Nature of a Red Cell Sensitizing Substance from Streptococci¹

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ABSTRACT

JACKSON, ROBERT W. (Purdue University, Lafayette, Ind.), AND MERWIN MOSKOWITZ. Nature of a red cell sensitizing substance from streptococci. *J. Bacteriol.* **91**: 2205–2209. 1966.—A method for purifying a streptococcal antigen which sensitizes red cells to agglutination by antiserum is described. The antigen, when purified by this method, is almost exclusively composed of glycerophosphate and D-alanine. The ratio of alanine to glycerophosphate varies from 1:5 to 1:3. The glycerophosphate is polymerized and is thus a teichoic acid. The polyglycerophosphate appears to be the antigenic determinant for agglutination. D-Alanine is readily removed by mild base and appears to be necessary for the attachment of the teichoic acid to red cells. Quantitative removal of alanine does not affect the ability of the polymer to absorb antibody from serum.

In a previous report, the separation of an antigen from *Streptococcus pyogenes* and a method for separating this antigen from bacterial cells was described (10).

In this study, the chemical nature of the antigen has been elucidated, and the relationship of its biological activity to its chemical structure at least partially defined. The chemical composition of the sensitizing substance is like that of the bacterial teichoic acids and is obviously related to the polyglycerophosphate isolated by McCarty (8) from group A streptococci.

MATERIALS AND METHODS

The growth and preparation of cells, the making of extracts, and the titration of sera and sensitizing substance were described in the preceding paper (10). Strain 1-RP41, a β -hemolytic group A streptococcus, was used as the source of sensitizing substance in these studies.

Purification of red cell sensitizing substance. Purification of the sensitizing antigen was achieved by paper chromatography followed by paper electrophoresis. A 15- to 20-ml amount of dialyzed crude extract with an average dry residue of 250 to 300 μ g/ml was placed in a narrow band along the short dimension of 8- by 16-inch chromatograms of What-

man 3 MM paper. The chromatograms were then developed by descending chromatography for 9 to 12 hr with the organic phase of 4:1:5 *n*-butanol-glacial acetic acid-water (solvent A). The sensitizing substance does not migrate in acidic organic solvents; therefore, after washing the developed chromatogram with acetone, the origin was removed and the sensitizing substance was eluted in approximately 10 volumes of distilled water, relative to the original volume placed on the paper. The elutriant was reduced in volume by pervaporation. A volume equivalent to 10 to 15 ml of the original crude extract was then placed along an origin 10 cm from the cathodic end of 13- by 46-cm electrophoretograms prepared from Whatman 3 MM paper.

Electrophoresis was accomplished in a carbon tetrachloride cell at 1,000 v and 10 ma in formate buffer (pH 3.5) for 1 hr. The sensitizing substance is not electrophoretically homogeneous and is found in a wide band from just in front of the origin to just behind the first of two ultraviolet-absorbing bands. The concentration of sensitizing activity is not linear within this band, but in fact exhibits a rather broad but relatively high maximum approximately midway between the two limits of the band. The area of maximal concentration was eluted in approximately 10 volumes of distilled water and was concentrated to convenient volumes by pervaporation.

Material purified by this scheme gave, upon being adjusted to the original volume of the crude extract, equivalent titers to the crude material, was sedimented as a single component in the ultracentrifuge, and was nondialyzable.

Chemical analysis. Determination of phosphorus (or phosphate) was done by the Allen method (1).

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Nitrogen measurement followed the procedure of Lang (5). The method for determination of pyruvic acid was adapted from Friedmann and Haugen (3). Quantitative estimation of amino acids followed the procedure of Spies (15). Estimation of glycerophosphate is adapted from Reeves (14) and MacFadyen (7).

Production of the dinitrofluorobenzene (DNP) derivative of sensitizing substance. Extracts containing hemagglutinating activity of known titer were adjusted with solid NaHCO_3 to pH 7.8. An equal volume of this solution was added to a volume of 5% alcoholic DNP. This mixture was stirred at 17 C with a vortex stirrer. Stirring was continued for 6 hr, at which time the mixture was acidified with HCl to below pH 3.0 and was then extracted four to five times with ether until extractions were colorless. The aqueous layer was then titrated for hemagglutinating activity, and the remainder was chromatographed with solvent A.

Chromatography. Except for the identification of amino acids, all chromatography was of the descending type on Schleicher and Schuell 507 paper for qualitative analysis and on Whatman 3 MM paper for preparative purification. The solvents employed included the organic phase of *n*-butanol-glacial acetic acid-water, 4:1:5 (solvent A); isobutyric acid:1 N NH_4OH :1 N ethylenediaminetetraacetate (EDTA), 100:60:1.6 (solvent B); *n*-butanol-pyridine-water, 3:2:1.5 (solvent C); and *i*-propanol-acetic acid-pyridine-water, 8:1:8:4 (solvent D).

Identification of amino acids was accomplished with ascending chromatography by use of a two-dimensional system, with development first with *n*-butanol-acetic acid-water, 3:1:1 (solvent E), followed by *i*-butanol-2-butanone-biethylamine-water, 40:40:4:20 (solvent F).

Reagents for identification of substances included periodate-permanganate for glycerol and other diols (6), ammonium molybdate reagent for phosphate esters (2), aniline hydrogen phthalate for sugars (12), and ninhydrin for amino acids and amino sugars (0.4% ninhydrin in methyl cellosolve containing 0.02% stannous chloride, buffered at pH 5.0 with 5% 0.2 M citrate buffer).

RESULTS

Chemical analysis of sensitizing substance. Prolonged hydrolysis in either strong base (1 N NaOH) or strong acid (4 N HCl) at 100 C of purified (see Materials and Methods) sensitizing substance results in complete destruction of sensitizing activity and yields products that migrate identically on paper chromatograms with glycerol, α -glycerophosphate, inorganic phosphate (with solvents A, B, C, and D), and alanine (with solvents E and F). Under the conditions of hydrolysis, the glycerophosphate is certain to be a mixture of the α and β isomers. When hydrolysis was carried out in relatively weak acid (0.1 N HCl) for a period of less than 30 min at 100 C, the products consisted almost entirely of alanine

and glycerophosphate. Table 1 shows the determinations of the two major products plus phosphorus and nitrogen content of representative solutions of hydrolyzed sensitizing substance. The data indicate that the sensitizing substance is composed principally, if not exclusively, of glycerophosphate and alanine, and provide for one alanine for each three to five glycerophosphates. It thus has a formal composition similar to the teichoic acid of *Lactobacillus casei* (4).

Polymerized state of glycerophosphate. That the sensitizing substance does not dialyze and has a comparatively high sedimentation coefficient ($\sim 13S$) indicates that it is of macromolecular dimensions. Alanine can be quantitatively removed from sensitizing substance by treatment with 15% NH_4OH at room temperature (25 C) for 30 min, yet neither the product after removal of alanine nor the sensitizing substance gives a discernible reaction with ammonium molybdate of paper chromatograms. However, the acid hydrolysis product of these gives the characteristic phosphate reaction. In view of the simple composition of the substance, this suggests that glycerophosphate exists as a polymer. Further support for this view is obtained from the infrared-absorption spectrum of purified sensitizing substance in KBr pellets (Fig. 1) from which evidence for the absorption expected for ionic phosphate groups ($1,150\text{ cm}^{-1}$ to $1,180\text{ cm}^{-1}$) is missing.

Conclusive evidence for the polymeric state of glycerophosphate was provided by subjecting a sample of sensitizing substance to the action of *Escherichia coli* alkaline phosphatase. This enzyme caused the release of an average of 1.56% of the total phosphorus. On the assumption that each polymer is not branched and has but one terminal phosphate, this corresponds to a molecular weight of 10,754 for the polyglycerophosphate. Addition of the alanine would increase this to between 11,898 and 12,602.

TABLE 1. Quantitative analysis of purified sensitizing substance

Determination	Sample 1	Sample 2
	μg	μg
Sensitizing substance (avg wt) . . .	1,513	184
Alanine (avg wt)	217	17
Glycerophosphate (avg wt)	1,283	154
Nitrogen: theory	34.2	2.74
determined	31.3	3.1
Phosphorus: theory*	232	27.9
determined	210	34.0
Glycerophosphate/alanine	3.1	4.6

* Based on glycerophosphoric acid.

FIG. 1. Infrared-absorption spectrum of purified sensitizing substance.

Esterified nature of alanine. The ease with which alanine can be removed from the polymer suggests that it is present as an ester, as with other teichoic acids (4). This is further indicated by the fact that sensitizing substance is reactive to ninhydrin both in solution and on paper chromatograms, indicating a free amino group. The esterified nature of alanine is still more strongly indicated by the infrared absorption spectrum of purified sensitizing substance (Fig. 1). The strong absorption at $1,765\text{ cm}^{-1}$ is characteristic of carbonyl stretching of esters with α -electronegative substituents. Moreover, the absorptions at $3,450\text{ cm}^{-1}$, $3,000\text{ cm}^{-1}$, and $1,645\text{ cm}^{-1}$ are not unreasonable for N-H stretching and deformation frequencies of primary amines. The evidence taken together is strongly indicative that the alanine exists in the polymer as a carboxylic acid ester.

Configuration of alanine. Samples of alanine obtained from sensitizing substance were subjected to the action of pig-kidney D-amino acid oxidase and to L-amino acid oxidase in *Crotalus adamanteus* venom. These samples yielded an average of $0.76\text{ }\mu\text{moles}$ of pyruvic acid from $0.69\text{ }\mu\text{moles}$ of alanine when treated with D-amino acid oxidase, but treatment with L-amino acid oxidase failed to yield detectable amounts of pyruvic acid. Thus, within the limits of the assay system, the alanine present in sensitizing substance is entirely the D-amino acid form.

Sensitizing substance in the hemagglutination reaction. Hemagglutination results, in this instance, from a three-component system; therefore, the sensitizing substance must react with two other components, which suggests the possibility of two distinct combining capacities. Earlier work demonstrated that polyglycerophosphate precipitates with specific antiserum (8) and that, although it cannot sensitize red cells, it can inhibit serum from combining with sensitized red

cells (16). It thus seems probable that the polymerized glycerophosphate portion of the sensitizing substance is responsible for the reaction with serum in the hemagglutination process. In support of this idea, it was possible to show that removal of alanine by NH_4OH leaves unaffected the ability of sensitizing substance to inhibit antiserum (Table 2). Removal of alanine destroys the ability of sensitizing substance to sensitize red cells, indicating specific involvement of alanine in the attachment of sensitizing substance to red cells. To further elucidate the involvement of alanine, the sensitizing substance was treated with fluorodinitrobenzene (FDNB) as a means of blocking the amino group. This treatment produced a marked reduction in the hemagglutination titer (Table 3). However, treatment of sensitizing substance with FDNB not only blocks the amino group, but also removes nearly half of the alanine. Hence, at least half of the reduction observed may be due to loss of the amino acid.

These data are in accord with the view that the polyglycerophosphate portion of the sensitizing substance is responsible for its antigenic

TABLE 2. Effect of alanine removal from the sensitizing substance on its ability to inhibit hemagglutination of sensitized red cells

Serum dilution	Serum	Serum plus sensitizing substance	Serum plus sensitizing substance (alanine removed)
1	4+, 4+	4+, 4+	4+, 4+
2	4+, 4+	3, 4	4, 4
4	4+, 4+	2, 3	3, 2
8	4+, 4+	0, 0	0, 0
16	4, 4	0, 0	0, 0
32	3, 2	0, 0	0, 0
64	0, 0	0, 0	0, 0

TABLE 3. *Sensitizing titers of the streptococcal sensitizing substance after treatment with fluorodinitrobenzene*

Dilution factor	Untreated sensitizing substance	Treated substance	
		Sample 1	Sample 2
100	4+, 4+	0, 0	3, 3
1,000	4+, 4+	0, 0	0, 0
2,000	4+, 4+	0, 0	0, 0
4,000	4, 3	0, 0	0, 0
8,000	3, 3	0, 0	0, 0

TABLE 4. *Comparison of alanine and glycerophosphate contents in sensitizing titers of extracts from two strains of group A streptococci**

Determination	Strain	
	1-RP41	Difco A
Alanine ($\mu\text{g/ml}$).....	17	1.5-2
Glycerophosphate ($\mu\text{g/ml}$)...	154	128
Sensitizing titer.....	16,000	1,000

*The extracts were prepared under identical conditions.

character and that attachment to red cells and subsequent hemagglutination in the presence of antiserum requires the presence of the D-alanine ester and probably directly involves the amino group.

Alanine and the sensitizing titer. The sensitizing titers of antigen preparations from various strains of streptococci vary widely, suggesting the possibility that this may be the result of quantitative differences in the alanine esterified to the polyglycerophosphate, as well as to quantitative differences in the amount of sensitizing substance in the various strains. To obtain a partial answer, a comparison was made of the amount of alanine released by NH_4OH and the amount of glycerophosphate present in crude extracts from 1-RP41 and from a group A streptococcal strain (Difco). As seen in Table 4, extracts made under identical conditions gave nearly comparable amounts of glycerophosphate from each strain, but a 10-fold difference in the amounts of alanine is observed. This correlates very strikingly with the sensitizing titers, which differ by at least an order of magnitude.

DISCUSSION

This study establishes that the red cell sensitizing substance, which has been variously called nonspecies specific substance (13), the Hickey antigen (16), and the heterogenetic streptococcal

antigen (11), is composed principally, if not exclusively, of D-alanine and glycerophosphoric acid. It further establishes that the glycerophosphate exists as a polymer, hence the sensitizing substance belongs to the class of substances called teichoic acids. The reactivity of the sensitizing substance with ninhydrin, the infrared absorption data, and the ease of release of alanine are all consistent with the view that alanine is present as a carboxylic acid ester. To prove that all of it is esterified would require quantitative conversion of alanine to one of its amides. The ease with which it is removed, however, makes such conversion difficult, if not impossible. Baddiley (4) reported the half-life of alanine hydroxamate formation in 1 N NH_2OH in similar teichoic acids to be close to that found for the esterified amino acids of transfer ribonucleic acid. Apparently the α -phosphoryl groups "activate" the ester bond, making it quite labile.

As prepared in this study, the sensitizing substance contained one alanine for each three to five glycerophosphates. Much higher ratios have been found in other teichoic acids, and the ease with which alanine is lost suggests that the actual ratio is one when associated with the intact cell. On the other hand, there is no compelling reason to believe that this must be the case. Other glycerol residues are bound to sugar residues, thus making it impossible to achieve a 1:1 ratio of esterified alanine to glycerophosphate. It could very well be that the method of extraction causes scission of such bonds, with attendant release of the sensitizing substance.

This work confirms the fact that polyglycerophosphate is responsible for the reactivity of the sensitizing substance with antiserum, and further reveals that D-alanine is necessary for sensitization of erythrocytes. Moreover, the observed hemagglutination titers of various extracts may be a reflection of the ratio of esterified alanine to glycerophosphate.

During the preparation of this manuscript, a paper by McCarty (9) appeared which generally confirmed our chemical analysis of the sensitizing substance, but which presents evidence that alanine is also involved in determining the serological specificity of the substance in precipitin reactions.

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