Heterogenetic Antigens of Gram-Positive Bacteria¹

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Received for publication 30 October 1965

ABSTRACT

CHORPENNING, FRANK W. (The Ohio State University, Columbus), AND MATTHEW C. DODD. Heterogenetic antigens of gram-positive bacteria. *J. Bacteriol.* **91**:1440-1445, 1966.—Soluble antigens obtained by various methods from gram-positive bacteria were used to modify erythrocytes whose hemagglutinating reactions with immune rabbit sera and normal human sera were then studied. Antigens from all gram-positive organisms studied except corynebacteria altered red cells, causing them to react with specific bacterial antisera and with normal human sera; however, cross-absorption and inhibition tests indicated that at least three different specificities were involved. One of these antigens seemed to be similar to Rantz's streptococcal NSS, which is shared with *Staphylococcus aureus* and *Bacillus* spp., and is therefore heterogenetic. Another was found in streptococci but was apparently not present in *S. aureus* and *Bacillus* spp. A third antigen, also heterogenetic, appeared to be shared by several species of *Bacillus* and by *S. aureus*, but not by streptococci or any gram-negative bacteria. The third antigen was heat-stable at pH 8.0, and appeared to be essentially polysaccharide in nature. Normal human sera varied in their content of antibodies which reacted with erythrocytes modified by extracts from gram-positive bacteria. Whereas some sera reacted very broadly with red cells modified by extracts of practically any gram-positive organism, other sera agglutinated only cells which had been modified by streptococcal antigen.

Rantz et al. (8, 9) demonstrated that broth culture filtrates of groups A, C, and G streptococci, enterococci, *Staphylococcus aureus*, pneumococci, and *Bacillus* spp., as well as acid extracts of streptococci, modify sheep erythrocytes so that they are hemagglutinated and lysed by human sera. Heterogenetic antigens common to a *Streptococcus* and to *S. aureus* were detected, and Neter et al. (6) showed that common antigens exist in the cells of *S. aureus* and *B. subtilis*.

This report concerns immunological comparison of the erythrocyte-modifying antigens referred to above and their further comparison with antigens of a *Bacillus* sp. isolated from the donor bottle involved in a bacteriogenic transfusion reaction (3).

MATERIALS AND METHODS

Bacterial species. The bacteria studied included the *Bacillus* sp. (372-56) previously reported by Chorpenning and Dodd (3), *B. cereus* (220), *Bacillus* sp. (Congo strain-Starr), *B. sphaericus* (62-61), *Corynebacterium* sp. (477), *B. cereus* (ATCC 7064), *B. pan-*

tothenticus (ATCC 14576), *Corynebacterium* sp. (H), *B. brevis* (142), *B. stearothermophilus* (26), *Streptococcus pyogenes* (367), *Staphylococcus aureus* (269), *Proteus vulgaris* OX2 (184), *P. vulgaris* OX19 (187), *P. vulgaris* OXK (182), and *Salmonella typhosa* (320). All cultures were stored on nutrient agar at 5 C, except for *S. pyogenes*, which was maintained in cooked-meat broth.

Preparation of antigens. Cultures were grown on nutrient agar for 3 to 5 days at 33 C, except for *B. stearothermophilus* (grown at 45 C) and *S. aureus*, *S. pyogenes*, *P. vulgaris*, and *S. typhosa* (incubated 24 hr at 37 C). Suspensions of bacteria in phosphate-buffered 0.85% NaCl solution were boiled for 30 min (distilled water being added to maintain volume) and then centrifuged to remove bacteria; the supernatant fluids were frozen for later use as modifying antigens. In certain instances, partially purified antigens were precipitated from these supernatant fluids by adding 10 to 15 volumes of acetone, allowing the mixture to stand for several days at 5 C, removing the supernatant acetone, drying the precipitate at 40 C, and resuspending the precipitate in buffered saline.

Since both Rantz et al. (8) and Neter et al. (6) reported that Rantz antigen (NSS) is inactivated by heating for 15 min at pH 7.2 or higher, some of the bacillary antigens, suspended in phosphate buffers at various pH values, were heated at 100 C for 30 min; this suspension was then centrifuged, and the super-

¹ Some preliminary observations were presented at the annual meeting of the American Association of Immunologists, Atlantic City, N.J., 16-20 April 1963.

nant liquids were used to modify erythrocytes. The degree of modification was assayed by testing the cells with portions of the same dilutions of reactive sera.

Antigens from group A *S. pyogenes* were prepared according to Rantz et al. for comparison: (i) filtrates were prepared from cultures grown in tryptose-yeast extract-dextrose broth at 37 C for 24 hr; (ii) supernatant fluids of broth cultures were obtained by centrifugation three separate times at $3,500 \times g$ for 30 min; and (iii) acid extracts of the sedimented cells were prepared by extraction with 2 M hydrochloric acid at 100 C for 10 min. The acid extracts were neutralized with 1 N sodium hydroxide. All antigens were heated at 56 C for 15 min before use.

Staphylococcal antigens were prepared by essentially the method of Neter et al. (6), except that the bacteria were grown on beef heart infusion agar and were suspended in phosphate-buffered saline at pH 6.7 instead of pH 7.3.

Chemical analysis of the antigen. The partially purified bacillary antigens and some supernatant fluids of crude antigens were analyzed chemically for carbohydrate by the anthrone test (10), for total uronic acid by Dische method A, for protein by the biuret (4) and Folin-Ciocalteu tests (5), and for nitrogen by the micro-Kjeldahl method. Dry weights of various purified antigen preparations were determined by first dialyzing the antigen solution against frequent changes of distilled water in the cold (5 C) and then drying measured volumes at 105 C for 5 days.

Modification of erythrocytes with bacterial antigens. Twenty volumes of antigen solution were added to washed, packed human group O erythrocytes; the mixture was incubated for 30 min at 35 C; the cells were then washed three times, and suspended in buffered saline (pH 6.7) to a concentration of 2%. Control suspensions consisted of cells prepared in the same manner but with buffered saline instead of antigen solution (except for controls of the NSS antigen, which were made with sterile broth).

Preparation of bacterial antisera. Antisera were prepared by injecting albino rabbits intraperitoneally at 2-day intervals with seven 1-ml amounts of the

boiled bacterial suspension, containing about 1 billion organisms per milliliter. The rabbits were bled by cardiac puncture; the serum was heated at 56 C for 30 min, and was absorbed with an equal volume of washed group O erythrocytes for 1 hr at room temperature and 18 hr at 5 C.

Cross-absorption procedure. Erythrocytes modified by antigens of the bacteria to be cross-tested were washed three times, packed, mixed with an equal volume of the serum being absorbed, and incubated at room temperature for 1 hr, followed by further incubation at 5 C for 18 hr. The absorbed serum was removed by centrifugation, and was then frozen.

Serological tests. Hemagglutination tests were performed with modified cells suspended (2%) in phosphate-buffered (pH 6.7) physiological saline solution. Equal amounts (usually 0.05 ml) of serum and modified cells or control cell suspensions were mixed and incubated at 5 C for 30 min. The mixtures were centrifuged lightly and examined microscopically for agglutination.

Inhibition tests were performed by adding an equal amount (0.05 ml) of the antigen to serial dilutions of the appropriate antiserum and incubating the mixtures for 5 min at room temperature followed by 15 min at 5 C. After incubation, 0.05 ml of a 2% suspension of modified erythrocytes was added, and the mixture was again incubated at 5 C for 30 min. The mixture was centrifuged lightly and examined microscopically for agglutination. In each experiment, tubes containing sterile broth (used in preparing certain antigens) or buffered saline were included as controls.

RESULTS

Nature of the antigen. Since cell-free extracts in water, saline, and phosphate solutions would still modify erythrocytes, the bacillary antigen is soluble in these solvents. The antigen was lost when passed through a Seitz filter, apparently by adsorption to the filter pad.

The antigen was also soluble in ethylene glycol and diethylene glycol, but relatively insoluble in ethyl alcohol, acetone, and mixtures of ethyl

TABLE 1. Relation of carbohydrate content to antigen titer*

Lot no.	Reciprocal of antigen dilution	Carbohydrate	Lowest active quantity
		$\mu\text{g/ml}$	$\mu\text{g/ml}$
2-2	8	33	4.0
3-1	4	18	4.5
4-1	2	8	4.0
5-1	8	37	4.5
6-1	16	46	2.9
6-3	8	42	5.2
6-7	8	41	5.1
7-1	32	150	4.7

* The standard curve used for making carbohydrate determinations was prepared with D-glucose.

TABLE 2. Analysis of purified antigens*

Lot no.	Carbohydrate	Uronic acid	Nitrogen	
			Folin-Ciocalteu	Kjeldahl
6-1	46	3.0	0.7	NT†
6-3	42	2.4	0	NT
6-7	41	3.5	0.5	1.6
7-1	150	15.0	2.7	8.0
7-1‡	83	8	1.5	4.4

* Standards were D-glucose, D-glucuronic acid, and Versatol A. The results are expressed as micrograms per milliliter.

† Not tested.

‡ Expressed as percentages of dry weight.

TABLE 3. Capacity of extracts from gram-positive bacteria to produce erythrocyte modification

Species from which extract was prepared	Hemagglutination with	
	Human sera (normal)	Anti-372-56 (immune rabbit)
<i>Bacillus</i> sp. (Congo strain-Starr)	+	+
<i>Bacillus</i> sp. (372-56-Chorpenning)	+	+
<i>B. brevis</i> OSU 142	—	+
<i>B. cereus</i> FAAL 220	+	+
<i>B. cereus</i> ATCC 7064	+	+
<i>B. pantothenicus</i> ATCC 14576 ..	+	+
<i>B. sphaericus</i> FAAL 62-61	—	+
<i>B. stearothermophilus</i> OSU 26 ..	NT*	+
<i>Streptococcus pyogenes</i> OSU 367	+	+
<i>Staphylococcus aureus</i> OSU 269 ..	+	+
<i>Corynebacterium</i> sp. (H)	—	—
<i>Corynebacterium</i> sp. (FAAL 477)	—	—

* Not tested.

alcohol and acetone. The highest yields of precipitated antigen were obtained with a fairly large excess of acetone alone. The precipitated material modified cells, was soluble in water, and was a homogeneous, white, amorphous mass. There was no significant loss of modifying capability when the antigen was boiled for 30 min at pH 7.4 or 8.0.

Chemical analyses of eight different purified antigen preparations indicated that carbohydrate was a major constituent and was related on a weight basis to modifying activity (Table 1). The minimal amount of carbohydrate needed to produce modification was calculated to average 4.35 µg/ml. Small amounts of uronic acid were present. Protein was not present in amounts detectable by the biuret test, and the Folin-

Ciocalteu test yielded results below the dependable range (except with the most concentrated antigen preparation). The nitrogen content, as determined by the micro-Kjeldahl method, was about three times that detected by the Folin-Ciocalteu test. Dry-weight determinations of several preparations were compared with the carbohydrate determinations. The relative proportions of the various constituents of the most highly purified antigen (no. 7-1) were determined (Table 2) as percentages of the dry weight. Representative results with other lots of antigen are also shown in Table 2 for comparison.

Presence of heterogenetic antigen in other species. Modification by crude boiled extracts from eight strains (seven species) of *Bacillus* and four other gram-positive species was assessed by testing with a panel of normal human sera and immune rabbit sera. Modification occurred with all of the gram-positive organisms used except for two species of *Corynebacterium* (Table 3). Each modified cell exhibited cross-reactivity with rabbit antisera to *Bacillus* sp. 372-56.

The reactions of red cells modified by bacillary antigens with rabbit antisera to the same antigens are shown in Table 4. These tests were made with early immune sera to antigens injected intraperitoneally, and thus were expected to exhibit a minimum of cross-reactivity. Yet, cross-reactivity occurred with every antiserum in which reactivity with homologous antigen was demonstrated. This heterologous reactivity was most pronounced with cells modified by *B. pantothenicus* and *B. cereus*. Modification with *B. brevis*, *B. sphaericus*, and *B. stearothermophilus* was of a low order, although *B. brevis* was antigenic (since it produced an antiserum which reacted quite well with *B. pantothenicus* and *B. cereus* cells).

Absorption of antisera to three different species with red cells modified by *Bacillus* sp. 372-56 removed the reactivity for cells modified by *Bacillus* 372-56 and *B. cereus*, and partially removed the reactivity for *B. pantothenicus* cells

TABLE 4. Cross-reactions of various *Bacillus* strains and antisera*

Antiserum	<i>Bacillus</i> 372-56	<i>B. pantothenicus</i>	<i>B. cereus</i>	<i>B. brevis</i>	<i>B. sphaericus</i>	<i>B. stearothermophilus</i>
<i>Bacillus</i> sp. 372-56	8	32	32	1	4	2
<i>B. pantothenicus</i>	1	8	8	0	NT†	NT
<i>B. cereus</i>	0	2	4	0	0	0
<i>B. brevis</i>	0	16	8	2	NT	NT
<i>B. sphaericus</i>	0	0	0	0	0	0
<i>B. stearothermophilus</i>	0	0	0	0	0	0

* Results are expressed as the reciprocal of the lowest serum dilution showing hemagglutination.

† Not tested.

TABLE 5. Cross-absorption of bacillary antisera by *Bacillus*-modified red cells*

Serum	Source of modifying antigens		
	<i>Bacillus</i> 372-56	<i>B. pantothenicus</i>	<i>B. cereus</i>
<i>Anti-Bacillus</i> 372-56			
Unabsorbed.....	4	8	16
372-56 absorbed.....	0	1	0
<i>B. pantothenicus</i> absorbed....	0	0	0
<i>B. cereus</i> absorbed.....	0	1	0
<i>Anti-B. pantothenicus</i>			
Unabsorbed.....	2	4	8
372-56 absorbed.....	0	4	0
<i>Anti-B. cereus</i>			
Unabsorbed.....	0	2	4
372-56 absorbed.....	0	1	0

* Results are expressed as the hemagglutination titer (reciprocal of serum dilution).

TABLE 6. Hemagglutination titers (reciprocal of serum dilution) of cells modified by 372-56, NSS, and *Staphylococcus aureus* antigens

Serum	Cells		
	372-56	NSS	<i>S. aureus</i>
<i>Rabbit</i>			
Anti-372 (no. 1091).....	16	16	16
Anti-372 (no. 1099).....	32	32	64
Anti- <i>S. aureus</i>	8	16	16
Anti- <i>S. epidermidis</i>	0	0	2
Normal no. 615.....	0	0	0
Normal no. 619.....	0	0	0
<i>Normal human</i>			
No. 81662.....	8	4	8
No. 31863.....	8	8	4
No. 40066.....	0	16	0
No. 40067.....	0	16	0

(Table 5). Absorption with cells modified by *B. pantothenicus* and *B. cereus* also removed heterologous antibody.

Comparison with antigens studied by Rantz and Neter. When erythrocytes modified with the NSS antigen and the *S. aureus* antigen of Neter et al. were tested in parallel with those modified by the heterogenetic antigen from *Bacillus* 372-56, similar reactions were frequently observed with immune rabbit sera but were not always present with normal human sera (Table 6).

Absorption of cross-reactive normal human sera and immune rabbit sera with *Bacillus* 372-56 cells removed the reactivity for all three cell types (Table 7); this was true also for human serum

TABLE 7. Absorption of cross-reacting sera with cells modified by 372-56, NSS, and *Staphylococcus aureus**

Serum	Cells		
	372-56	NSS	<i>S. aureus</i>
<i>Human no. 81662</i>			
O absorbed.....	8	4	8
372 absorbed.....	0	0	0
NSS absorbed.....	2	0	1
<i>S. aureus</i> absorbed.....	0	0	0
<i>Rabbit anti-372</i>			
O absorbed.....	32	32	64
372 absorbed.....	0	0	0
NSS absorbed.....	4	0	4
<i>S. aureus</i> absorbed.....	0	2	0

* Results are expressed as the hemagglutination titer (reciprocal of serum dilution).

absorbed with *S. aureus* cells. NSS cells were not so efficient in removing all antibody.

When the various antigens were used in an attempt to inhibit the reactions of a specific anti-NSS, only the NSS supernatant and filtrate antigens were effective (Table 8). Both of these antigens inhibited at least 16 hemagglutinating units of specific anti-NSS. Fourfold dilution of the NSS supernatant fluid still produced a partial inhibitory effect. The *Bacillus* 372-56 and *S. aureus* antigens, which did not inhibit the specific anti-NSS activity, were quite active for immunization, modification, and absorption of anti-*Bacillus* 372-56 antibodies.

Antigens of *P. vulgaris* and *S. typhosa* failed to inhibit the hemagglutination of *Bacillus* 372-56 cells by specific antiserum.

DISCUSSION

Water-soluble antigens, extractable from many gram-positive bacteria, are apparently capable of modifying erythrocytes so that they react with homologous bacterial antisera, and with some normal human sera, and cross-react with antisera to other gram-positive bacteria. Erythrocyte-modifying antigens occurred in all members of the genus *Bacillus* studied, as well as in selected species of *Streptococcus* and *Staphylococcus*. As reported by others, this work suggests the presence of a common antigen in gram-positive bacteria, including *B. brevis*, although Anzai et al. (1) had indicated that *B. brevis* did not modify erythrocytes so as to react with anti-*S. aureus* rabbit serum. The data from cross-absorption experiments support this suggestion, since most of the reactivity for cells modified by a particular antigen was removed by cells modified

TABLE 8. Failure of 372-56 and *Staphylococcus aureus* antigens to inhibit hemagglutination by anti-NSS

Inhibitor	Anti-NSS serum dilution					
	1:1	1:2	1:4	1:8	1:16	1:32
NSS supernatant fluid	—	—	—	—	—	—
NSS filtrate	—	—	—	—	—	—
NSS acid extract	4+	4+	3+	±	—	—
372 (crude)	4+	4+	4+	3+	1+	—
372 (purified, no. 6-1)	4+	4+	4+	3+	1+	—
372 (purified, no. 6-7)	4+	4+	4+	2+	1+	—
<i>S. aureus</i> supernatant fluid	4+	4+	3+	2+	—	—
Sterile broth	4+	4+	3+	1+	—	—
Saline-phosphate	4+	4+	4+	3+	1+	—

with a heterologous antigen. However, there must be at least one heterogenetic antigen in *Bacillus* 372-56 and *S. aureus* extracts which is not found in NSS, since NSS cells fail to absorb completely antibodies reactive with both 372-56 and *S. aureus* cells. Results with other sera demonstrated clearly that the *Streptococcus*-modified NSS cells also possessed an antigen distinct from the one shared by other gram-positive organisms. Thus, some human sera agglutinated NSS cells but failed to agglutinate 372-56- or *S. aureus*-modified cells. The antibody involved in these reactions is distinguished from anti-372-56 by the failure of *S. aureus* and 372-56 antigens to inhibit its action, although NSS extracts produced such inhibition.

The Rantz acid extract of *S. pyogenes* did not inhibit the reaction between filtrate-modified NSS cells and anti-NSS; this indicates that the extract did not contain the same antigen, either because the two methods extract different antigens or because the NSS was lost.

Differences suggesting that more than one antigen was being observed are shown by the following comparisons. (i) The extracts were obtained from different organisms, and the extraction methods were different. (ii) NSS treatment "browned" erythrocytes, but 372-56 treatment did not. (iii) The bacillary extract from 10-day cultures was as strong as that from cultures of 1 to 4 days. Rantz et al. (8) stated that the shorter period is desirable and that NSS disappeared from 9-day cultures. (iv) *Bacillus* 372-56 antigen remained active after treatment at 100 C for 30 min at pH 8.0, whereas NSS was inactivated by such conditions (8). (v) Data to be published separately indicate that the optimal pH for extraction of 372-56 antigen was pH 6.7, whereas that for NSS is from pH 1.7 to 3.8 (8). However, human cells were employed and Rantz used sheep cells, which may account for some of the fore-

going differences. Bokkenheuser et al. (2) showed that considerable differences in hemagglutinability of modified cells exist among different species. (vi) The chemical nature of the antigens appears to differ. The bacillary antigen is probably a complex nitrogen-containing polysaccharide; Neter et al. (7) suggested that the antigen of Rantz et al. is polyglycerophosphate. Analyses indicate that the bacillary extract is principally carbohydrate, with uronic acid and nitrogen as possible additional constituents. Presumably, the antigen is not protein, since it is not denatured by boiling; however, the possibility remains that aromatic amino acids (Folin-Ciocalteu test) are part of the antigenic complex.

The fact that the 372-56 heterogenetic antigen is distinct from the heterogenetic antigens found in *Proteus* and *S. typhosa* is shown by the lack of inhibition by such antigens.

The chemical nature of the antigen suggests that it could easily be present in many foods, or at least that similarities of groups, linkages, or spatial arrangements could be common. Further, it is obvious that the exposure of the population to gram-positive bacteria is very high and might easily be 100%. Thus, it can be readily seen that the origin of the "normal" antibodies which react with 372-56 cells may be from such stimuli.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service grant HE-06124-02 from the National Heart Institute.

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