Immunology of bovine heart valves

II. Reaction with connective tissue components

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In the previous paper we examined the evidence for a cross-reaction between heart valves and the C-polysaccharide of Group A streptococci. In this paper we examine the reactions of sera raised against whole heart valves with the chemical fractions of the valves to see if the fractionation techniques separate the antigenic components. Also we examine the reaction of the sera with cryostat sections of human tissue to see in which tissues of the human body antigenic determinants shared with the valves are present. In this immunofluorescent study the reactions were compared with those given by antistreptococcal sera to see if there was any overlap in the distribution of the antigens and hence the possibility of cross-reactions. Finally the valve fractions were examined in the macrophage migration test in collaboration with Dr. D. A. Rajapakse to see if there was any evidence of involvement of the antigens in rheumatic fever.

Material and methods

These are largely those of the previous paper. The immunofluorescent techniques are those of Kingston and Glynn (1971), which differ only in detail from those of Holborow and Johnson (1967). The conjugate was Hylands' goat antirabbit γ -globulin labelled with fluorescein and absorbed with human heart powder. The tissues were obtained at *post mortem* after accident or suicide, small portions being frozen in thin polythene bags with solid CO₂ and alcohol within 12 hrs of death. They were stored at about -30°C. The following tissues were studied: myocardium, mitral valve, lymph node, kidney, and skin. The sections used were 6 μ . cryostat sections, air dried, unfixed, and unwashed. The tissues were obtained from an apparently healthy young adult male of blood group O who was accidentally drowned. Additional myocardium came from a number of sources of which the blood group was generally not known. Absorptions were carried out with 10 mg of the valve fraction per ml. neat serum. The serum was rotated for 2 hrs at room temperature and then held stationary at 4°C. overnight. The serum was centrifuged and the absorption repeated. Macrophage migration

experiments were carried out in collaboration with Dr. D. A. Rajapakse by the technique of Rajapakse and Glynn (1970). This technique uses a mixed system of guinea-pig peritoneal exudate cells and human peripheral blood lymphocytes.

Results

REACTIONS OF VALVE FRACTIONS WITH ANTIBHV SERA

These were examined by double diffusion in agar.

The CTC extracts isolated with either technique gave three to five lines with antiBHV sera, the number of lines being reduced to two with sera which had been absorbed with dried bovine serum (100 mg./ml.).

The crude soluble collagen (CSC) fractions gave patterns very similar to those given by CTC extracts. As has been discussed in the previous paper, CSC-D gave a faint non-specific line with all sera. The solutions of this particular fraction for double diffusion purpose were made by dissolving it in 8 M urea and dialysing against buffered saline pH 7.2.

The CTC extract and crude soluble collagen contained at least one antigen in common which gave a fused identity line.

VSGP (valvular structural glycoprotein), isolated according to the original procedure, gave two or three lines with homologous sera, of which one or two persisted when sera absorbed with bovine serum were used. VSGP-D gave only one line with one of the lines given by VSGP. VSGP (but not VSGP-D) gave a weak non-specific line with all sera, probably owing to the collagen present.

AntiBHV sera were absorbed with different fractions and the absorbed sera reacted with the fractions. Double absorption with CTC at concentration 10 mg./ml. removed all antibodies against any valvular fraction. Sera absorbed with CSC or CSC-D still gave one line when tested against CTC or VSGP fractions. VSGP absorbed sera gave single line with CTC and CSC and no line with VSGP-D. VSGP-D absorbed sera were still able to give two or three

lines either with CTC or CSC and one line with VSGP. Thus the VSGP-D fraction seemed to lack an antigen present in the VSGP, CTC and the CSC and CSC-D fractions.

It seems to us that these results could be explained by the following scheme:

CTC	contains antigens a, b, c
CSC (& CSC-D)	contains antigens a, b
VSGP	contains antigens b, c
VSGP-D	contains antigens c

THE ABILITY OF ANTI-BHV SERA TO GIVE IMMUNOFLOUORESCENT STAINING OF HUMAN TISSUES

As well as studying the reactions of anti-BHV sera with various human tissues, a simultaneous comparison was made with the staining patterns of antistreptococcal sera to see if there were any antigens recognized by both groups, and if so where they were to be found. A more detailed account of the reactions of the antistreptococcal sera is given in Kingston and Glynn (1971) and is summarized in brief in Table I of the previous paper.

The anti-BHV sera tested were taken at different times during the course of immunization. All these sera stained connective tissue wherever this was tested, the appearance being that of reticulin (histologically defined) and basement membrane staining as described by Midgley and Pierce (1963) and Loewi (1967). Additional staining reactions were

noticed, especially in sera after prolonged immunization. These additional reactions were much easier to see after the sera had been absorbed with various valvular fractions (see below) which removed the reticulin/basement membrane staining.

Mitral valve

Anti-BHV sera stained reticulin round the muscle fibres, the connective tissue plate of the valve and the endocardium. Staining of the connective tissue was not uniform: there was marked intensification of the staining of the outer edge, but this layer appeared to include more than the endothelium. The additional staining involved fibroblasts and sarcolemmal/subsarcolemmal zones of the myocardium. By contrast, none of the antistreptococcal sera gave any staining of the connective tissue, other than of the fibroblasts and endothelium (especially Sera 4, 13, and 18). Strong sarcolemmal/subsarcolemmal staining was given by Sera 4, 10, and 18, and this was quite different in appearance from the reticulin reaction of the anti-BHV sera.

Myocardium

As with the mitral valve sections, the anti-BHV sera stained reticulin round the muscle fibres (Fig. 1). [Figs 2 and 3 (overleaf) show sarcolemmal/subsarcolemmal staining by antistreptococcal Serum 10 for comparison.]

The connective tissue components of the arterial walls were also stained. The additional staining

FIG. 1 Myocardium, anti-BHV serum. Staining of reticulin round muscle fibres. $\times 220$

FIG. 2 *Myocardium, antistreptococcal Serum 10. Sarcolemmal/subsarcolemmal staining fibres, no connective tissue staining. $\times 220$*

FIG. 3 *As (2), high power. $\times 500$*

reaction observed was the sarcolemmal/subsarcolemmal reaction. The antistreptococcal sera reacted as described for the mitral valve section, but in addition the smooth muscle and endothelium of the arterioles was stained, especially by Sera 4, 13, and 18.

Kidney

The antiBHV sera stained the glomerular tuft, Bowman's capsule, and the intertubular material (Fig. 4). The later sera stained the glomerulus strongly, the appearance being that of basement membrane

FIG. 4 *Kidney, antiBHV serum. Basement membrane and other staining of glomerular tuft, staining of Bowman's capsule and peritubular connective tissue. $\times 380$*

staining with additional material, whereas the early sera stained the glomerulus only weakly. The anti-streptococcal sera (especially 4, 10, and 13) reacted with the glomerulus only (Fig. 5), except that the smooth muscle and endothelium of arterioles were

stained. Sera 4 and 10 gave a generalized staining of the glomerulus, and Serum 13 a defined staining which differed in appearance from that of basement membrane staining.

FIG. 5 *Kidney, antistreptococcal Serum 13. Staining of glomerular tuft. No staining of Bowman's capsule or peritubular connective tissue. Some autofluorescence of tubular epithelium. $\times 500$*

Other tissues

AntiBHV sera were also tested on skin, liver, and lymph node. The patterns of staining produced were

those of connective tissue antisera, and quite distinct from those given by the streptococci.

Thus, while antistreptococcal Serum 13 stained the

FIG. 6 Liver, antiBHV serum. Staining of basement membrane of sinusoids. $\times 360$

FIG. 7 Liver, antistreptococcal Serum 13. Staining of Kupffer cells only. $\times 440$ (From Kingston and Glynn, 1971)

FIG. 8 Liver, preimmune Serum 13. Autofluorescence of intracellular granules only. $\times 440$ (From Kingston and Glynn, 1971)

Kupffer cells in the liver sinusoids (Fig. 7), the anti-BHV sera outlined the complete basement membrane (Fig. 6).

The antiBHV sera gave brilliant staining of the reticulin fibres outlining the germinal centres in the lymph node (Fig. 9), which was not given by any of the antistreptococcal sera.

ABSORPTION EXPERIMENTS

Absorption of antiBHV sera with bovine serum did not affect the staining reactions, except that the intertubular staining in the kidney became more defined. Absorption with CTC, CSC, or VSGP at 10 mg./ml. serum abolished the connective tissue staining reaction. Thus the reticulin staining round the muscle fibres in sections of heart valves and myocardium was abolished, leaving the sarcolemmal/subsarcolemmal staining as described by Kaplan, Meyeserian, and Kushner (1961) much more clearly visible. Similarly, staining of the connective tissue plate and endocardium of the valves was abolished, leaving more obvious the staining of fibroblasts and endothelial cells. In the kidney staining round the tubules and in the glomerulus was abolished.

Absorption of antiBHV sera with disrupted suspensions of *Str. pyogenes* Type 6 matt did not affect the reticulin and basement membrane staining

reactions of the sera tested in lymph node, heart valve, and kidney, except that with the latter an irregular staining of the nuclei became visible, possibly owing to the action of the bacterial enzymes.

MACROPHAGE MIGRATION EXPERIMENTS

VSGP, CTC, CSC, and sodium hydroxide extract fractions were examined in macrophage migration experiments in the mixed system containing guinea-pig macrophages and human peripheral blood lymphocytes either from normal volunteers or from rheumatic fever patients. It was hoped that this might provide evidence of the role of valvular antigens in rheumatic fever.

Valvular structural glycoprotein was examined in three different migrations, two of them using lymphocytes from rheumatic fever patients, the third normal lymphocytes. A cytotoxic effect was observed at concentration 1/500 to 1/5000. At further dilutions the cytotoxic effect disappeared but there was no sign of inhibition of macrophage migration.

Crude soluble collagen, CTC, and sodium hydroxide fraction were tested in four, three and two migrations respectively, either with normal or rheumatic fever patients' lymphocytes. All of them were highly cytotoxic up to a dilution of 10^{-6} .

FIG. 9 Lymph node, antiBHV serum. Staining of reticulin fibres round germinal centre. (Periphery of field not fully illuminated). $\times 260$

Discussion

Our study of the various chemical fractions of heart valves suggests that the procedures of Goldstein, Halpern, and Robert (1967) are of only limited value in fractionating the antigens. VSGP-D was less of a mixture than the others. The CTC extract appeared to contain most of the heart valve antigens, as judged by its ability to absorb out the connective tissue staining of antiBHV sera, and by the results of double diffusion in gel. Since CTC extract is readily prepared under mild conditions, this suggests that it might be a useful starting point for a study of the connective tissue antigens.

Our immunofluorescent studies show that heart valves are closely immunologically related to connective tissue in other parts of the body. More interesting conclusions follow from the comparison between the staining patterns given by antiBHV and antistreptococcal sera. Our antistreptococcal sera stained the cellular components of connective tissue only. There was thus no evidence of the type of cross-reaction postulated by Goldstein and others (1967). However the antiBHV after prolonged immunization did stain cellular components, the antibodies responsible presumably being evoked by the cellular components of the heart valves (fibroblasts, muscle, and endothelial cells). Since these cellular components have antigens in common with *Str. pyogenes* such sera might well react with *Str. pyogenes* and were in fact found to do so (see previous paper). However the earlier antiBHV sera which stained strongly those areas where VSGP is found, but did not stain the cellular components, did not stain *Str. pyogenes*. Thus we consider that the staining reactions of antiBHV sera also provide evidence against the VSGP/C-polysaccharide cross-reaction. It is interesting to note that absorption with either CTC, CSC, or VSGP abolished the connective tissue staining reactions of antiBHV sera, but not the cellular staining reactions. Thus these extracts either contain only a small proportion of cellular antigens (CTC), or the antigens have been destroyed (CSC, VSGP).

Our limited study of these fractions in the macrophage migration inhibition test, using peripheral blood lymphocytes from two rheumatic fever patients, found no evidence that VSGP was involved in the pathogenesis of the disease. In view of the work of Murphy (see Murphy, 1960; Becker and

Murphy, 1969), it is anyway possible that it is muscle and not connective tissue antigens which are concerned in the damage to heart valves and endocardium in rheumatic fever.

Summary

Chemical extraction of bovine heart valves by the procedures of Goldstein and others (1967) gave three soluble fractions. The water-soluble material extracted with buffered calcium chloride (CTC extract) contained all the antigens which could be detected by double diffusion in agar. The valvular structural glycoprotein (VSGP) fraction prepared by the modified (drastic) procedures, *i.e.* VSGP-D, contained only one.

The patterns of immunofluorescent staining given on human tissues by sera raised against whole bovine heart valves (antiBHV sera) were compared with those given by antistreptococcal sera. The former stained connective tissue and basement membranes throughout the body, and these staining reactions could be abolished by absorption with CTC extract. By contrast, the antistreptococcal sera stained only various cellular components as described in detail by Kingston and Glynn (1971). These cells were also stained weakly by antiBHV sera from rabbits which had received a prolonged course of immunization, a reaction made more obvious by absorption of the sera with CTC extract. It is suggested that this is due to the cells (fibroblasts and myocardial fibres) present in the valves, and that there is no cross-reaction between streptococci and the non-cellular components of heart valves.

Four fractions were tested for their ability to cause inhibition of macrophage migration. Crude soluble collagen, CTC extract, and a fraction extracted with NaOH were all highly cytotoxic. VSGP was cytotoxic up to 1/5000, and gave no inhibition of macrophage migration at higher dilutions when tested with peripheral blood lymphocytes from two patients with rheumatic fever. Thus we could find no evidence that this antigen was involved in the pathogenesis of rheumatic fever.

Based on the diffusion experiments and also the abolition of the connective tissue staining of antiBHV sera, we consider that the CTC extract is a potentially useful source of connective tissue antigens.

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