Somatic Antigens of *Streptococcus* Group E

I. Comparison of Extraction Techniques

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Received for publication 12 January 1970

Eleven *Streptococcus* group E strains, representing serotypes I, II, III, IV, V, and "untypable" isolates, were extracted by formamide, trichloroacetic acid, and hydrochloric acid under various conditions in an effort to determine the best method for recovering maximum amounts of group and type antigens. The group antigen was found to be relatively stable, and adequate amounts for identification purposes were recovered by a wide spectrum of conditions. Type-specific antigens were relatively labile, and were destroyed at low pH in acid hydrolysis or by prolonged heating in formamide hydrolysis. The best single procedure for recovering both type and group antigens from *Streptococcus* group E was formamide hydrolysis for 30 min at 180 C.

A substantial number of *Streptococcus* group E (SGE) isolates recovered from swine affected with streptococcal lymphadenitis [jowl abscess (4, 5, 10, 14)] cannot be serotyped (1, 15). Such "untypable" isolates may represent serotypes for which typing antiserums are unavailable, or they may be deficient in or devoid of type antigen. Obtaining adequate type antigen for serotyping deficient SGE could be directly related to the efficiency of antigenic extraction.

Several methods of extracting streptococcal antigens have been reported (8, 9, 11, 12). However, the relative efficiency and the optimal parameters of each method for extracting the type and group antigens of SGE have not been described to our knowledge.

The work reported here was undertaken to define a more nearly optimal technique of recovering the somatic antigens of SGE. The results should be useful in the study of streptococcal lymphadenitis of swine inasmuch as these antigens may be related to immunogenesis and pathogenesis of the disease.

**MATERIALS AND METHODS**

**Bacteria.** Eleven SGE isolates, representing five serotypes and two isolates of unknown serotype, were studied. Seven of the isolates were recovered from swine affected with streptococcal lymphadenitis and four were reference strains. The strains, serotypes, and origins of the SGE studied are summarized in Table 1.

Freeze-dried SGE was hydrated and streaked onto blood-agar (5% citrated sheep blood in tryptose phosphate agar). The plates were incubated in a candle jar for 48 hr at 37 C and were subcultured to tryptose phosphate agar "stabs." The stabs were incubated for 48 hr and stored at 4 C. Four 500-ml volumes of Todd-Hewitt broth (Difco), inoculated from each stab culture, were incubated for 16 to 18 hr at 37 C and were determined to be pure by blood-agar streak plate subculture. Each of the four 500-ml volumes of broth culture was subcultured to a 2,000-ml volume of tryptose broth (2% tryptose, 1% dextrose, and 0.0018% phenol red) and incubated at 37 C. The desired pH was maintained by adding 5 N NaOH (approximately once per hour) and a volume of 50% dextrose solution equal to the amount of NaOH employed was added to reestablish a dextrose concentration of 1% as described by Boszormenyi et al. (2). Cultures were monitored and adjusted for 6 to 8 hr, incubated at 37 C overnight without further adjustment, determined to be pure, phenolized (1% weight per vol), and incubated for 24 hr at 4 C. Phenolized bacteria were sedimented by centrifugation (Sharples Centrifuge, Equipment Division of Pennsalt, Warminster, Pa.), washed three times in physiologic saline solution (PSS), freeze dried, and stored in a desiccator jar at room temperature.

**Antiseria.** *Streptococcus* group C grouping antiserum was prepared with strain K64/0/14 as described previously (1). SGE typing antiserums were prepared as described previously with strain K-131 (serotype II), EM-1 (serotype IV), and TG-9 (serotype V). *Streptococcus* strains K64/0/14 and K-131 were obtained from Rebecca Lancefield (The Hospital of the Rockefeller Institute of Medical Research, New York, N.Y.), and strains EM-1 and TG-9 were recovered during an etiologic study of streptococcal lymphadenitis (1).

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1. Published as journal paper 3943 by the Purdue University Agricultural Experiment Station.
2. Present address: Amdal Co., P.O. Box 98, Berlin, Md. 21811.
Table 1. *Streptococcus* group E studied

| Strain     | Serotype | Source  |
|------------|----------|---------|
| Reference strains |          |         |
| K-129*    | I        | Milk    |
| C24A*     | I        | Swine abscess |
| K-131*    | II       | Milk    |
| 4678b     | III      | Milk    |
| Field isolates of known serotype |          |         |
| OSC-1     | II       | Swine abscess |
| EM-1      | IV       | Swine abscess |
| CA-10     | IV       | Swine abscess |
| TG-9      | V        | Swine abscess |
| TG-19     | V        | Swine abscess |
| Field isolates of unknown serotype | Unknown | Swine abscess |
| NNJ-10    | Unknown  | Swine abscess |
| PA-21     | Unknown  | Swine abscess |

* Obtained from R. C. Lancefield, the Hospital of the Rockefeller Institute of Medical Research, New York, N.Y. Strains K-129 and K-131 were recovered from milk by Brown et al. (3), and C24A was recovered from a swine abscess by Stafseth and Clinton (14).

† Procured from Richard Shuman, the National Animal Disease Laboratory, Ames, Iowa [designated serotype III by Moreira-Jacob (13)].

‡ Recovered during an etiological study of streptococcal lymphadenitis of swine (1).

*Streptococcus* grouping antiseraums were prepared as follows with SGE strains K-129 and C24A. (These strains, obtained from Dr. Lancefield, were reported to be serotype I (13) and were used in an effort to produce type 1 antiserum, but only grouping antibodies were recovered.) Todd-Hewitt broth cultures (18 hr) were centrifuged and the bacteria were dried with acetone. A 5-mg amount of dried SGE was mixed with 5 mg of *Mycobacterium tuberculosis* strain H37Ra (Difco) and added to 5 ml of Freund's incomplete adjuvant. The mixture was emulsified in a TenBroeck grinder, heated for 30 min at 60 C, and inoculated into Holstein-Friesian heifers of approximately 800 lb. Bilateral intradermal inoculations of 0.25 ml were made over the anterior upper third of the scapulae and over the flanks just anterior to the stifle joints. Approximately 100 ml of heat-inactivated (60 C for 30 min) saline suspension of bacteria (optical density of 1.0) was inoculated intravenously on postinoculation days 55, 94, and 108.

Serums, collected every other day after the last intravenous inoculation, were tested against homologous and heterologous streptococcal antigens by the tube-prefiniment test. Antiserums of satisfactory potency and specificity were obtained 7 days after the final intravenous inoculation. Approximately 2 liters of serum was collected from each of two cattle (having received SGE strains C24A and K-129, respectively).

The sera were filter sterilized, treated with thimerosal (Merthiolate, Eli Lilly Co., Indianapolis, Ind.; final concentration of 1:10,000), and stored at -20 C.

Adsortion of antiserum. Antiserus to SGE group antigens and SGE type IV antigens were rendered specific by adsorption as described previously (1). Type II antiserum (prepared with SGE strain K-131) was adsorbed with acetone-precipitated antigen from SGE strain EM-1 (serotype IV). A 1-ml amount of different concentrations of antigen solution was precipitated with 10 volumes of acetone and air-dried. The dried precipitate was mixed with 1 ml of serum and incubated for 1 hr at 37 C. The serum was clarified by centrifugation and tested for specificity. The least amount of antigen required to render the antiserum specific was determined and an appropriate amount was used to adsorb 100 ml of antiserum.

Preparation of antigens: HCl hydrolysis. A modification of Lancefield's HCl hydrolysis technique (11) was employed. Samples (250 mg) of freeze-dried bacteria were hydrated with deionized water, collected by centrifugation, suspended in 3 ml of PSS, and adjusted to pH 0.8, 1.3, 1.8, and 2.3. Each suspension was heated in a boiling-water bath for 10 min, cooled, and centrifuged. The supernatant fluids were removed, neutralized with NaOH (using phenol red indicator), clarified by a final centrifugation, and diluted to 5 ml. The sediments were extracted a second time in the same manner.

Trichloroacetic acid hydrolysis. A modification of Ikawa's trichloroacetic acid hydrolysis technique (9) was used in a manner similar to the HCl procedure, except that bacterial suspensions were acidified with trichloroacetic acid and extracted at 90 C (water bath) for 15 min.

Formamide hydrolysis. A modification of Fuller's procedure (8) was employed. Samples (250 mg) of freeze-dried bacteria were hydrated with deionized water, collected by centrifugation, and extracted with 15 ml of formamide at 180 C (in an oil bath) for 15, 30, 60, and 120 min. Each hydrolysate was cooled and mixed with 37.5 ml of acid alcohol (95% absolute ethanol and 5% 2 N HCl). Resulting mixtures were centrifuged, and 75 ml of acetone was added to each of the supernatant fluids. Carbohydrates remaining in solution after the first acetone treatment were removed by adding an additional 75 ml of acetone. The carbohydrate precipitates were removed by centrifugation and dissolved in 5 ml of PSS, and the solution was centrifuged. The resulting solutions were neutralized with NaOH (using phenol red indicator) and clarified by a final centrifugation.

RESULTS

Hydrochloric acid hydrolysis (Table 2). The greatest yield of group antigen occurred, in general, when SGE was extracted at pH 0.8. In contrast, maximal amounts of type antigen were recovered, in most cases, when the streptococci were hydrolyzed at pH 1.8. Sediment from the first hydrolysis was reextracted and antigen yield was one-half to one-eighth of that recovered during the first extraction. As in the initial ex-
TABLE 2. Antigen titers obtained by hydrochloric acid hydrolysis

| Titera | Strain | Serotype | Titer at extraction pHb |
|--------|--------|----------|-------------------------|
|        |        |          | 0.8 | 1.3 | 1.8 | 2.3 |
| Group antigen | K-129e | I | 160 | 160 | 40 | 20 |
| | C24Ae | I | 320 | 80 | 40 | 20 |
| | K-131 | II | 160 | 40 | 40 | 10 |
| | OSC-1 | II | 160 | 160 | 40 | 10 |
| | 4678e | III | 320 | 80 | 40 | 10 |
| | EM-1 | IV | 320 | 40 | 20 | 20 |
| | CA-10 | IV | 80 | 80 | 40 | 20 |
| | TG-9 | V | 80 | 40 | 40 | 40 |
| | TG-19 | V | 160 | 80 | 20 | 20 |
| | NNJ-10e | Unknown | 320 | 80 | 20 | 10 |
| | PA-21e | Unknown | 160 | 80 | 40 | 10 |
| Type antigen | K-131 | II | 160 | 160 | 160 | 40 |
| | OSC-1 | II | 80 | 80 | 80 | 10 |
| | EM-1 | IV | 20 | 80 | 80 | 40 |
| | CA-10 | IV | 1 | 1 | 80 | 40 |
| | TG-9 | V | 10 | 20 | 320 | 320 |
| | TG-19 | V | 10 | 40 | 160 | 160 |

* Titers represent reciprocal of highest dilution yielding a positive tube-precipitin reaction (incubated for 15 min at room temperature).

b Extractions were conducted at 100 C for 10 min.

c Not tested for type antigen due to lack of appropriate antiserums.

Trichloroacetic acid hydrolysis (Table 3). As in HCl extraction, maximal amounts of group antigen were obtained at pH 0.8, and a maximal amount of type antigen at pH 1.8.

TABLE 3. Antigen titers obtained by trichloroacetic acid hydrolysis

| Titera | Strain | Serotype | Titer at extraction pHb |
|--------|--------|----------|-------------------------|
|        |        |          | 0.8 | 1.3 | 1.8 | 2.3 |
| Group antigen | K-129e | I | 40 | 40 | 20 | 10 |
| | C24Ae | I | 40 | 20 | 10 | 1 |
| | K-131 | II | 40 | 20 | 20 | 1 |
| | OSC-1 | II | 40 | 10 | 10 | 1 |
| | 4678e | III | 40 | 20 | 10 | 1 |
| | EM-1 | IV | 80 | 20 | 20 | 1 |
| | CA-10 | IV | 1 | 1 | 80 | 40 |
| | TG-9 | V | 80 | 20 | 40 | 10 |
| | TG-19 | V | 40 | 20 | 20 | 10 |
| | NNJ-10e | Unknown | 40 | 20 | 10 | 10 |
| | PA-21e | Unknown | 40 | 20 | 20 | 1 |
| Type antigen | K-131 | II | 80 | 20 | 40 | 40 |
| | OSC-1 | II | 40 | 10 | 40 | 20 |
| | EM-1 | IV | 80 | 20 | 20 | 20 |
| | CA-10 | IV | 10 | 10 | 10 | 1 |
| | TG-9 | V | 80 | 80 | 160 | 160 |
| | TG-19 | V | 40 | 80 | 160 | 160 |

* Titers represent reciprocal of highest dilution yielding a positive tube-precipitin reaction (incubated for 15 min at room temperature).

b Extractions were conducted at 90 C for 15 min.

c Not tested for type antigen due to lack of appropriate antiserums.
TABLE 4. Antigen titers obtained by formamide hydrolysis

| Titer*         | Strain | Serotype | 15 | 30 | 60 | 120 |
|----------------|--------|----------|----|----|----|-----|
| Group antigen  | K-129a | I        | 320| 320| 640| 640 |
|                | C24Aa  | I        | 160| 320| 640| 640 |
|                | K-131  | II       | 160| 320| 640| 320 |
|                | OSC-1  | II       | 160| 320| 640| 160 |
|                | 4678b  | III      | 160| 320| 640| 640 |
|                | EM-1   | IV       | 320| 640| 640| 640 |
|                | CA-10  | IV       | 160| 320| 640| 160 |
|                | TG-9   | V        | 160| 640| 640| 160 |
|                | TG-19  | V        | 160| 640| 640| 160 |
|                | NNJ-10c| Unknown  | 160| 160| 320| 640 |
|                | PA-21c | Unknown  | 160| 160| 1280|640 |
| Type antigen   | K-131  | II       | 40 | 320| 320| 20  |
|                | OSC-1  | II       | 40 | 160| 80 | 10  |
|                | EM-1   | IV       | 160| 160| 80 | 10  |
|                | CA-10  | IV       | 20 | 10 | 10 | 0   |
|                | TG-9   | V        | 320| 320| 1  | 1   |
|                | TG-19  | V        | 160| 160| 10 | 1   |

* Titors represent reciprocal of highest dilution yielding a positive tube-precipitin reaction (incubated for 15 min at room temperature).

b Extractions were conducted at 180 C.

c Not tested for type antigen due to lack of appropriate antiserums.

K-131 and OSC-1) and serotype IV (strains EM-1 and CA-10) occurred when they were extracted at pH 0.8, whereas titers of serotype V (strains TG-9 and TG-19) were greatest when hydrolyzed at pH 1.8. Antigen titers obtained in the second trichloroacetic acid extraction were similar to those of the first extraction and indicated that the primary trichloroacetic acid extractions were relatively less efficient in removing antigens than the primary HCl extractions.

Formamide hydrolysis (Table 4). Maximal amounts of group antigen occurred, in most cases, when the streptococci were hydrolyzed for 60 min. Strain NNJ-10 was an exception in that 120 min hydrolysis yielded the most antigen. Titers of type antigen, in general, were highest when the bacteria were extracted for 30 min. Solutions of carbohydrates prepared by adding a second volume of acetone had little antigenic activity.

Comparison of hydrolytic methods. The types of titers obtained by HCl, trichloroacetic acid, and formamide hydrolysis are compared in Table 5. The most nearly optimal set of conditions for recovering type antigen from different isolates was employed with each procedure (i.e., pH 1.8 for HCl, pH 0.8 or 1.8 for trichloroacetic acid, and 30 min at 180 C for the formamide technique). The formamide method was equal or superior to the others with respect to recovering maximal amounts of type antigen. A minor exception was CA-10 which gave comparatively low yields of type antigen throughout the various trials.

Comparison of data regarding recovery of group antigen also indicated that the formamide method was equal or superior to the other techniques. Relatively large amounts of group antigen were obtained by all of the procedures, and extracting conditions were not as critical as for recovering type antigens.

TABLE 5. Comparison of the efficacy of recovering Streptococcus group E type antigen by hydrochloric, trichloroacetic acid, and formamide hydrolysis

| Strain | Serotype | HCl | Trichloroacetic acid | Formamide |
|--------|----------|-----|----------------------|-----------|
| K-131  | II       | 160 | 80                   | 320       |
| OSC-1  | II       | 80  | 40                   | 160       |
| EM-1   | IV       | 80  | 80                   | 160       |
| CA-10  | IV       | 20  | 10                   | 10        |
| TG-9   | V        | 320 | 160                  | 320       |
| TG-19  | V        | 160 | 160                  | 160       |

* Titer represents reciprocal of highest dilution yielding a positive tube-precipitin reaction (incubated for 15 min at room temperature). HCl extraction, 10 min at 100 C at pH 1.8; trichloroacetic acid extraction, 15 min at 90 C at pH 0.8 or 1.8; formamide extraction, 30 min at 180 C.
**DISCUSSION**

Serological studies of SGE isolates recovered from swine affected with streptococcal lymphadenitis have revealed that a certain percentage are untypable. Deibel et al. found that 85 of 99 SGE isolates recovered from abscesses of swine were serotype, IV (6). Fourteen isolates could not be serotyped and were believed to be lacking in the type antigen. Yao et al. observed that 42 of 47 SGE isolated from swine were type IV (15). The remaining five isolates were believed to be devoid of type substance. Armstrong and Payne (1) studied 51 SGE isolates that could not be typed on initial examination. Eighteen of the 51 isolates became typable when large numbers of bacteria were extracted under various conditions. The results suggested that some of the so-called untypable isolates might be deficient in type antigen rather than devoid of the substance, and that a more nearly optimal extraction procedure might allow additional SGE isolates to be serotyped.

The relative efficiency of the three antigen extraction methods may be compared since the extracts of 250 mg of freeze-dried bacteria were diluted to a final volume of 5 ml in each procedure. Group antigen was present in larger quantities than the type antigen, and different hydrolysis conditions were required for recovering maximal amounts of the two substances. Adequate quantities of group antigen, for serologic identification, were recovered under a variety of hydrolysis conditions. Extraction conditions required for maximal yields of type antigen were more critical. The most nearly optimal set of conditions for recovering SGE antigens in general was formamide hydrolysis for 30 min at 180 C. These conditions probably would be adequate for obtaining type antigens from the majority of SGE isolates. However, individual differences among isolates probably exist, and untypable isolates should be subjected to formamide hydrolysis at 180 C for various time periods before assuming that a given isolate is devoid of type antigen.

DeMoor and Thal (7) reported that an antigen common to Streptococcus groups P and U was recovered by formamide but not by HCl hydrolysis. They viewed the cross-reacting antigen as being the true group antigen; i.e., they suggested that the original classification was based on the erroneous assumption that type-specific antigens recovered from prototype strains represented group-specific antigens. They also suggested that the common group antigen may have been acid labile. In light of the present study, it seems more likely that the conditions of acid hydrolysis employed in their study may have been too mild to release group-specific substance. Our data seem to indicate that the type antigen is peripheral and that the group antigen is more deeply situated.

**ACKNOWLEDGMENT**

This investigation was supported by cooperative agreement 12-14-100-9379 (45) with the Agricultural Research Service, U.S. Department of Agriculture.

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