On Hemagglutination Procedures Utilizing Isolated Polysaccharide and Protein Antigens

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Here are presented the technological details of new tests that may prove useful in epidemiological field studies of typhoid fever, diphtheria, and a variety of other conditions that remain as basic public health problems.

The adsorption of bacterial antigens on erythrocytes and the subsequent agglutination of such antigen-treated erythrocytes by specific antibody was first reported in 1948.[1] In the intervening five years the hemagglutination technic has found wide acceptance and has been applied to a number of bacterial antigens. The development of this test has coincided with current intensive investigations concerned with the isolation and characterization of bacterial antigens, and in certain instances it has been a useful adjunct in these studies.

It is commonly recognized that antigens which can be successfully adsorbed on normal erythrocytes, while they may be derived from a number of bacterial genera, appear to have one common attribute—all are polysaccharides. Protein antigens, on the other hand, are not adsorbed by normal erythrocytes, unless, as observed by Boyden,[2] erythrocyte receptors are first modified by treatment with tannic acid. Red blood cells thus modified before treatment with protein antigens are agglutinated by antiprotein antibody.

With the use of these two procedures it now is possible to adsorb on erythrocytes any of a large array of purified bacterial antigens, protein as well as polysaccharide, and to utilize antigen-modified erythrocytes in a variety of immunological problems. It is the object of this report to elucidate certain factors concerned in this phenomenon, to present data on antigens not previously studied in this reaction and, finally, to point out certain limitations, as well as the broad utility of this procedure.

Hemagglutination tests with purified antigens were carried out employing methods which have been described in the literature.[2, 3] Polysaccharide antigens were adsorbed on washed sheep erythrocytes by incubating mixtures of appropriate quantities of antigen and 10 per cent erythrocyte suspension for two hours at 37° C. The antigen-treated erythrocytes were washed three times and diluted to a 1 per cent suspension. On the other hand, protein antigens in appropriate concentration were adsorbed on sheep erythrocytes previously treated with 0.005 per cent tannic acid according to the technic of Boyden.

The antigens employed in this investi-

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Table 1—Activity of Purified Antigens in the Sensitization of Erythrocytes for Specific Hemagglutination

	Antigens Derived from	Antigen Designation	Prepared by:	Chemical Composition	μg of Antigen Required for Sensitization of 1 ml. of 1 per cent Erythrocytes: For Detectable Agglutination	For Maximal Agglutination
Polysaccharides	Salmonella typhosa	O	Webster, Landy [4]	Lipopolysaccharide	0.01	2
	Serratia marcescens	P-25	Shear [5]	"	0.4	2
	Pseudomonas aeruginosa	PC-9	Ginger [6]	"	0.4	2
	Proteus vulgaris	OXK	Hilleman [7]	"	0.4	4
	Pasteurella tularensis	Schu	Wright [8]	"	0.8	4
	Diplococcus pneumoniae	SSSIII	Heidelberger [9]	Acidic polymer	2.0	10
	Escherichia coli	Vi	Webster, Landy [10]	"	0.01	1
					μg/ml. of 2.5 per cent RBC	
Proteins	Pasteurella pestis	Capsular protein	Amies [11]	Protein	0.16	4
	Pasteurella pestis	Toxin	Ajl [12]	"	0.8	4
	Corynebacterium diphtheriae	Toxoid	National Drug Co.	"	0.6	15
	Ovalbumin	..	Worthington Labs.	"	0.16	20

gation were of high purity. Many had been well characterized, and all were known to be essentially free of cross-reacting components. Their activity in specifically modifying erythrocytes was determined by treating 1 ml. volumes of a 10 per cent suspension with quantities of antigen ranging by fivefold increments from 100 micrograms to 0.00032 μg. Information pertaining to the source and chemical type of the purified antigens is recorded in Table 1. Results of antigen titrations in terms of the quantity of antigen per unit volume of erythrocytes required to yield detectable, as well as maximal agglutination (to the titer of antiserum), are also shown. The sera employed in these tests were prepared by immunizing rabbits with intact organisms from which the antigen had been derived and with the isolated antigens where they proved to be antigenic in rabbits. Sera against both the intact organism and the isolated antigen were available for Salmonella typhosa, Serratia marcescens, Pseudomonas aeruginosa, Escherichia coli, and Pasteurella pestis. Essentially identical antigenic activity was observed in tests with sera developed against these isolated antigens and their respective intact bacilli.

With the possible exception of the pneumococcus Type III polysaccharide, a rather surprising degree of uniformity was observed in the antigenic activity of polysaccharides and for the group of protein antigens as well. The similarity was most pronounced in the quantities of polysaccharides required to sensitize erythrocytes for maximal agglutinative activity (to titer of specific antisera). The fairly consistent picture of antigenic activity for each of the two groups of

antigens was quite unexpected since these products not only were isolated from biologically diverse microbial species but, in addition, had been prepared in 10 laboratories by different investigators and by widely different isolation procedures. The similarities in antigenic potency of the lipopolysaccharides, for example, suggest that all antigens of a given chemical structure, regardless of their generic origin, might exhibit the same activity quantitatively, if they were prepared by identical procedures. This is in contrast to reports by other workers which indicated that the amount of antigen required to sensitize red cells has varied considerably from one antigen preparation to another. In view of the data presented in Table 1, this variation may have been due to the fact that reactive components of the antigens tested represented only a portion of the total specimen.

In an effort to obtain information concerning uptake of these antigens by erythrocytes and the nature of receptors, a number of experiments were performed which may extend our information concerning the hemagglutination phenomenon. Normal sheep erythrocytes were exposed to the polysaccharide antigens listed in Table 1 at concentrations which, as previously established, yielded maximal agglutinative activity. Sensitization was effected with the antigens individually, consecutively, and simultaneously. In the consecutive or stepwise sensitization of red blood cells, the incubation time was reduced from two hours to one-half hour for practical considerations. In this procedure, washed red cells were exposed initially to a single antigen. Following incubation and washing an aliquot of these sensitized cells was removed for test and a second antigen was added to the remainder. The latter were also incubated and washed, and an aliquot of the washed cells, now sensitized with two antigens, was removed for test before a third antigen was added to the remainder. The procedure was repeated for each of the antigens employed. Cells sensitized with each of the antigens individually, consecutively, and simultaneously were added to appropriate dilutions of the homologous antisera, and the tests were incubated and read in the usual manner. The results of this experiment are presented in Table 2. The data reveal that the serological reactivity of erythrocytes treated with six polysaccharide antigens was identical regardless of whether the antigens were adsorbed individually, simultaneously, or consecutively. A similar experiment conducted with four protein antigens and employing tannic acid-treated erythrocytes, yielded comparable results, i.e., simultaneous or consecutive sensitization yielded hemagglutination titers which were the same as those obtained with erythrocytes sensitized with these antigens individually. Moreover, it was observed that tannic acid-treated erythrocytes retained their ability to adsorb polysaccharide as well as protein antigens. Sensitization with both types of antigens could also be accomplished simultaneously or in either sequence.

To determine whether it was possible to saturate all receptors on the red cell surface with a single antigen and thus block the adsorption of other antigens, aliquots of washed normal red cells were exposed to concentrations of 20 and of 5,000 μg (per ml. of 10 per cent cell suspension) of polysaccharide from S. marcescens, incubated for two hours, and washed. Twenty μg represented the smallest quantity of antigen providing maximal agglutination (for 1 ml. of 10 per cent cell suspension), while 5,000 μg represented a 250-fold excess of the marcescens polysaccharide. These two antigen-treated red cell suspensions were then exposed to a mixture of five other polysaccharide antigens, incubated for two hours, washed with saline, and

Table 2—Serological Reactivity of Erythrocytes Following Multiple Adsorption of Six Polysaccharide Antigens

Antisera Specific for Indicated Organisms	Antigens Employed for Adsorption	Reciprocal of Hemagglutination Titer Following: Adsorption of Homologous Antigen Alone	Consecutive Adsorption of Indicated Antigens: 0	0 Vi	0 Vi Schu	0 Vi Schu PC-9	0 Vi Schu PC-9 P-25	0 Vi Schu PC-9 P-25 SSSIII	Simultaneous Adsorption of All Antigens
Salmonella typhosa	0	2,560	2,560	2,560	2,560	2,560	2,560	2,560	2,560
Escherichia coli	Vi	640	0	640	640	640	640	640	640
Pasteurella tularensis	Schu	1,280	0	0	1,280	1,280	1,280	1,280	1,280
Pseudomonas aeruginosa	PC-9	320	0	0	0	320	320	320	320
Serratia marcescens	P-25	1,280	0	0	0	0	1,280	1,280	1,280
Diplococcus pneumoniae	SSSIII	1,280	0	0	0	0	0	1,280	1,280

1 ml. of a 10 per cent sheep erythrocyte suspension was treated with 20 μg of each antigen either individually, consecutively, or simultaneously as indicated

set up in hemagglutination tests with the six homologous antisera. Maximal serum titers for each antigen were obtained with red cells treated with excess as well as the predetermined adequate quantity of marcescens polysaccharide. This indicated that it had not been possible to saturate cell receptors with a high concentration of a single antigen. These observations suggest that different receptors or possibly different areas of the erythrocyte may be involved for each antigen. Consequently, the possibility of blocking out other antigens by a high concentration of any single one is unlikely.

In these experiments, involving 11 purified antigens, inhibition by one antigen of uptake of any other by erythrocytes was not encountered. However, factors which significantly affect the adsorption of certain antigens by erythrocytes (and their subsequent reactivity in hemagglutination tests) have been observed in the course of these studies. For example, the lipopolysaccharide from S. typhosa (0 antigen—endotoxin) as isolated from the viable bacterial cell contains more than 30 per cent of bound lipid [4] and is poorly adsorbed by erythrocytes. Heating, alkali treatment, or a combination of the two, releases as much as 50 per cent of this bound lipid and is accompanied by an increase in activity of the antigen of from 100- to 1,000-fold in effectiveness in sensitizing erythrocytes.[13] Thus, the initial product exhibits limited activity at a level of 10 μg per ml. of 1 per cent erythrocyte suspension, while after alkali treatment as little as 0.01 μg is effective. This was the only case encountered of interference with the uptake of polysaccharide antigens by erythrocytes. On the other hand, our studies with proteins indicate that the sensitization of tannic acid-treated erythrocytes with these antigens is far more susceptible to interference by external factors. Certain divalent cations, such as Fe, Ca, and Mg, interfered markedly with the uptake of plague capsular protein by tannic acid-treated erythrocytes.[14] The adsorption of diphtheria toxoid on tannic acid-treated red cells was only slightly affected by these

cations, but certain commercially prepared toxoids were shown to contain components, whose nature and composition is unknown, which interfered with the adsorption of pure toxoid on erythrocytes.[15] The inhibitors in such toxoids generally could be removed by electrodialysis.

Space limitations do not permit the detailing of other factors that may interfere with or complicate this useful serological procedure. However, it cannot be stressed too strongly that antigens of high purity are the principal requisites for satisfactory results. In this respect the test is no better than the quality of the antigen preparations employed. This is not meant to imply that useful information is not obtainable with bacterial extracts or culture filtrates. Such antigenic preparations have been employed by some investigators with excellent results. However, it is important to be mindful of the fact that most, if not all, bacterial species possessing a dominant antigen probably contain an undetermined number of minor, uncharacterized antigenic components as well. The presence of these secondary or minor antigenic components generally is not manifested in bacterial agglutination tests, but these antigens may be extracted from cells along with the principal antigen and thus be "uncovered" or rendered reactive. It already has been pointed out that a number of antigens may be simultaneously adsorbed on erythrocytes. Consequently, where relatively crude antigen preparations are utilized to sensitize erythrocytes, it becomes mandatory to establish the specificity of the hemagglutination test by appropriate absorption controls.

The hemagglutination technic has been applied advantageously to a variety of immunological problems in these laboratories and it may be appropriate to conclude by identifying certain of these studies. Erythrocytes treated with Vi antigen have been utilized to detect Vi antibody in the sera of typhoid carriers[3] and to measure Vi antibody response in field trials of experimental antityphoid immunizing agents. The isolated Vi antigen employed in the hemagglutination test offered advantages of greater stability, sensitivity, and specificity over available Vi bacterial agglutination reagents. Typhoid O antigen (endotoxin) adsorbed on red cells was found to equal or exceed the conventional bacterial antigen in terms of sensitivity and specificity in reacting with antibody.[13] Hemagglutination inhibition tests with this lipopolysaccharide may provide an exceedingly sensitive measure of free antigen (endotoxin) in the blood or urine of typhoid patients. The capsular protein of the plague bacillus adsorbed on tannic acid-treated red cells yielded a specific serological reagent which, in tests with sera from patients suffering from pneumonic plague, was 20–50 times as sensitive as the complement fixation test employing the same antigen.[14] The hemagglutination procedure was therefore capable of detecting plague antibody at levels very much lower than those required for fixation of complement. Finally, a hemagglutination test using diphtheria toxoid adsorbed on tannic acid-treated erythrocytes has been employed to measure the level of antitoxin in human sera. These sera also were assayed for antitoxin by the rabbit intradermal neutralization test. The resultant values appeared to be well correlated and when the data were appropriately analyzed the hemagglutination titers and units antitoxin were found to be linearly related. The hemagglutination procedure may therefore prove to be an acceptable alternate method for the measurement of diphtheria antitoxin.[15]

Our studies suggest that erythrocytes treated with purified bacterial antigens may, under appropriate conditions, pro-

vide useful tools in a variety of procedures applicable in public health laboratories. In many instances they offer substantial advantages over present methods, since purified antigens in the isolated state are uniform, stable, and readily standardized. Hemagglutination tests employing these materials are sensitive and simple serological procedures. Consequently, it seems likely that they will enjoy even wider use in the future than they have in the past.

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Maximum Utilization of Hospital Personnel

In order to help hospitals increase the effectiveness of their personnel, the Health Resources Advisory Committee of the Office of Defense Mobilization has prepared and made available for distribution a handbook entitled "Mobilizing Your Personnel Resources for Better Patient Care." A copy has been sent to the administrators of each hospital registered by the American Hospital Association, and, in the case of larger hospitals, to the chairman of the governing body, the chief of medical staff, and the director of nursing. The suggestions aim to alleviate the added strain upon an already short supply of health personnel that may be caused by a defense emergency. Two additional pamphlets are planned, the next to describe hospitals' experiences in organizing methods improvement programs. The handbook is available from the Government Printing Office, Washington, D. C., 25¢.