

β -Hemolytic Streptococci with Group A and Type II Carbohydrate Antigens¹

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ABSTRACT

JABLON, JAMES M. (National Children's Cardiac Hospital, Miami, Fla.), BETTY BRUST, AND MILTON S. SASLAW. β -Hemolytic streptococci with group A and type II carbohydrate antigens. *J. Bacteriol.* **89**:529-534. 1965.—Ten strains of β -hemolytic streptococci with unusual somatic antigens were isolated from excised tonsils or throat cultures or both. Acid extracts of these strains reacted with commercial group A and group F antisera, but gave no reaction when tested with 35 type-specific group A antisera. Serum cross-absorption and agar-gel diffusion studies established the identity of the reactive antigens as the specific carbohydrates of group A and of type II. The latter antigen has been found in many strains of group F streptococci. The organisms do not possess the group-specific carbohydrate of group F, and the reaction with the commercial group F antiserum is due to the presence of type II antibody in the antiserum. The organisms give a stronger reaction to fluorescein-conjugated type II antiserum than to group A. However, the organisms have only one group-specific carbohydrate, that of group A, and, tentatively, should be classified as such.

Hemolytic streptococci are divided into serological groups based on the presence of an immunologically distinct carbohydrate, the C polysaccharide, in the cell wall (Lancefield, 1928, 1933, 1941). The C polysaccharide of more than one serological group is usually not found, naturally, in any one strain of *Streptococcus*. In 1958, during an investigation of the epidemiology of the β -hemolytic streptococci in Miami, Fla., several strains were isolated which reacted to both group A and group F streptococcal antisera (Saslav et al., 1958). To verify the reactions, acid extracts of the organisms were tested with three group A antisera (two different commercial preparations and one prepared in our laboratory) and two group F antisera (different commercial preparations); all gave positive reactions. If these reactions were engendered by group-specific polysaccharides, this would indicate the unusual occurrence of more than one group-specific carbohydrate in a single strain. Since the original isolation, these organisms have been repeatedly subcultured on artificial media, and extracts continue to give the same reactions, indicating the stability of the antigenic components. This unusual reactivity prompted the present in-

vestigation into the nature of the somatic antigens of these organisms.

MATERIALS AND METHODS

Source of streptococcal strains. Ten streptococcal strains which reacted with commercial group A and group F antisera were isolated from throat cultures or excised tonsils or both. For convenience, these cultures will be designated as AF for the remainder of this report. One of these cultures, AF 953, was used in all of the subsequent studies. To rule out the possibility of mixed cultures, they were repeatedly streaked on Blood Agar Base (Difco) containing 4% defibrinated sheep blood, and isolated colonies were picked and grown in Todd-Hewitt broth (Difco). Extracts of the cultures all gave positive precipitin reactions with the group A and group F antisera. Group F II strain was obtained as a group F culture from Max D. Moody, Communicable Disease Center, U.S. Public Health Service, Atlanta, Ga. This culture was found to possess group F and type II carbohydrate antigens. Group F₀ strain (I 57) possessed the group F antigen but did not have any type antigen. Two type II strains were used. One of these, H 189, together with culture I 57, was obtained through the courtesy of H. Ottens and K. C. Winkler, University of Utrecht, Netherlands. The second culture of type II was isolated here. It was presumed to be a group F culture because of its reaction with commercial group F antiserum, but later it was found to be a type II culture without the group F antigen.

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Extracts for grouping and typing. The cultures were grown in 40 ml of Todd-Hewitt broth, and acid extracts were prepared by the Lancefield (1928) method and tested by the microprecipitin technique of Swift, Wilson, and Lancefield (1943) for group and type.

Group A type antisera were obtained through the courtesy of John F. Winn, Communicable Disease Center, U.S. Public Health Service, Atlanta, Ga.

Preparation of nongroup A antisera. Three antisera were prepared: (i) AF antiserum, with culture AF 953 as a vaccine; (ii) group F₀ antiserum, with culture I 57 as a vaccine; (iii) type II antiserum, with culture H 189 as a vaccine. The cultures were grown in Todd-Hewitt broth for 18 hr and heated in a water bath at 60 C for 30 min. The bacterial suspensions were washed twice with 0.86% NaCl solution and standardized to a density of 25 to 30% optical transmittance in a Coleman Junior colorimeter at 610 m μ . Rabbits were given intravenous injections on alternate days for 4 weeks, 0.5 ml per injection the first week and 1.0 ml for the remaining 3 weeks. After 1 week of rest, trial bleedings showed that strong, specific antisera had been produced.

Fluorescein-conjugated antiserum. Part of the type II and F₀ antisera were conjugated with fluorescein isothiocyanate according to the technique of Moody, Ellis, and Updyke (1958).

Effect of enzymes. Trypsin (Microbiological Associates, Inc., Bethesda, Md.) equivalent to 2.5% was used in a 1:10 dilution with saline, adjusted to pH 7.8; pepsin, 1:10,000 (Difco), was used in a concentration of 1% in 0.01 N HCl, adjusted to pH 2.0. Strains of group A, group F₀, F₁₁, type II, and AF streptococci were grown in 750 ml of Todd-Hewitt broth for 18 hr, centrifuged, and suspended in 25 ml of 0.86% salt solution. The suspensions were heated in a water bath at 60 C for 30 min. A 5-ml sample of each culture suspension was removed and centrifuged, and the organisms were resuspended in 5 ml of trypsin or pepsin and left at room temperature for 18 hr. The trypsin suspensions were then placed in a boiling-water bath for 10 min to inactivate the trypsin; the pepsin suspensions were inactivated by adjusting the pH to 7.2. The suspensions were centrifuged, washed with sterile 0.86% salt solution, and acid extracts were prepared for testing with appropriate antisera.

Serum cross-absorption tests. Commercial group A and group F streptococcal antisera and the antisera prepared against AF 953, group F₀, and type II streptococci and the homologous cultures were used. The cultures were grown in 40 ml of Todd-Hewitt broth for 18 hr, heat-killed at 60 C for 1 hr in a water bath, centrifuged, and the sedimented organisms were washed twice in 0.86% saline. A 1-ml amount of each antiserum was used to suspend the organisms, and the antiserum-organism suspensions were incubated at 37 C for 1 hr, with occasional shaking, and then placed in the refrigerator at 4 C for 18 hr. The suspensions

were centrifuged, and the clear supernatant antisera were tested with acid extracts of the cultures used for absorption to ascertain that complete absorption had taken place. The absorbed antisera were used in the cross-absorption tests.

Agar diffusion plates. Agar (1%, Difco) was prepared in 0.76% saline and tubed, 25 ml per tube. Petri plates were poured by use of one tube per plate. After the agar hardened, well-patterns were cut with an 8-mm cork borer, and the wells were filled with acid extracts of the cultures or with antisera. The plates were then placed in a refrigerator, and after 6 to 7 days they were examined for precipitation bands and photographed.

RESULTS

Typing the AF streptococci. Group A streptococci have been subdivided into more than 40 serological types based on the presence of a type-specific M protein in the cell wall. Acid extracts of the AF streptococci in the present study reacted with group A antiserum, placing them within group A. An attempt was made to see if they would fall into one of the specific types of group A. Thirty-five type-specific group A antisera were used, but the extracts did not react to any of them. (The following type antisera were used: 1 to 6; 11, 12, 14, 15, 17, 18, 19; 22 to 26; 28 to 33; 36 to 44; 46, 47.)

Reactivity of the AF antiserum. The antiserum resulting from the immunization of rabbits with culture AF 953 gave strong precipitin reactions with acid extracts of the AF cultures and with several group F cultures. However, it did not react with extracts of group A cultures, even though the group A antigen was present in the AF culture used as a vaccine, as evidenced by positive precipitin tests with group A antiserum. Attempts to increase the reactivity of the antiserum by putting the rabbits through a second series of injections failed to alter the reactivity of the antiserum. It reacted with the AF and some group F cultures but not with group A.

Effect of proteolytic enzymes. The antigens responsible for the group specificity of streptococci are carbohydrates. The reactivity of the present strains with group A and group F streptococcal antisera, therefore, must be due to either group-specific carbohydrates or to cross-reactions of a nonspecific nature. To rule out nonspecific proteins, heat-killed cultures (60 C for 30 min) of the AF-reacting streptococci were treated with trypsin or pepsin for 18 hr, and acid extracts were prepared. Group A and group F streptococcal strains and their antisera were included in the testing as controls. Enzyme treatment did not alter the reactivity of the organisms, indicating that the reactive fractions were carbohydrates.

Identity of the somatic antigens of AF streptococci by cross-absorption tests. The reactions of extracts of the AF streptococci indicated the presence of at least two antigens, one that reacted with group A antiserum and one that reacted with group F antiserum. However, the extracts did not react with any of the group A type antisera. An attempt was made, therefore, to see if they could be typed within group F. In contradistinction to group A where typing is based on a protein, the M protein (Lancefield, 1928), typing in group F is dependent on a carbohydrate which is distinct from the group-specific carbohydrates. On this basis, group F was subdivided into four types (Bliss, 1937), and, more recently, a fifth type has been reported (Ottens and Winkler, 1962).

There are several important considerations with regard to the group and type antigens of group F: (i) both antigens may be present in a single strain; (ii) the group antigen may be present without an accompanying type antigen; (iii) the type antigen may be present without the group antigen; (iv) the type antigen may be present in another group. In the latter two categories the strains cannot be classified as group F.

To determine the type of the AF streptococcal strain used in the present study, the commercial group F antiserum was tested for group and type antibodies by the microprecipitin technique by use of acid extracts of several strains of group F containing known group and type antigens. Surprisingly, a preliminary study showed that the antiserum contained type II antibodies but no group F antibodies. This was corroborated by carrying out cross-absorption tests with the prepared antisera against AF, F₀ (no type antibodies), and type II (no group antibodies) as well as the commercial group F antiserum. Group A antiserum and group A streptococci were included in the tests. The results are presented in Table 1.

The results show that the group A and the AF-reacting streptococci completely absorb the group A antibodies from the group A antiserum indicating an identity of the group A antigen in the two strains. The AF and type II streptococci completely absorb the antibodies from the commercial group F and type II antisera, and the group F₀ strain does not do so. This suggests an antibody identity between the commercial group F and the type II antisera. This was verified by further studies with the agar-gel double-diffusion technique of Ouchterlony (1949).

Identity of the somatic antigens of the AF streptococci by agar-gel diffusion. Initially, acid ex-

TABLE 1. *Effects of cross-absorption on antiserum precipitin reactions*

Antiserum	Absorbed with (cultures)	Streptococcal culture (acid extracts)				
		A	F ₀	F _{II}	Type II	AF
Group A (commercial)	Unabsorbed	+	-	-	-	+
	A	-	-	-	-	-
	F ₀	+	-	-	-	+
	F _{II}	+	-	-	-	+
	Type II	+	-	-	-	+
Group F (commercial)	Unabsorbed	-	-	+	+	+
	A	-	-	+	+	+
	F ₀	-	-	+	+	+
	F _{II}	-	-	-	-	-
	Type II	-	-	-	-	-
Group F ₀	Unabsorbed	-	+	+	-	-
	A	-	+	+	-	-
	F ₀	-	-	-	-	-
	F _{II}	-	-	-	-	-
	Type II	-	+	+	-	-
Type II (H 189)	Unabsorbed	-	-	+	+	+
	A	-	-	+	+	+
	F ₀	-	-	+	+	+
	F _{II}	-	-	-	-	-
	Type II	-	-	-	-	-
AF (953)	Unabsorbed	-	-	+	+	+
	A	-	-	+	+	+
	F ₀	-	-	+	+	+
	F _{II}	-	-	-	-	-
	Type II	-	-	-	-	-
	AF	-	-	-	-	-

tracts of group A, group F, and AF streptococci were tested for reactivity to their antisera (Fig. 1 and 2). In Fig. 1, the two vertical wells were filled with antisera A and AF, and the horizontal wells were filled with extracts of group A and AF streptococci. In all the figures the antisera are labeled with capital letters and the extracts with lower-case letters. The group A extract showed no reaction to the AF antiserum, indicating that the antiserum did not contain antibodies to group A even though the AF streptococci, which were used to prepare the antiserum, contained the group A antigen as shown by a reaction band identical to that of the group A extract to the group A antiserum. In the reactivity of the AF antiserum with the AF extract, two reactive bands, one major and one minor, were evident, indicating the presence of two antigens and antibodies. The major band was due to the reaction of the type II antigen and antibody. This is apparent from Fig. 3 to 6.

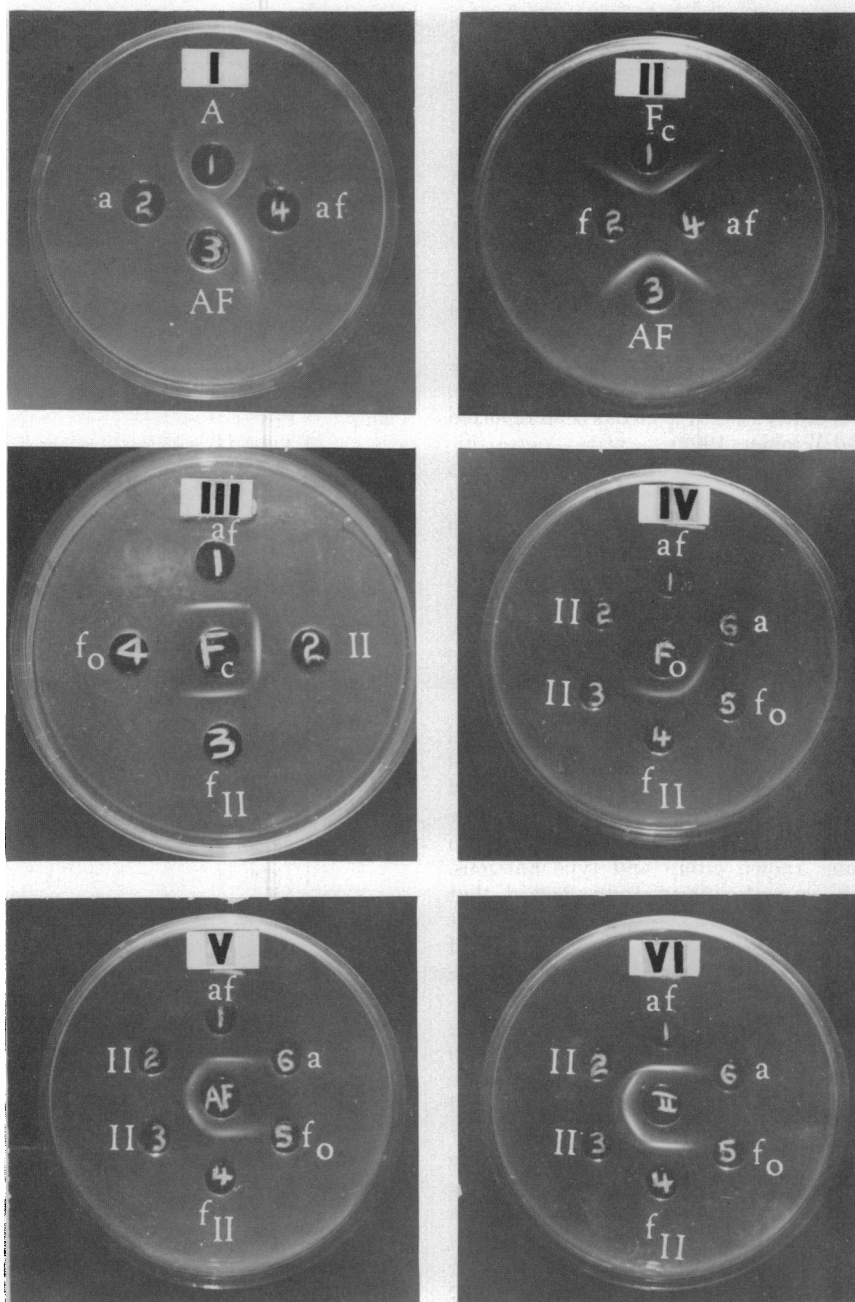


FIG. 1 to 6. Agar-gel diffusion reactions of group and type antigens and antibodies. Antisera are in capital letters; antigens (streptococcal acid extracts) are in lower case letters. A = streptococcal group A antiserum; AF = antiserum to AF culture 953; F_c = commercial group F antiserum; F_0 = antiserum to culture 157 containing antibodies to group F, but lacking type antibodies; II (Fig. 6, center well) = antiserum to type II; a = extract of group A Streptococcus; af = extract of Streptococcus AF 953; f = extract of group F Streptococcus later found to contain group F and type II antigens; f_0 = extract of culture 157, containing group F antigen, but lacking type antigens; f_{II} = extract containing group F and type II antigens; II (in peripheral wells) = extract containing type II antigen, but lacking group F antigen.

However, the significance of the minor band remains to be elucidated.

In Fig. 2, group F (commercial) and AF antisera were placed in the vertical wells and acid extracts of group F and AF streptococci were placed in the horizontal wells. The major reactant fractions to both antisera show continuous identical bands indicating an identity of antigens and antibodies.

The previous results from the cross-absorption tests indicated that the commercial group F antiserum contained only type II antibody. To check the identity of the reacting antigens, acid extracts of the AF, type II, group F_{II}, and group F₀ streptococci were tested against the commercial group F antiserum (Fig. 3). The antiserum is in the center well and the extracts are in the peripheral wells. The three extracts of the AF, type II, and F_{II} streptococci had an identical reactive fraction, and that of F₀ did not react. This indicated that the commercial group F antiserum contained type II antibody, not group F.

The above extracts were also tested against F₀ antiserum (Fig. 4). In this series of tests, extracts of group A and a second strain of type II were included as controls. The F₀ and F_{II} extracts showed an identical reaction band, and the extracts of AF and type II showed no reaction, indicating that they do not contain group F antigen. The group A control extract also showed no reaction.

The major antigen of the AF *Streptococcus* is type II. This is shown in Fig. 5 and 6 where the AF and type II antisera, respectively, were placed in the central wells. The peripheral wells in both figures contained acid extracts of AF, type II (two strains), group F_{II}, group F₀, and group A streptococci. The identity of the reactivity of the extracts to both antisera indicated that the major antigenic component of the AF strain was the type II antigen. Group F₀ and group A extracts did not react.

Reaction of the AF streptococci to fluorescein-conjugated antisera. Group A streptococci are usually identified by precipitin reaction of streptococcal extracts with group-specific antiserum. In recent years, group A antiserum has been tagged with fluorescein isothiocyanate (Moody et al., 1958) and used in the identification of group A streptococci by the fluorescent-antibody (FA) technique. A modification of this technique was used by Cole and Hahn (1962) to identify the locale of specific antigen in the cell wall of the *Streptococcus*.

In the present study, the AF streptococci

were stained with fluorescein-conjugated group A antiserum. The organisms showed only a slight, irregular fluorescence; control group A streptococci showed a strong fluorescence. The AF streptococci were then stained with fluorescein-conjugated type II antiserum. They gave a brilliant fluorescence, but the control group A streptococci did not fluoresce. Since both antigens are present in the AF streptococci, this may be an indication that the type II antigen in some way overshadows or suppresses the reaction to the group A antigen. This may explain why the AF antiserum does not have group A antibodies.

DISCUSSION

The unusual strains of β -hemolytic streptococci in the present study contain both group A and type II carbohydrate antigens in or on the cell wall. On original isolation, acid extracts of these strains gave positive precipitin tests with commercial preparations of group A and group F streptococcal antisera. This was interpreted to mean that the strains possessed two group-specific carbohydrate antigens, one to group A and one to group F. Ottens and Winkler (1962), however, showed that different commercial group F antisera contained antibodies either to type II or to group F and type II in combination. Investigation showed that the commercial group F antiserum used here in the precipitin tests with these unusual strains of streptococci contained antibodies to type II, not group F. The strains under study, therefore, contained only a single group carbohydrate antigen, that of group A. However, the major antigen of these strains, with regard to antibody production, is the type II carbohydrate antigen. When the organisms are stained with fluorescein-conjugated antisera, they show a strong fluorescence with type II but only an incomplete, spotty fluorescence with group A. Evidently the group A antigen is not as reactive on the surface of the organisms as is the type II antigen. However, the organisms can absorb group A antibody; therefore, sufficient antigen must be available for this reaction. But either the quantity is not sufficient for the stimulation of antibody production when the whole organism is used as a vaccine, or the type II antigen is present as a major antigen and suppresses the reaction to the group A antigen and only type II antibody is produced. The observation was made by Bliss (1937) and Ottens and Winkler (1962) that in group F, when type and group antigens are present in a single strain, the type antigen usually predominates when

the strain is used as a vaccine for antibody production. This observation may apply to the AF streptococci in the present study.

The type II carbohydrate antigen is common among strains of group F. However, it has also been reported in one strain of group T (Ottens and Winkler, 1962). The present report of the type II antigen in strains of streptococci containing the group A antigen indicates the possibility that it may also be present in other groups. The significance of the presence of this antigen, either alone or in combination with a variety of group antigens, is not apparent at the moment. However, it should be recognized that its presence may result in confusing cross-reactions with commercial group F antisera which contain antibodies to type II.

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LITERATURE CITED

- BLISS, E. A. 1937. Studies upon minute hemolytic streptococci. III. Serological differentiation. *J. Bacteriol.* **33**:625-642.
- COLE, R. M., AND J. J. HAHN. 1962. Cell wall replication in *Streptococcus pyogenes*. *Science* **135**:722-744.
- LANCEFIELD, R. C. 1928. The antigenic complex of streptococcus haemolyticus. III. Chemical and immunological properties of the species-specific substance. *J. Exp. Med.* **47**:481-491.
- LANCEFIELD, R. C. 1933. A serological differentiation of human and other groups of hemolytic streptococci. *J. Exp. Med.* **57**:571-595.
- LANCEFIELD, R. C. 1940-1941. Specific relationship of cell composition to biological activity of hemolytic streptococci. Harvey Lectures, Ser. 36, p. 251-290.
- MOODY, M. D., E. C. ELLIS, AND E. L. UPDYKE. 1958. Staining bacterial smears with fluorescent antibody. IV. Grouping streptococci with fluorescent antibody. *J. Bacteriol.* **75**:553-560.
- OTTENS, H., AND K. C. WINKLER. 1962. Indifferent and haemolytic streptococci possessing group-antigen F. *J. Gen. Microbiol.* **28**:181-191.
- OUCHTERLONY, O. 1949. Antigen-antibody reactions in gels. *Acta Pathol. Microbiol. Scand.* **26**:507-515.
- SASLAW, M. S., M. SAUL, S. A. JENKS, AND J. M. JABLON. 1959. β -Hemolytic streptococci from several sources reactive in both groups A and F antisera. *J. Bacteriol.* **77**:519.
- SWIFT, H. F., A. T. WILSON, AND R. C. LANCEFIELD. 1943. Typing group A hemolytic streptococci by M precipitin reactions in capillary pipettes. *J. Exp. Med.* **78**:127-133.