IMMUNOLOGIC RELATION OF STREPTOCOCCAL AND TISSUE ANTIGENS

II. CROSS-REACTION OF ANTISERA TO MAMMALIAN HEART TISSUE WITH A CELL WALL CONSTITUENT OF CERTAIN STRAINS OF GROUP A STREPTOCOCCI,

BY MELVIN H. KAPLAN, M.D., AND MARY LOUISE SUCHY
(From the Department of Medicine, Metropolitan General Hospital, and Western Reserve University School of Medicine, Cleveland)

PLATE 69
(Received for publication December 11, 1963)

A cross-reaction between antisera to cell walls of certain Group A streptococcal strains and human heart tissue has been demonstrated by complement fixation and immunofluorescence (1, 2). The streptococcal cross-reactive antigen was a protein, which could be extracted from cell walls by acid hydrolysis and partially purified by neutral salt fractionation and ion exchange chromatography. The related antigen or antigens in heart tissue was shown by immunofluorescence to be localized to subsarcolemmal sarcoplasm or sarcolemma of cardiac myofibers and to smooth muscle elements of vessel walls and endocardium. It was present also in skeletal muscle fibers but not in other organs. A similar distribution was observed in rabbit organs.

The present report describes the reverse cross-reaction: goat antisera to human or rabbit heart exhibited a precipitin reaction with extracts of streptococcal cell walls. The cross-reactive antigen was found present in the same neutral salt and chromatographic fractions of cell wall extracts as described previously with antistreptococcal sera.

Materials and Methods

Goat Antisera to Human and Rabbit Heart Tissue.—The immunizing preparations consisted of washed homogenates of normal human or rabbit heart incorporated in alumina gel adjuvant, as previously described (3). Normal human heart tissue was provided at postmortem from individuals who had died from accidental or traumatic causes. Rabbit heart tissue was obtained with sterile precautions from healthy albino rabbits supplied by local dealers. The hearts were cut into segments of approximately 10 gm and stored in the frozen state at −30°C.

* This work was performed under grants-in-aid from the National Heart Institute, United States Public Health Service (HI-3726).

643
On the day of injection, a segment of myocardium was thawed and minced in sterile cold saline. After washing three times in cold saline, the mince was mixed with 3 volumes of buffered saline, 0.01 M phosphate, pH 7.4, and ground in a Potter-Elvehjem homogenizer. The resulting homogenate was centrifuged at 2500 rpm for 20 minutes and the sedimented tissue made up in saline to a 20 per cent suspension (v/v). An equal volume of sterile 20 per cent aluminum hydroxide gel, pH 7.0, was added to give a final 10 per cent suspension of tissue in the adjuvant mixture. Penicillin, 100 units per ml, and streptomycin, 0.1 mg per ml, were incorporated in the mixture to ensure bacteriostasis.

Young goats were injected twice weekly with 8.0 ml of each of the heart tissue preparations in multiple subcutaneous sites in both axillary and inguinal regions. After repeated monthly courses of injection alternating with monthly rest periods, cross-reactive sera were obtained at approximately 5 months after onset of immunization. All serologic tests, including both capillary and agar gel diffusion tests, were performed with the globulin fraction separated from these antisera at half-saturation ammonium sulfate. The globulin fraction was from 2 to 3 times concentrated with respect to the initial serum and was preserved with 1:20,000 merthiolate.

Streptococcal Preparations.—Cell walls were derived from mass cultures of the following strains: Tripp, Type 5; Has, Type 19; and Rydz, Type 12. The cells were grown in tryptic-soy or trypticase-soy broth and washed twice with normal saline. They were then heat-killed at 60°C for 30 minutes, washed 5 times in distilled water, and ruptured in a Mickle apparatus in the cold with the aid of No. 13 ballotini beads (4). After discarding the sediment obtained by centrifuging at 1500 rpm for 10 minutes, the cell walls were separated by centrifuging at 3600 g for 90 minutes (5). The cell walls were washed three times with distilled water, twice with 0.1 M sodium phosphate, pH 7.4, twice with distilled water, and were then extracted three times for 10 minutes at pH 2.0, at 95°C following the procedure of Lancefield and Perlmann (6).

Cell wall antigen was also extracted by shaking with glass beads according to the procedure described by Slade and Vetter (7). A 20 per cent suspension (v/v) of washed cell walls in saline was mixed with an equal volume of No. 13 ballotini beads, a few drops n-tributyl phosphate added to prevent foaming, and the mixture shaken in a Mickle disintegrator at maximal amplitude at 4°C for a total period of 2 hours, with intermittent cooling to prevent excessive heating. After centrifugation at 20,000 g for 2 hours, the supernatant was filtered through an ultrafine sintered glass filter and dried by lyophilization.

Agar Gel Diffusion.—Agar diffusion was carried out by the Ouchterlony procedure (8) in 10 x 60 mm glass Petri dishes containing 10 ml 0.7 per cent washed bacto-agar in a solution of 0.85 per cent NaCl, 0.04 M sodium barbiturate, and 0.3 M glycine at pH 8.0. Merthiolate 1:10,000 was employed as preservative. The wells were 9 mm in diameter arranged hexagonally around a central well at a distance of 8 mm between wells. Precipitation patterns were developed at room temperature for at least 7 days. The plates were washed for 5 days, dried under filter paper, and stained with amidoblack 10 B. To prevent distortion of the agar during drying, slits were cut from the wells to the edge of the plate, and from the central well to one of the outer wells.

Block Electrophoresis.—Serum proteins were fractionated by preparative zone electrophoresis on g.e. 427, a polyvinyl chloride resin, as supporting medium (9) with barbital buffer, pH

---

1 Supplied by Difco Laboratories, Inc., Detroit. We are grateful to Mr. Aaron Lane, Difco Laboratories, Inc., for supplying frozen cells from mass cultures of the Tripp and Has strains for this work.

2 Supplied by Baltimore Biological Laboratories, Baltimore.

3 Supplied by B. F. Goodrich Chemical Co., Cleveland.
8.6, ionic strength 0.1. Four to 8 ml of serum were applied to a block 45 x 21 x 1.2 cm, and electrophoresis performed at 4°C for 45 hours at a constant voltage of 600 v and a current of 50 to 60 ma. The block was cut into 1 cm segments and protein recovered by stirring the supporting medium in 5 ml saline and filtering under suction through sintered glass filters. Protein content was determined by a modified Folin-Ciocalteu procedure (10). The eluates making up the separate peaks were pooled, dialyzed for 48 hours against cold 0.02 M NaCl, and concentrated by lyophilization. Each dried fraction was dissolved in 2.0 ml saline.

Column Chromatography.—The procedures employed for chromatographic separation of cell wall cross-reactive antigen by gradient elution from DEAE (diethylaminoethyl) and CM (carboxymethyl) cellulose columns have been previously described in detail (2). The eluates comprising each of the peaks developed in the chromatogram were pooled, dialyzed for 48 hours against cold 0.02 M NaCl, and concentrated by lyophilization.

Protein concentration of streptococcal fractions was estimated by a modified Folin-Ciocalteu procedure (10) calibrated by micro-Kjeldahl with rabbit γ-globulin as reference standard.

RESULTS

Cross-Reaction of Goat Anti-Human and Rabbit Heart Sera with Streptococcal Cell Wall Preparations.—Acid extracts of cell walls derived from strains Tripp, type 5; Has, type 19; and Rydz, type 12, were tested at various concentrations against both goat anti-human and anti–rabbit heart globulin preparations by capillary precipitin tests and agar gel diffusion. Cell wall preparations from the Tripp and Has strains yielded positive tests with both immune globulin preparations, while extracts from the Rydz strain were non-reactive (Figs. 1 and 2). Gel diffusion plates revealed a single band of precipitation between the Tripp and Has cell wall extracts and the globulin fractions of both antiheart sera. The precipitation lines given by antihuman globulin with these two extracts of different serologic type showed a pattern of complete fusion without spur formation (Fig. 1). Precipitation lines obtained with the anti–human heart globulin were considerably less intense than with anti–rabbit heart globulin and could not be satisfactorily reproduced photographically. Reaction with these antiheart globulins could be demonstrated with extract at a minimum concentration of 3 mg protein per ml. No reaction was obtained with normal preimmune goat globulin or with a goat hyperimmune serum globulin prepared against whole rabbit serum.

Absorption of antihuman globulin with dialyzed tryptic-soy or trypticase-soy media at amounts up to 10 mg per 0.1 ml globulin did not affect the serologic reaction with cell wall antigen. In direct tests with these media as antigens, no precipitation was observed. Precipitation with cell wall extracts could thus not be attributed to constituents derived from the medium.

Saline extracts of washed cell walls from strains Tripp and Has prepared by grinding or shaking with glass beads also produced precipitation with anti–rabbit and anti–human heart sera. It was established that the precipitation reaction with both acid extracts and saline extracts of ground cell walls was associated with the γ-globulin fraction of these antisera separated by zone
electrophoresis (Text-fig. 1, Figs. 3 and 4). The relative positions and curvatures of the precipitation lines with these two cell wall extracts indicated a more rapid diffusion and a smaller size of the antigen in the acid-hydrolyzed preparation than that in the ground cell wall preparation (11).

At high concentrations of the acid extract preparation, i.e. 50 mg protein per ml, it was noted that a very faint precipitation was also given with the goat \(\alpha_2\)-globulin fraction of normal and immune serum (Fig. 3), whereas saline extracts of ground cell walls at this same concentration did not exhibit reaction with any serum protein fraction other than immune \(\gamma\)-globulin (Fig. 4). This reaction with the acid extract preparation was possibly related to precipitation phenomena which have been observed to occur between acid extracts of Group A streptococcal cells and normal serum and plasma proteins by Wood and Schramm (12) and Kantor (13). Since all agar diffusion tests were performed with the globulin fraction separated at half-saturation ammonium sulfate, this non-specific precipitation was avoided.

Fractionation of Cell Wall Extracts.—After treatment with ribonuclease (2), the cell wall acid extract was fractionated with ammonium sulfate, and the reactive antigen found concentrated in the fraction insoluble at 0.3 to 0.7 saturation (Table I). Following chromatography of this latter fraction on DEAE cellulose with gradient elution from 0.005 to 0.3 \(\text{m}\) phosphate, pH 8.0 (2), the active material was found associated with the second peak, i.e. that eluted by the gradient buffer, referred to as chromatographic fraction II. The eluates comprising this second peak were pooled and subjected to chromatography on CM cellulose with gradient elution from 0.005 to 0.3 \(\text{m}\) Na acetate,
pH 5.5. Most of the precipitating activity was present in the second peak (fraction IV), but some slight activity was present also in the first peak. Chromatographic fraction IV was reactive with anti-rabbit heart globulin at a concentration of 1.0 mg protein per ml. These results are summarized in Table I. Absorption of the antiheart globulin preparations with washed cell walls, 10 mg per 0.1 ml globulin, completely abolished precipitation reaction with these active fractions. Specific absorption with active fractions similarly abolished precipitation.

**TABLE I**

*Precipitation Reaction of Goat Antiheart Globulin with Cell Wall Antigen of a Type 5 Strain (Tripp)*

<table>
<thead>
<tr>
<th>Streptococcal preparation</th>
<th>Precipitation in agar gel with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anti-rabbit heart globulin</td>
</tr>
<tr>
<td>Acid extract of washed cell walls</td>
<td>+</td>
</tr>
<tr>
<td>(a) Fraction at 0 to 0.3 sat. ammonium sulfate</td>
<td>Faint</td>
</tr>
<tr>
<td>(b) Fraction at 0.3 to 0.7 sat. ammonium sulfate</td>
<td>+</td>
</tr>
<tr>
<td>(c) Fraction at 0.7 to 1.0 sat. ammonium sulfate</td>
<td>0</td>
</tr>
</tbody>
</table>

Fraction b subjected to chromatography on DEAE cellulose:
- Peak 1 from DEAE cellulose (fraction I) | 0 | 0 |
- Peak 2 from DEAE cellulose (fraction II) | + | + |

Chromatographic fraction II subjected to rechromatography on CM cellulose:
- Peak 1 from CM cellulose (fraction III) | Faint | 0 |
- Peak 2 from CM cellulose (fraction IV) | + | + |

Antigen preparations were employed at a concentration of 10 mg protein per ml.

**DISCUSSION**

The presence of a streptococcal antigen immunologically related to human heart tissue has been shown in this work by the demonstration of a cross-precipitin reaction between goat anti-human and anti-rabbit heart sera and extracts of cell walls of certain streptococcal strains. The immune nature of this reaction was supported by association of the precipitation with the γ-globulin fraction of the goat antiheart sera, by negative reaction with normal preimmune goat globulin, and by specific absorption of the precipitin with purified preparations of the cell wall antigen. Further, the related antigen was found in the same neutral salt and chromatographic fractions of cell wall extracts previously shown to contain cross-reactive antigen by immunofluorescence inhibition.

In previous work employing immunofluorescence inhibition of antistreptococ-
cal sera, cross-reactive antigen was shown to be present in several type 5 and 19 strains but not in strains of other types tested, including strain Rydz, Type 12. Similar results were obtained with the cross-precipitation reaction, thus providing additional evidence of the variation of this constituent in the cell walls of different streptococcal strains. It is probable that the cross-precipitation reaction given by antihuman serum could serve as the basis of a sensitive procedure for routine detection of this antigen in different strains.

The biologic significance of cross-reactions between bacterial and viral antigens and host tissue constituents in relation to problems of infection, resistance, and disease has only recently been appreciated (cf. references 14-16). Rowley and Jenkin (16) have proposed that in some instances cross-reactions may play a role in the virulence of infectious agents. In particular they attributed the diminished opsonic capacity and reticuloendothelial clearance of *Salmonella typhimurium* in the mouse to a cross-reactive relationship between this organism and mouse tissue constituents. In analogy to the above, the possible relation of cross-reactive antigen in the Group A streptococcus to the virulence of this organism merits similar investigation. Of more direct relevance to the relationship of streptococcal cross-reactive antigen to heart tissue is the possibility that autoimmunity to heart might be induced following infection by streptococcal strains containing cross-reactive antigen. Evidence indicating that this mechanism for induction of autoimmunity does occur in man is presented in the paper which follows.

**SUMMARY**

Further evidence of a cross-reactive relationship between a cell wall antigen of certain Group A streptococcal strains and mammalian heart tissue is provided by the demonstration that goat antisera to washed human or rabbit heart tissue homogenates exhibit precipitation with cell wall extract preparations. A single line of precipitation was observed in agar gel diffusion tests. Precipitating activity of goat antihuman sera was related to the γ-globulin fraction in zone electrophoresis. The cell wall antigen was demonstrated in cell wall extracts of a Type 5 and Type 19 but not of a Type 12 strain. Antigenic activity was concentrated in the same neutral salt and chromatographic fractions of acid extracts of cell wall previously reported to exhibit this activity by immunofluorescent inhibition of the cross-reaction of antistreptococcal serum with heart tissue.

**BIBLIOGRAPHY**

an immunologic cross-reaction with human heart tissue, *J. Immunol.*, 1963, 90, 595.


EXPLANATION OF PLATE 69

Fig. 1. Agar Gel Diffusion. Central well: goat anti-rabbit heart globulin; peripheral wells: acid extracts of cell walls of 1, strain Tripp, Type 5; 2, strain Has, Type 19; 3, strain Rydz, Type 12. Antigen concentration in each case was 10 mg protein per ml. Remaining 3 wells are empty.

Fig. 2. Plate HJ. Central well: goat anti-rabbit heart globulin; wells 1, 2, and 3: cell wall extract preparation of strain Tripp at concentrations of 50, 12.5, and 3.1 mg per ml. Wells 4, 5, and 6 contain a second preparation of Tripp cell wall extract at the same concentrations, respectively. The cell wall extracts were partially purified by ribonuclease digestion and fractionation between 0.3 and 0.7 saturation ammonium sulfate. In the original plates before staining a faint reaction was observed in wells 3 and 6, at the 3.1 mg per ml concentration.

Fig. 3. Plate IK. Reaction of zone electrophoretic fractions of goat anti-rabbit heart serum with acid extract preparation of cell walls of strain Tripp. Central well: cell wall acid extract, 50 mg per ml; 1, γ-globulin fraction; 2, β-globulin fraction; 3, α2-globulin fraction; 4, α1-globulin fraction; 5, albumin fraction (see Text-fig. 1).

Fig. 4. Plate IJ. Reaction of electrophoretic fractions of goat anti-rabbit heart serum with the saline extract of ground cell walls of strain Tripp, fractionated between 0.3 and 0.7 saturation ammonium sulfate; central well: cell wall extract, 50 mg per ml; peripheral wells: electrophoretic fractions as described in Fig. 3. Single line with γ-globulin fraction in 1 is close to antigen well with curvature toward the latter in contrast with position and curvature given by acid extract preparation in Fig. 3.