Studies on the Pathogenesis of Rheumatic Heart Disease
An immunological relationship between the polysaccharide of
group A streptococcus and the glycoprotein of heart valve

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It is well known that acute rheumatic fever and rheumatic heart disease occur following antecedent group A streptococcal infections. Many investigations suggested that the pathogenesis of rheumatic heart disease is of an immunological nature. Kaplan\(^1\) first demonstrated the existence of cross-reactive antigen between the streptococcal M-protein and some constituents of myocardium. Then Goldstein\(^2\) proved a specific immunological relationship between group A streptococcal polysaccharide and structural glycoprotein in human and bovine heart valves. Our present investigations were designed to isolate and purify the antigenic substance from bovine heart valves and to demonstrate the cross-reactivity between group A streptococcal polysaccharides and constituents of bovine heart valves. Further, the presence of gamma globulin in valve of patients with rheumatic valvular disease who had valve replacement was examined.

MATERIALS AND METHODS

1. Antigens
   a) Streptococcal strains: Group A, type 5 streptococci were kindly supplied by Dr. Melvin Kaplan. Streptococcal cell walls were prepared by Braun cell homogenizer according to the procedure of Bleiweis et al.\(^3\)
   Group specific polysaccharides: The cell walls were treated with pronase E according to Heyman\(^4\) and then treated with ribonuclease according to the procedure of Mc Carty.\(^5\) For the extraction of polysaccharides from cell walls, minor modifications of the method of Fuller were employed.
   b) Bovine heart valves: Bovine heart valves were obtained from slaughter house and frozen immediately to \(-20^\circ\)C.
   i) Soluble extract of bovine heart valves: According to Goldstein's technique valves were homogenized in calcium-tris-citrate (CTC) buffer at pH 7.2 and extracted with continuous stirring for 24 hours at \(4^\circ\)C. After 7 extractions and centrifugations, supernatant was dialyzed against saline for 48 hours and then distilled water for 24 hours. After centrifugations ethanol was added to clean supernatant in a final concentration of 20%. Precipitate was dissolved with 0.1M tris-HCl, and then the solution was passed through a column of Sephadex G 150\(^6\)
   ii) Enzymatic digestion of bovine heart valves: According to Shibata’s technique valves were suspended in saline and digested with crystalline trypsin for 3 hours at \(37^\circ\)C after adjusting to pH 8.2 with 0.1M borate buffer every 30 minutes. After inactivation of trypsin, the mixture was centrifuged at 27,000rpm for 35 minutes. Supernatant was dialyzed and lyophilized. These materials were dissolved in saline and digested with pronase P at \(37^\circ\)C for 3 hours or 24 hours after adjusting to pH 7.8 with 0.1M tris-HCl and passed through a column of Sephadex G-200.\(^7,8\)
2. Antibodies
   a) The anti-streptococcal sera were obtained from rabbits by injecting suspension of heat killed group A streptococci.
   b) The anti-bovine heart valve sera were prepared from rabbits by injecting the total homogenates of the valves emulsified in an equal

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Japanese Circulation Journal Vol. 39, April 1975 439
volume of Freund's complete adjuvants and its titer was checked by capillary precipitin tests.
3. Determination of immunological reaction
   a) The immunodiffusion (immunoelectrophoresis) was performed according to the procedure of Graber. The slides were layered with 15 ml of 1% agar in 0.05 ion strength veronal buffer, pH 8.6. Electrophoresis was run at 3 mA per 1 cm for 90 minutes at 4°C, and then the slides were stained with amidoblock B.
   b) The immunofluorescence was examined by the indirect staining technique employing sheep anti-rabbit gamma globulin conjugate absorbed with mouse organ powder! Each section of bovine heart valves was exposed to the anti-bovine valve sera absorbed with mouse organ powder for 3–4 hours, washed several times for 10 minutes with staining buffer, and then treated with fluorescent anti-rabbit gamma globulin for 30 minutes at 37°C and for 30 minutes at room temperature.
   Sections of fresh specimens of valves obtained from patients with rheumatic heart disease at valve replacement were stained for gamma globulin by rabbit anti-human IgG labelled with FITC.
   c) In the absorption of sera with bovine heart valve homogenates, 0.2 ml of serum was added to sample of 0.1 ml packed valve homogenates, and after thorough mixing, the tubes were incubated at 37°C for 30 minutes, and overnight in the cold. Absorbed sera were separated by centrifugation.
4. Fractionation
   Columns of Sephadex G 150 in CTC treated materials and Sephadex G 200 in trypsin-pronase treated materials were employed in attempted purification procedures. The column was 25.0 x 2.2 cm, and the flow rate was 5 ml/cm²/hr. In former experiments 2 ml and in latter 2.5 ml of effluent fractions were collected in each tube. Optical density of each fraction was checked at 280 μm by UV spectrophotometer and sugar content was determined by anthron technique. Analysis of the monosaccharide composition was carried out by gaschromatography. Nippon Electric Model GC-MS was used.
5. Determination of the streptococcal antibodies in sera and the immunoglobulin levels of patients with rheumatic heart diseases
   The serum titer of antistreptolysin O was measured by the method of Rantz and Randall. Streptococcal antiyaluronicidase was assayed by the turbidimetric method of Harris and Harris,
antistreptokinase determinations were made by the agglutination method of Ishii, with minor modifications. Antistreptozyme determinations were made by the slide agglutination method with antigen supplied by Wampole Laboratories. This antigen contains streptolysin O, streptokinase, hyaluronidase, DNA-ase and NAD-ase. The concentrations of immunoglobulins, IgG, IgA, IgM and C3 were determined on the quantitative immunodiffusion plate prepared by the Meloy Laboratories.

RESULTS

Experiment 1. On fractionation of soluble CTC extract of bovine heart valves by Sephadex G 150, two peaks (I and II) were obtained (Fig. 1). The fractions distributed in the first peak showed high optical density at UV 280 μm and strong precipitin reaction against anti-valvular sera by capillary precipitin tests. This fraction also

Japanese Circulation Journal  Vol. 39, April 1975
exhibited a single precipitin line against antisera in cathodal side by immunoelectrophoresis. However, the fractions distributed in the second peak exhibited low optical density and weak precipitin reaction against anti-valvular sera. Therefore, the active materials were found associated with the first peak.

Experiment 2. Fractionation of bovine heart valve extracts treated with trypsin and pronase by Sephadex G 200 for 3 or 24 hours yielded two peaks (I and II) at UV 280 nu. The precipitin reaction given by anti-valvular sera with the fractions distributed in first peak was more intense than the fractions of the second peak by capillary precipitin tests (Fig.2 and 3). The monosaccharide composition of each fraction was analysed by gaschromatography. The main sugars were glucose and galactose and rich in the second peak. A ratio of glucose: galactose was about 1:2 in the first peak. The content of galactose showed the tendency of increase at the end portion of the first peak. Especially at the second peak of materials which were treated with pronase for 24 hours the remarkable

*Japanese Circulation Journal* Vol. 39, April 1975
increase of galactose was observed and a small amount of glucosamine and mannose were detected. The first peak of these materials reacted with anti-valvular sera by immunoelectrophoresis and two precipitin lines were obtained.

Experiment 3. As determined by immunofluorescent procedures, anti-valvular sera exhibited intense reaction with bovine valve sections (Fig. 4). Immunofluorescent staining of valves was remarkably inhibited by previous absorption of anti-valvular sera with purified streptococcal polysaccharides (Fig. 5). Moreover, absorption of anti-valvular sera with streptococcal polysaccharides inhibited the precipitation reaction with the fraction in the first peak of bovine valve extracts treated with trypsin and pronase when tested by immunoelectrophoresis (Fig. 6). Anti-group A streptococcal sera yielded a single band of precipitation with streptococcal polysaccharides by immunoelectrophoresis. Previous absorption of antisera with homogenates of bovine valves resulted a marked reduction of the precipitation reaction with streptococcal polysaccharides (Fig. 7).

Experiment 4. Out of sections of valves from 5 patients with rheumatic heart disease, mitral insufficiency in 2 and aortic stenosis in 3, gamma globulin was demonstrated in following patient. A 22-year-old female admitted on 14 April, 1973 to the Kyoto University Hospital with a history of sore throat, migratory polyarthritis, and dyspnea. She had a previous history of rheumatic fever at 16. Physical findings included cardiomegaly, apical systolic murmur and heart rate of 120 per minutes. Electrocardiogram showed atrial fibrillation. The throat cultures were negative for group A streptococci. Laboratory findings included an antistreptolysin O titer of
Fig. 7. Immunoelectrophoretic pattern developed with anti-group A streptococcal sera.
Reservoir: upper: anti-group A streptococcal sera, unabsorbed lower: anti-group A streptococcal sera, absorbed with bovine valve homogenates
Trench: group A, type 5, streptococcal polysaccharides.

Table 1

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Fig. 8. Immune response of patients with acute rheumatic fever with mitral insufficiency. M.Y. 22Y. Female.

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extracts treated with pronase which share immunological properties with group A streptococcal polysaccharides.\textsuperscript{11} We have confirmed the existence of cross-reactive antigen between group A, type 5 streptococcal polysaccharides and the urea extract of bovine heart valves by the immunoelectrophoretic-inhibition test\textsuperscript{12} while little or no absorbing activity was demonstrable in either M-protein or membrane. The further studies demonstrated that the fractions eluted from soluble extracts of bovine heart valves on fractionation by Sephadex G 150 had also antigenicity against anti-bovine valve sera, and moreover anti-valvular antibodies reacted with these soluble antigens were partially absorbed with streptococcal polysaccharides.\textsuperscript{13} Recently, Shibata has demonstrated that rat glomerular basement membrane treated with trypsin and pronase released glycoprotein which contained monosaccharides and had potency to produce the nephrototoxic antibodies. Our present studies revealed the evidence that the treatment of bovine heart valves with trypsin and pronase produced the antigens reacted with anti-valvular sera. Among the fractions eluted on column fractionation of valve extracts by Sephadex G, most of the precipitating activity was found in the first peak, but weak activity was also detected in the second peak. These active materials contained monosaccharides, mainly glucose and galactose, and a small amount of glucosamine and mannose, and also the cross-reactive antigenic activity as shown by immunological inhibition tests. Further, absorption of anti-group A streptococcal sera with valve homogenates remarkably inhibited precipitation reaction with streptococcal polysaccharides. These results suggested that dialyzable glycoprotein of valves possessed the cross-reactive antigens with streptococcal polysaccharides. Goldstein indicated that glucosamine occupies the important position as cross-reactive antigen between valvular glycoprotein and streptococcal polysaccharide. Ayoub's\textsuperscript{14} studies disclosed that the antibody levels to streptococcal carbohydrate were elevated in sera of patients with acute rheumatic valvulitis, and the elevated antibody levels persisted for periods of at least 1 year and up to 20 years after the last acute attack. In general, the relevance of group A streptococcal infections to the pathogenesis of rheumatic fever was strongly supported by observations of the elevated titers of streptococcal antibodies. One patient presented in our studies showed high
titers of antibodies to streptococcal extracellular products, high concentrations of immunoglobulins in sera, and gamma globulin in valve. It is stated that these deposits of gamma globulin were related to the severe cardiac failure exhibited clinically. Lannigan demonstrated that gamma globulin located in the endocardium and subendocardium of rheumatic heart disease by the ferritin-labelled antibody technique and the heaviest concentrations of ferritin were in relation to collagen fibrils. From these facts it is considered that group A streptococcal polysaccharide might be involved in the production of rheumatic valvulitis following group A streptococcal infections.

**Summary**

The immunization of rabbits with bovine heart valve homogenates induced the formation of antibody which reacted with CTC soluble extracts and also proteolytic enzyme treated fractions. Analysis by fractionation of materials revealed 2 peaks and both peaks contain monosaccharides. The first peak was more antigenic to anti-valvular sera. The cross-reactivity between the valvular glycoprotein and group A streptococcal polysaccharides was confirmed by the immunological inhibition tests. Widespread deposits of gamma globulin were observed in valve of patients with recurrent rheumatic valvulitis who had valve replacement. This work was performed under scientific-grants-in-aid from the Ministry of Education. Portions of this study were presented at the 38th annual meeting of the Japanese Circulation Society, Yamaguchi, April, 1974.

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**REFERENCES**


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