fed. Proc.,* 1959, v18, 588.

15. Neter, E., Cohen, E., Westphal, O., Lüderitz, O., Hart, E. P., PROC. SOC. EXP. BIOL. AND MED., 1957, v96, 803.

16. Neter, E., Cohen, E., Westphal, O., Lüderitz, O., *J. Immunol.,* 1959, v82, 85.

17. Rantz, L. A., Randall, E., Zuckerman, A., *J. Infect. Dis.,* 1956, v98, 211.

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New Observations on Pneumococcal Transformations *in vivo.** (24991)

ROSS H. HALL† AND GEORGE O. GALE (Introduced by T. H. Jukes)
Lederle Labs. Division, Am. Cyanamid Co., Pearl River, N. Y.

The first genetic transformation of pneumococcal types was reported by Griffith(1), who inoculated mice subcutaneously with a mixture of live unencapsulated pneumococci and a vaccine of heat-killed encapsulated pneumococci. The mice developed an infection due to encapsulated pneumococci of the same type as the strain from which the vaccine had been prepared. Since that time most of our information concerning genetic transformation of bacteria has come from studies *in vitro,* although there has been some extension of Griffith's technic *in vivo,* as for example by Austrian(2). We were interested in using purified deoxyribonucleic acid (DNA) preparations for transforming experiments *in vivo.* Previously, only vaccines of intact cells had been used. This report describes results of using partially purified DNA preparations *in vivo,* as well as a modification of Griffith's original technic.

Methods and materials. 1. Strains of *Diplococcus pneumoniae.* SV1, Type I strain virulent.‡ A-66, Type III strain virulent. A-66 1,000, Type III, a mutant obtained from strain A-66 in one step, resistant to 1000 γ/

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† Present address, Roswell Park Memorial Inst., Buffalo, N. Y.

‡ Virulence of pneumococci seems to depend directly on amount of polysaccharide capsule(3).