JOURNAL OF BACTERIOLOGY, Nov. 1969, p. 641-646

Vol. 100, No. 2
Printed in U.S.A.

# Adsorption of $^3$H-Fatty Acid Esters of Streptococcal Groups A and E Cell Wall Polysaccharide Antigens by Red Blood Cells and Their Effect on Hemagglutination

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Received for publication 14 July 1969

The streptococcal group A and E cell wall polysaccharide (PS) antigens were esterified under identical conditions with four fatty acid chlorides (lauroyl, myristoyl, palmitoyl, and stearoyl), varying from 12 to 18 carbon atoms. With group A PS, it was shown that the four resulting esters varied in their ability to sensitize red blood cells (RBC) to agglutination in the presence of specific antiserum. The most active was palmitoyl (16C) followed by myristoyl (14C). The least active was the lauroyl ester (12C). One-tenth as much palmitoyl ester was required as stearoyl group A PS ester. Such variation in the ability to sensitize RBC was not demonstrated with the group E esters, with the exception of the lauroyl ester which was the least active. Removal of *N*-acetylglucosamine from the esterified and the nonesterified group A PS by enzyme action resulted in a significant loss of serological activity of both antigens. No appreciable difference in the rate or total loss of activity was found in either case. It was demonstrated that both tritium-labeled stearic and palmitic acids and their respective PS esters were adsorbed in significant amounts to RBC. The results indicate that the esterified antigens were adsorbed to the RBC because of the presence of the fatty acid in the PS ester. Attempts to block the receptor sites on the red cell by presensitizing the cells with fatty acid were negative. Likewise, the adsorbed ester did not prevent the uptake of fatty acid at the levels tested. Tritium-labeled esterified group A PS and group E PS were used to show that the amount of antigen required to produce maximal agglutination was the same when cells from the same individual were used, whereas this was not the case when cells from different individuals were used. The amount of antigen required to produce maximal agglutination varied from one batch of sheep RBC to another. Once the optimal concentration of antigen was reached, any additional adsorption did not increase the titer of agglutination.

Lipid-free, nonacidic polysaccharides (PS) from the bacterial cell wall, such as streptococcal group carbohydrate antigens, will not sensitize untreated red blood cells (RBC) in order for passive agglutination to occur in the presence of specific antiserum. In recent studies, Hammerling and Westphal (2) and Slade and Hammerling (7) reported that such PS after esterification with a fatty acid, such as stearic acid, will sensitize RBC and agglutination can be obtained. It was shown (7) that the procedure can be used to determine the antibody levels in sera from men and animals with group A or E streptococcal infections. It was indicated that a similar procedure might be applied to other antigenic polysaccharides.

The authors also noted that different amounts of esterified group A- and group E-specific PS, possessing approximately equal ester contents, were required to produce optimal sensitization. It was suggested (7) that individual antigen molecules may not contain the same quantity of ester and that the stearic acid chain exerts steric hindrance, depending on the structure of the molecule.

The present study was undertaken to obtain direct evidence of adsorption of the esterified PS to the RBC. It was also of interest to determine the effect of the length of the fatty acid chain and the possible effect of steric hindrance by the fatty acid in the PS ester on sensitization of RBC

to hemagglutination. A preliminary report has already been presented (O. Pavlovskis and H. D. Slade, Fed. Proc. 28:663, 1969).

## MATERIALS AND METHODS

**Strains of streptococci.** The group A (type 3, Richards) cells were obtained through the courtesy of Difco. The group E (strain K 129) cells were grown on Todd-Hewitt broth plus a glucose-salts mixture (3). The cells were centrifuged, washed until the supernatant fluid was colorless, and then lyophilized.

**Extraction of antigen.** Group-specific PS cell wall antigen was extracted from whole cells by the trichloroacetic-phenol procedure of Slade (6).

**Esterification of antigen.** The fatty acid chlorides (lauroyl, palmitoyl, myristoyl, and stearoyl) used in the esterification of the polysaccharides were purchased from Eastman Organic Chemicals. The tritium-labeled stearoyl-9,10-$^3$H and palmitoyl-9,10-$^3$H chlorides were obtained from New England Nuclear Corp. Other reagents used in the esterification were prepared as reported earlier (2, 7).

The group A and group E PS were esterified by the procedure of Hammerling and Westphal (2) with certain modifications. A 25-mg amount of PS was esterified with 10 mg of the fatty acid chloride. After 3 days of rapid agitation of the solution in sealed tubes at room temperature, water was added to the mixture to destroy the excess chloride. The volume of the solution was then reduced and dialyzed against 10% ethyl alcohol for several days. The nondialyzable esterified PS was then lyophilized.

**Sensitization of sheep RBC.** Citrated sheep RBC were sensitized as reported previously (7). The RBC were obtained from a commercial source and were drawn from different animals.

**Adsorption and heating of sera.** All sera were held for 30 min at 56 C and then were adsorbed with one-tenth volume of washed sheep RBC for 30 min at room temperature.

**Antibody titration.** Antibody titrations were performed by the method of Slade et al. (7), by using as dilutant 0.85% saline containing 0.3% lyophilized whole normal rabbit serum. Antisera were prepared with rabbits by the injection of whole streptococcal cells according to Slade and Slamp (8) or were used as received from the National Communicable Disease Center. Precipitin tests were performed as reported previously (7).

**Preparation of N-acetylglucosaminidase.** The enzyme was prepared from the culture of a *Bacillus* strain according to McCarty (4). The culture was kindly provided by M. McCarty. The glucosamine was determined as reported previously (6).

**Counting procedure.** All samples were counted with a Beckman LS-100 liquid scintillation counter. The standard scintillation fluid consisted of 1,080 ml of toluene, 1,080 ml of dioxane, 650 ml of absolute alcohol, 240 g of naphthalene, 15 g of 2,5-diphenyloxazole, and 0.15 g of 1,4-bis-[2-(4 methyl-5-phenyloxazolyl)]-benzene.

Aqueous samples of no more than 25 μliters were added to 10 ml of scintillation fluid. All samples were counted in duplicate for 20 or 50 min. If background was greater than 1% of the total count, the necessary correction was applied. Corrections were made for quenching and for the efficiency of the counter.

## RESULTS

Slade et al. (7) demonstrated that the *O*-stearoyl derivatives of group A and group E PS sensitize the red cell and that sufficient receptor sites remain available to produce hemagglutination. It was shown that quantitative aspects of the antigen-antibody combination were affected by esterification; i.e., more esterified antigen was required at the equivalence point and less nitrogen was precipitated. This indicated that the stearic acid chain exerted steric inhibition on some of the active sites (7). It was of interest, therefore, to determine whether esters containing shorter fatty acid molecules would exert less steric hindrance and thereby change the quantitative aspects of the method.

The two group-specific PS were esterified, under identical conditions, with each of four fatty acid chlorides: lauroyl, myristoyl, palmitoyl, and stearoyl. The resulting esters were compared by passive hemagglutination tests with group-specific antisera. In the case of group A PS, it was found that the esters differed in their ability to sensitize sheep RBC, since different amounts of the esterified PS were required to give maximal agglutination (Table 1). This variation in the sensitivity of the esterified PS appeared to be

TABLE 1. *PS antigen concentration responsible for maximal hemagglutination*[a]

| Ester | Group A | | | | Group E | | | |
|---|---|---|---|---|---|---|---|---|
| | Amt of ester (μg) | | Titer | | Amt of ester (μg) | | Titer | |
| | I | II | I | II | I | II | I | II |
| Stearoyl | 250 | 250 | 128 | 128 | 25 | 25 | 128 | 128 |
| Palmitoyl | 25 | 25 | 128 | 256 | 50 | 50 | 256 | 128 |
| Myristoyl | 50 | 100 | 512 | 256 | 25 | 50 | 128 | 128 |
| Lauroyl | 250 | 250 | 128 | 32 | 50 | 75 | 32 | 64 |

[a] The data of two separate experiments are given. Homologous rabbit antisera were used.

related to the length of the carbon atom chain of the fatty acid used in the esterification. The palmitoyl group A PS was about 10 times more active than the stearoyl PS, whereas the myristoyl group A PS was approximately 5 times as active as the A stearoyl derivative. Lauroyl was the least active of the four esters. It appeared that, of the fatty acid chlorides used, palmitoyl chloride yielded the most efficient esters.

This difference in the ability to sensitize sheep RBC was not observed, with one exception, with the group E esters (Table 1). The lauroyl group E ester was again the least active, whereas the other three group E esters were similar to palmitoyl and myristoyl group A PS, since approximately 25 to 50 $\mu$g was required for maximal agglutination.

The steric effect of the fatty acid moiety of the molecule was further investigated by treatment of both esterified and nonesterified PS with an *N*-acetylglucosaminidase. It has been demonstrated that this enzyme removes *N*-acetylglucosamine and the serological specificity from the group A PS (4). The enzyme-PS mixture was held at 25 C; at various times, samples were withdrawn, and the amount of glucosamine released and the antigen-antibody precipitin curves using specific antisera were determined. The results of one experiment are shown in Fig. 1. The rate of glucosamine released and the decrease in serological activity were approximately the same for both the esterified and the nonesterified antigen. The results indicate that, after 24 hr of incubation, the glucosamine released began to level off and 8% more glucosamine was released from the nonesterified antigen than from the stearoyl PS after 30 hr. At this time, approximately 43% of the serological activity of the ester and 56% of the activity of the nonesterified antigen remained. These results show that the action of the enzyme on the ester is similar to its action on the nonesterified PS and that the lipid moiety of the PS does not interfere to any appreciable extent.

It was of interest to investigate whether adsorption of the ester to RBC involved the fatty acid directly, or whether alterations in the PS molecule due to esterification was responsible for adsorption. The cells were sensitized in the usual manner, with the exception that either radioactive stearic or palmitic acids were used instead of esterified PS. The labeled acid adsorbed in significant amounts to the RBC (Table 2), indicating that the fatty acid is most likely responsible for the adsorption of the esterified antigen. There was a limited amount of fatty acid that could be adsorbed to the cell. If the fatty acid concentration was increased to any appreciable amount above

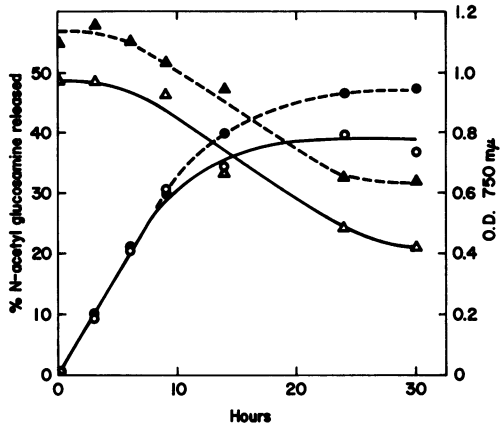


FIG. 1. *Effect of N-acetyl glucosaminidase on nonesterified (- - -) and stearoyl ester (——) of group A PS. Per cent N-acetylglucosamine released (○, ●). Change in serological activity (△, ▲) expressed as optical density.*

the concentration shown in Table 2, the cells lysed rapidly. The concentration of fatty acid at which significant lysis began depended on the cells and their age.

To obtain more precise information regarding the receptor sites on the red cell surface involved in the adsorption, several "blocking" experiments were done with unlabeled ester and tritium-labeled palmitic acid. The cells were first sensi-

TABLE 2. *Effect of presensitization of RBC by palmitoyl-A polysaccharide upon the adsorption of $^3$H-palmitic acid*

| Amt of PS for sensitization[a] | Amt of $^3$H-palmitic acid | Amt of palmitic acid adsorbed per RBC |
|---|---|---|
| $\mu$g | $\mu$g | $\mu$g |
| 0 | 0.38 | $0.118 \times 10^{-8}$ |
| 25 | | $0.085 \times 10^{-8}$ |
| 75 | | $0.117 \times 10^{-8}$ |
| 0 | 0.94 | $0.324 \times 10^{-8}$ |
| 25 | | $0.452 \times 10^{-8}$ |
| 75 | | $0.368 \times 10^{-8}$ |
| 0 | 2.29 | $0.721 \times 10^{-8}$ |
| 25 | | $0.537 \times 10^{-8}$ |
| 75 | | $0.913 \times 10^{-8}$ |
| 0 | 4.76 | $1.462 \times 10^{-8}$ |
| 25 | | $1.679 \times 10^{-8}$ |
| 75 | | $1.648 \times 10^{-8}$ |
| 0 | 6.67 | $1.924 \times 10^{-8}$ |
| 25 | | $2.100 \times 10^{-8}$ |
| 75 | | $2.191 \times 10^{-8}$ |

[a] A 25-$\mu$g amount of PS contains approximately 2 $\mu$g of palmitic acid. Single batch of RBC used.

tized with the unlabeled ester at two levels of palmitoyl group A PS (25 and 75 μg) and then washed four times with saline. These cells were then sensitized with tritium-labeled palmitic acid. Results of one such experiment are given in Table 2. It can be seen that the quantity of palmitic acid adsorbed was in proportion to the quantity of acid to which the red cells were exposed, even though the RBC had previously been exposed to the optimal ester concentration (25 μg) for hemagglutination. Microscopic examination of the cells showed that at high antigen concentration the cells often became misshapen and lysis occurred.

Similar results were obtained when the reverse experiments were done, that is, when cells were presensitized with radioactive palmitic acid, washed four times, and then sensitized with unlabeled palmitoyl group A PS. The highest level of palmitic acid used to presensitize the cells (Table 3) was more than double the concentration of the fatty acid moiety found in the esterified PS at levels giving maximal agglutination. The results (Table 3) show that there is no appreciable decrease in the agglutination titers due to "blocking" the adsorption sites with palmitic acid.

TABLE 3. *Passive hemagglutination titers of palmitoyl group A polysaccharide-sensitized RBC after presensitization with $^3$H-palmitic acid*

| Amt of $^3$H-palmitic acid | Amt of palmitoyl group A PS | Titer |
|---|---|---|
| μg | μg | |
| 0 | 5 | 128 |
| | 10 | 256 |
| 0.428 | 5 | 128 |
| | 10 | 256 |
| 0.889 | 5 | 256 |
| | 10 | 256 |
| 1.750 | 5 | 128 |
| | 10 | 512 |
| 2.669 | 5 | 64 |
| | 10 | 256 |
| 3.703 | 5 | 128 |
| | 10 | 128 |
| 4.487 | 5 | 128 |
| | 10 | 256 |
| 5.377 | 5 | 128 |
| | 10 | 128 |

Tritium-labeled palmitoyl and stearoyl group A and group E esters were used to study the quantitative adsorption of the esters by the RBC. The results of one such test are represented in Fig. 2. The correlation between the amount of antigen adsorbed per red cell and the agglutination titer of this experiment is shown in Fig. 2. Previous investigators have reported that there is an optimal concentration of the esterified PS for sensitization of RBC (2, 7). We have found, by use of radioactive esters, that the antigen adsorption increases asymptotically with respect to the titer as expected. The base line of the curve, however, varies depending on the RBC. Red cells from different individuals adsorb different amounts of antigen.

## DISCUSSION

In the present study, we have shown that, when streptococcal group A PS is esterified with fatty acids differing in the length of their carbon atom chain, the resulting esterified PS varies in its ability to sensitize sheep erythrocytes. This variation could be related to the carbon atom chain of the fatty acid. The optimal length of the chain which yielded the most active antigen was 16 carbon atoms.

The group E PS esters, with the exception of the lauroyl ester, did not show such a distinct variation in their sensitivity. Differences between

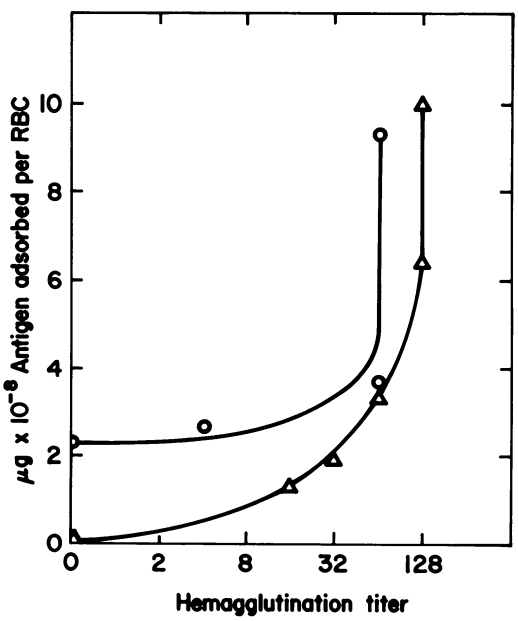


FIG. 2. *Hemagglutination titers of sensitized sheep RBC in relation to $^3$H-esterified group E PS adsorbed by the RBC. Symbols: ○, stearoyl-E; △, palmitoyl-E.*

the two esterified group-specific PS have been noted previously by Slade et al. (7). There are also variations between esterified starch, dextran, and PS from different gram-negative bacteria (2). The reasons for this variation between the two group-specific PS are not known at the present time but are most likely related to the structure of the polysaccharide. It has been shown (H. D. Slade et al., Bacteriol. Proc., p. 46, 1965) that group E PS is a rhamnose-glucose polymer with a molecular weight of about 10,000, whereas the group A PS is a rhamnose-*N*-acetyl-glucosamine polymer (4). To explain the difference in the sensitizing ability of the two types of PS, the degree of cross-linkage and the number of hydroxyl groups available for esterification would have to be known. Since the two types of PS vary in their gross chemical composition, they probably also vary in their structure, most likely in the length and nature of the serologically specific side chain. One could account for the differences by postulating that (i) because of chemical and structural differences, some of the antibody combining sites may be masked by the fatty acids in one PS and not in the other; (ii) different numbers of antibody combining sites may be available in the two group-specific PS; and (iii) the different fatty acids can cause steric hindrance in varying degrees in the two types of PS.

It appears from Table 1 that the fatty acid moiety either has a masking effect on the antibody combining site or exerts steric hindrance, or both, since fatty acid chlorides having carbon chains of differing lengths gave antigens possessing different sensitivities. Also it appears from Fig. 1 that no significant steric effect was involved in the enzymatic release of the serologically specific part of the group A PS. Thus, it is not likely that any single factor is responsible for the differences between esterified group A and group E PS, but rather a combination of factors is involved. Finally, because of the procedures used for the extraction of the PS from the cell wall, the two PS molecules may be degraded into various heterogenous fragments which respond differently to the esterification procedure. That this may occur was also indirectly pointed out by Hammerling and Westphal (2), who stated that it is necessary to determine the optimal concentration of antigen for each preparation.

As noted earlier, direct evidence was needed to determine whether the esterified antigen adsorbed to the RBC via its lipid. The results presented in Table 2 clearly show that the fatty acid is adsorbed in significant amounts by the erythrocyte, indicating that the fatty acid of the PS ester is also responsible for the adsorption of the ester to the RBC. It would be of interest to know whether the esterified PS is adsorbed to receptors on the red cell or to the red cell per se. Several investigators (9) have shown, for example, that the lipopolysaccharide of *E. coli* $O^{86}$ adsorbs to receptor sites on human RBC. It has also been shown that RBC may have receptor sites for several different antigens (1, 5). If specific receptor sites are involved in the adsorption of the PS esters, it should be possible to block these sites. The results of these experiments, however, with tritium-labeled palmitic acid and unlabeled palmitoyl group A PS (Tables 2 and 3) were negative. There was no significant decrease in the uptake of radioactive palmitic acid when the cells were presensitized with esterified PS, nor was there a decrease in agglutination titers when cells were presensitized with palmitic acid. However, since only a limited amount of antigen can be adsorbed to the RBC before lysis occurs, the possibility exists that at the concentration of antigen used only a fraction of the receptors were involved. If this concentration of fatty acid involves only a small fraction of the receptors, effective blocking may not be possible by this technique. Another possibility is that the fatty acid molecules, regardless of whether alone or as a part of the PS molecule, can aggregate even when attached to the receptor. Only steric hindrance caused by the PS part of the molecule may finally inhibit the addition of more fatty acid molecules.

By use of labeled, esterified PS, we have demonstrated that, once the optimal amount of antigen has been adsorbed by the cell resulting in maximal agglutination, additional adsorption of antigen will not increase the hemagglutination titer. The minimal amount of antigen adsorbed by the RBC from different animals to produce maximal agglutination can vary (*unpublished data*). However, when RBC from the same source were used, the results were similar. It may, therefore, be assumed that the difference in the amount of the radioactive PS adsorbed by RBC from different animals is due to the variation in the number of receptor sites on the red cell. At antigen concentrations below those yielding maximal agglutination, the agglutination titers, however, were not necessarily linear to the amount of antigen adsorbed by the RBC.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grant HE-03709 from the National Heart Institute and by grants from the Office of Naval Research (NR 103-608), the Chicago-Illinois Heart Association, and the Grainger Fund. O.P. was a predoctoral trainee supported by Microbiology Training grant 5 T1-GM-724 from the National Institute of General Medical Sciences. H.D.S. was the recipient of Research Career Award

K6-GM-16284 from the National Institute of General Medical Sciences.

## LITERATURE CITED

1. Davies, D. A. L., M. J. Crumpton, I. A. Macpherson, and A. M. Hutchison. 1958. The adsorption of bacterial polysaccharides by erythrocytes. Immunology **1**:157–171.
2. Hammerling, U., and O. Westphal. 1967. Synthesis and use of *O*-stearoyl polysaccharides in passive hemagglutination and hemolysis. Eur. J. Biochem. **1**:46–50.
3. Hess, E. L., and H. D. Slade. 1955. An electrophoretic examination of cell free extracts from various serological types of group A hemolytic streptococci. Biochim. Biophys. Acta **16**: 346–353.
4. McCarty, M. 1956. Variation in the group-specific carbohydrate of group A streptococci. II. Studies on the chemical basis for serological specificity of the carbohydrates. J. Exp. Med. **104**:629–643.
5. Neter, E. 1956. Bacterial hemagglutination and hemolysis. Bacteriol. Rev. **20**:166–188.
6. Slade, H. D. 1965. Extraction of cell-wall polysaccharide antigen from streptococci. J. Bacteriol. **90**:667–672.
7. Slade, H. D., and U. Hammerling. 1968. Detection by hemagglutination of antibodies to group A and group E streptococci by the use of *O*-stearoyl derivatives of their cell wall carbohydrate-grouping antigens. J. Bacteriol. **95**:1572–1579.
8. Slade, H. D., and W. C. Slamp. 1962. Cell-wall composition and grouping antigens of streptococci. J. Bacteriol. **84**:345–351.
9. Springer, G. F., E. T. Wang, J. H. Nichols, and J. M. Shear. 1966. Relations between bacterial lipopolysaccharide structures and those of human cells. Ann. N.Y. Acad. Sci. **133**: 556–579.