

CARDIAC AUTOANTIBODIES

I. IMMUNODIFFUSION ANALYSIS OF MULTIPLE RESPONSES EVOKED HOMOLOGOUSLY AND HETEROLOGOUSLY*

BY S. P. HALBERT, M.D., S. E. HOLM, M.D., AND A. THOMPSON

(From the Department of Pediatrics, University of Miami School of Medicine,
Miami, Florida 33136)

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It has been known for some time that animals immunized with heterologous heart tissue may produce antibodies reactive with hearts of the species being immunized (1-6). These antibodies have also been shown by immunofluorescence to be reactive with the hearts of the *individual* antibody-producing animal (2). In the autoimmune reactions reported by Kaplan (6), absorption tests indicated that insoluble antigens were involved and that they were associated with fractions sedimentable at moderately high centrifugal speeds (56,000 *g*).

Immunization with pooled homologous hearts has sometimes proved ineffective in evoking cardiac antibodies (7) although, in a few instances, weak responses have been reported (8-11). Several techniques were used in these latter investigations for demonstrating such antibodies, but when immunodiffusion was employed in two studies, only a single antigen was detected. In one of these reports, the soluble antigen involved appeared to be present in a number of other organs, as well as heart (8). In the other instance, a single antibody induced homologously was reported to be restricted in its reactivity to heart (11).

The present report describes the successful production of rabbit antibodies to soluble rabbit cardiac antigens, either by immunization with pooled rabbit heart, or with heterologous heart (human, rat, and guinea pig). Up to five soluble antigens participated in these responses in some animals. In several cases, they were shown to be truly autoimmune, i.e., by reactions with the heart from the individual antibody-producing rabbit. Some of the antigens involved were found to be apparently restricted to heart, and cross-reactions with hearts from other mammalian species were encountered.

Materials and Methods

The animals used for immunization were 5-6 lb. white female New Zealand rabbits purchased from a local dealer. The rabbit, guinea pig, and rat tissues used for injection and test-

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ing were obtained from commercial sources (Pel-Freeze, Ark., and Pentex, Kankakee, Ill.) These tissues had been rapidly frozen after harvesting and were shipped in dry ice (-60°C), then stored in the frozen state at -20°C . Bovine hearts were obtained fresh from a local slaughterhouse.

In some instances, rabbits purchased locally were sacrificed, and fresh tissue extracts were utilized in immunodiffusion tests. The human tissues were obtained from normal adults of varying age and sex, within 6 to 18 hr after death by accident or other trauma.

The tissues were thoroughly homogenized in a Sorvall omnimixer (Sorvall, Norwalk, Conn.) in buffered saline (0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2) at a concentration of 600 mg wet weight/ml for routine testing and immunization purposes. For injection, the entire homogenates were mixed with equal volumes of complete Freund's adjuvant. The latter contained 1 part of Arlacel A (Atlas Chemical Industries, Wilmington, Del., lot 103B), 6 parts of mineral oil (Bayol 55, Humble Oil & Refining Co., Houston, Tex.), and 4 mg/ml of heat-killed lyophilized *Mycobacterium butyricum* (Difco, Detroit, Mich.).

After one or more preimmune sample bleedings, the antigens were injected intradermally into each animal at five widely separated sites of 0.2 ml each. Three such doses were given at two weekly intervals, followed by bleedings from the central ear artery at 7 and 10 days after the last injection. Subsequently, repeated booster doses of the same quantities were given regularly once a month with bleedings after 7-10 days. The serum samples were designated by an alphabetical series. The sera were harvested and stored in the frozen state without additives. Concentrates of the immunoglobulin fractions of the sera were prepared by precipitation with 50% saturated $(\text{NH}_4)_2\text{SO}_4$ at 4°C for 3 hr, and re-solution at $\frac{1}{4}$ the original volume.

Two directional immunodiffusion assays were performed on a microscale by a modification (12) of the Wadsworth technic (13). Bacto-agar (Difco) was used in a concentration of 1.5%, and the routine test antigens were either buffered saline extracts of the homogenized tissues or the entire homogenate, with equivalent results. Development was allowed to take place at room temperature, after preliminary comparisons failed to reveal any differences with the ultimate reactions after development at 4°C . Immunoelectrophoresis was carried out according to the technique described by Wadsworth and Hanson (14). In most instances, the precipitates were appropriately stained with amido black, which sometimes revealed faint lines otherwise difficult to see. All photographs were taken with the Cordis immunodiffusion camera (Cordis, Miami, Fla.).

Absorption of the rabbit antisera with plasma, to remove antibodies to these proteins, was regularly performed by adding samples of the antisera to lyophilized plasma of the appropriate species, at the rate of 60 mg/ml. After stirring to dissolve, the antisera were stored at 4°C for 1-4 days, then clarified by centrifugation. In those instances where subsequent absorptions with tissues were carried out, homogenates of the appropriate tissues were made in the high speed blender without added buffered saline. The paste-like brei was added to antisera at the rate of 500 mg wet weight/ml. After storage at 4°C for 2-4 days, with occasional stirring, the mixtures were clarified by cold centrifugation. In this way, dilution of antibody was kept to a minimum.

In order to ascertain that the antigen preparations used in the immunodiffusion tests contained all of the potential soluble autoantigens, several extraction procedures were tested. All extracts were clarified by ultracentrifugation at 59,000 g or more for at least 30 min at 4°C . Exposure of these solutions to centrifugal fields as high as 140,000 g left all detectable antigens in the supernate. After extraction of the finely homogenized rabbit heart with buffered saline and repeated washing of the insoluble residue, the final residue was further extracted with high concentrations of potassium chloride, under conditions shown to solubilize myosin (15). The remaining residues were further extracted with 0.3% sodium desoxycholate

at 4°C for 24 hr or with hot (80°C) glycine buffer at pH 2. In all instances, the autoantigens detectable with these antisera appeared predominantly in the first buffered saline extracts. Some were also found in the potassium chloride extracts, but they were fewer in number and appeared to be in lower concentrations than in the saline extracts. Negligible reactions were observed with the desoxycholate or the acid extracts of the saline insoluble residues. Concentrates of the cardiac antigens were prepared by precipitation with 0.66 saturated (NH₄)₂SO₄, and re-solution in 1/5 the original volume.

The streptococcal concentrates were prepared from growth of a Group A beta hemolytic strain (C203S) in an antigen-free medium. The medium was prepared from Bacto-soytone, Bacto-casitone, or Bacto-proteose-peptone No. 3 (Difco), after a short digestion of each with crystalline trypsin at 37°C, and ultrafiltration through the Amicon apparatus (Boston, Mass.). The membranes used allowed only substances below 1000 molecular weight to pass, and these ultrafiltrates were used as the medium base, with additional ingredients described earlier (16). Streptococcal cellular extracts were prepared by Mickle disintegration of thoroughly washed cells in the presence of fine ballotini glass beads.

TABLE I
Rabbits Developing Precipitating Antibodies to Soluble Rabbit Heart Antigens

Immunizing antigen	Positive vs. rabbit hearts / No. immunized	Bleedings positive vs. rabbit heart / No. of bleedings	Rabbits with maximum No. of rabbit cardiac antibodies*				
			0	1	2	3	4
Rabbit heart	10/14‡	27/58	4	5	2	2	1
Human heart	10/10	47/79	0	4	6	0	0
Rat heart	10/11	38/82	1	7	3	0	0
Guinea pig heart	4/6	13/32	2	2	1	1	0

* In routine tests with unconcentrated antiserum absorbed with the appropriate normal plasma, tested against homogenates of rabbit heart.

‡ Only those animals which had received the first series of 3 injections are included.

RESULTS

Incidence and Characteristics of Rabbit Cardiac Autoantibodies.—Many of the rabbits immunized with rabbit heart developed detectable precipitating antibodies to rabbit heart. As can be seen in Table I, 10 of the 14 animals given more than four immunizations responded with up to four immune systems which were detected in routine immunodiffusion tests against rabbit heart homogenates or extracts, and whole antiserum. In some of the animals, the first test bleedings were positive, while in others a more prolonged immunization appeared necessary. Of the 58 bleedings from these 14 rabbits, a total of 27 samples were positive. Examples of these reactions are shown in Fig. 1, where differences in antibody responses of several animals are illustrated. It is evident that the antibodies evoked were directed against the same cardiac antigens, although in different relative proportions. Concentrates of these antibodies showed that at least five systems were involved (Fig. 1 *b*).

In almost all cases, absorption with pooled normal rabbit plasma failed to reduce significantly the number of reactions against rabbit heart, as shown in Fig. 1. In a few instances, rabbits immunized with rabbit heart did reveal one

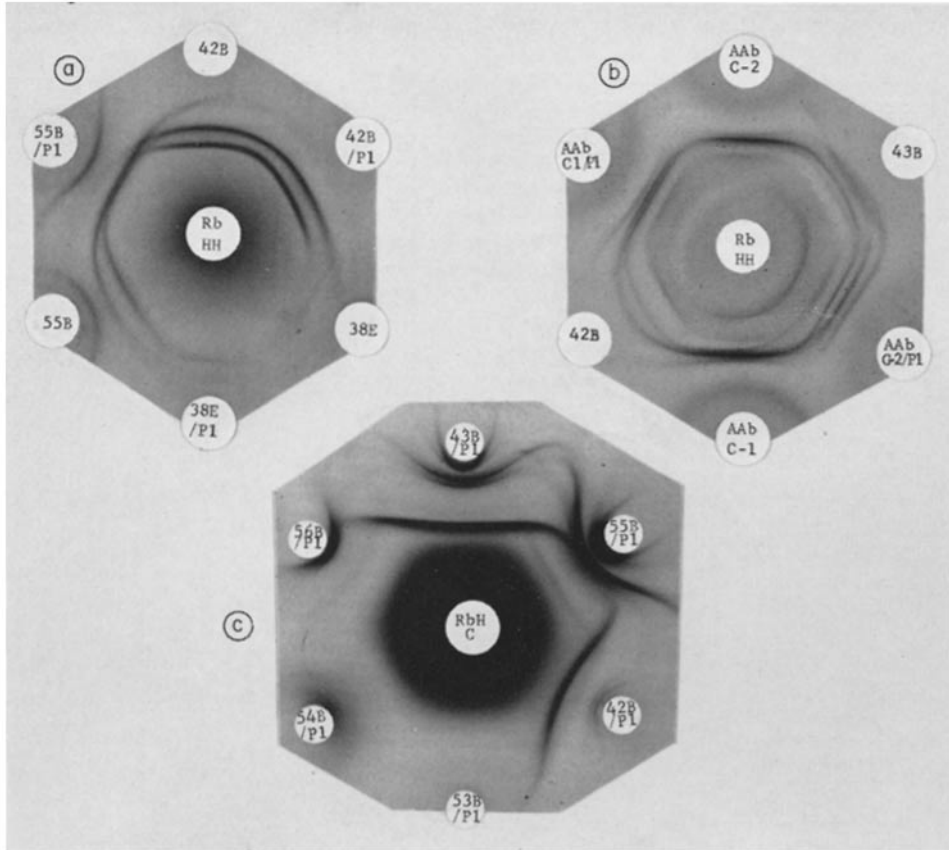


FIG. 1. Immunodiffusion reactions of rabbit anti-rabbit heart sera tested against rabbit heart. 42B, 38E, etc., rabbit number, antiserum sample. 42B/P1, etc., antiserum absorbed with lyophilized pooled normal rabbit plasma (60 mg/ml). RbHH, pooled rabbit heart homogenate (600 mg wet weight/ml). AAbC-1, etc., rabbit anti-rabbit heart antibody concentrate. AAbC-1/P1, etc., rabbit anti-rabbit heart antibody concentrate absorbed with lyophilized normal rabbit plasma (60 mg/ml). RbH C, rabbit heart extract concentrated by ammonium sulfate precipitation.

or two immune systems with pooled normal rabbit plasma, which were undoubtedly due to the presence of plasma protein allotypes (17) in the pooled rabbit heart homogenates used for immunization. This was confirmed by tests with these antiserum samples against individual normal rabbit sera, which

showed a very small per cent of positive reactions. However, even with the antisera revealing such reactions with pooled rabbit plasma, the number of systems detected with rabbit heart appeared unchanged after complete absorption with rabbit plasma. A series of tests performed with potent antisera and similarly prepared extracts of *fresh* rabbit heart showed reactions indistinguishable from those illustrated above. Comparison of these fresh extracts before and after freezing failed to reveal any differences in the immunodiffusion patterns.

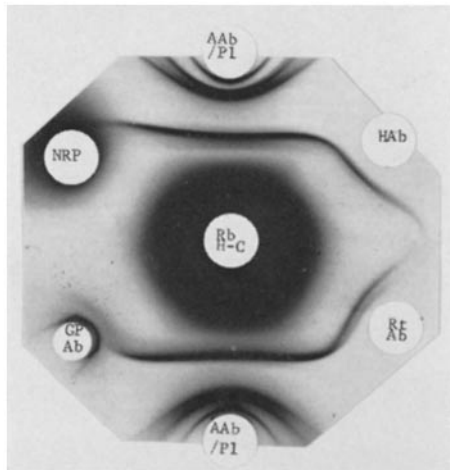


FIG. 2. Immunodiffusion comparison of rabbit cardiac antibodies evoked by immunization of rabbits with rabbit, human, rat, and guinea pig heart. In each case, the antisera were absorbed with correspondent pooled normal lyophilized plasma at 60 mg/ml.

RbH C, rabbit heart extract concentrated by ammonium sulfate precipitation. AAb/PI, rabbit anti rabbit heart autoantibody concentrate absorbed with rabbit plasma. HAb, rabbit antiserum to human heart (11E) absorbed with human plasma. RtAb, rabbit antiserum to rat heart (18E) absorbed with rat plasma. GPAb, rabbit antiserum to guinea pig heart (1D) absorbed with guinea pig plasma. NRP, normal pooled rabbit plasma.

The rabbits immunized with human, rat, or guinea pig heart tissue similarly revealed a high frequency of precipitating antibodies to rabbit heart extracts. 3 of these 27 animals showed three immune systems with rabbit heart in routine tests, while the majority of them demonstrated one or two. Roughly similar proportions of the postimmunization serum samples were positive for rabbit heart autoantibodies, as had been found in the rabbits immunized with rabbit heart.

The rabbit heart antibodies elicited by immunization with heterologous heart showed apparent "reactions of identity" with the antibodies evoked by immunization with rabbit heart. This is illustrated in Fig. 2, where it may be noted that spurs were not seen. This was always true in the cross-reactions

with the autoantibodies so produced. Conversely, the rabbit anti-rabbit heart sera showed similar immunodiffusion patterns with cardiac extracts from other species, again with apparent "reactions of identity" (see Fig. 3).

However, in the case of the cross-reactions of completely heterologous car-

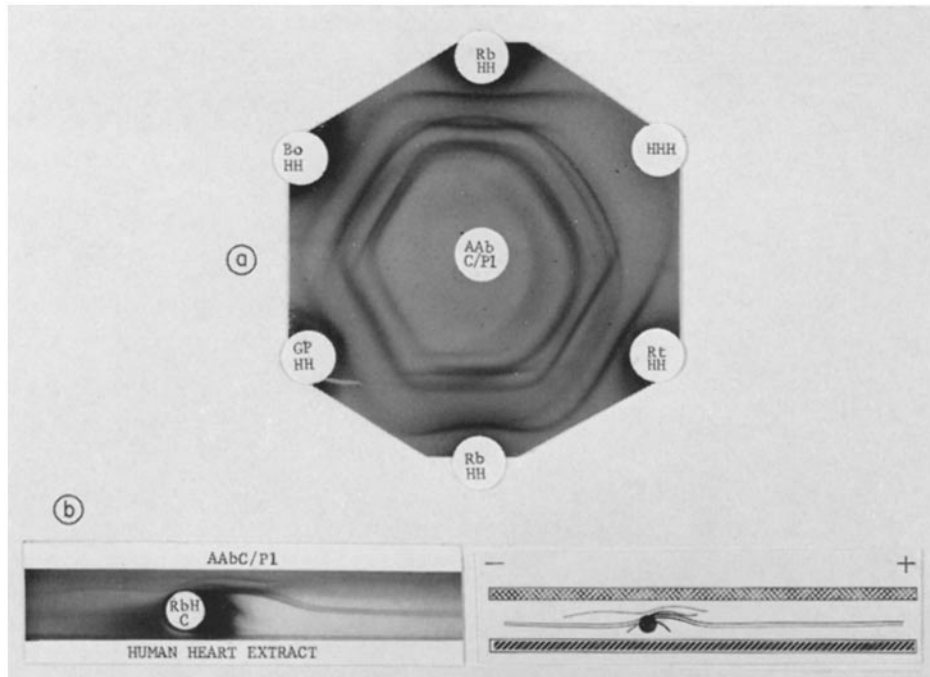


FIG. 3. Cross-reactions of rabbit anti-rabbit heart antibodies with cardiac antigens from other mammalian species. AAbC/Pl, rabbit anti-rabbit heart autoantibody concentrate absorbed with pooled lyophilized normal rabbit plasma (60 mg/ml)

RbHH, rabbit heart homogenate; HhH, human heart homogenate; RtHH, rat heart homogenate; GpHH, guinea pig heart homogenate; BOHH, bovine heart homogenate; all 600 mg wet weight/ml.

RbHC, rabbit heart extract concentrated by ammonium sulfate precipitation.

diac tissue antigens (e.g., anti-rat vs. rat and guinea pig hearts, and vice versa) suggestive spurs of "partial identity" were sometimes noted, as in Fig. 4. These patterns were usually rather complex, (as in Fig. 4 b) making conclusions of this nature uncertain.

It was of interest that rabbits immunized with guinea pig heart showed a high mortality rate in the early stages of immunization. The deaths which were encountered during the course of injections are summarized in Table II. It may be noted that almost all of the 10 rabbits which died after immuniza-

tion with guinea pig heart did so after the first to the third immunizing doses. Two groups of rabbits were immunized with guinea pig heart at different times, and separate pools of frozen guinea pig hearts from different commercial

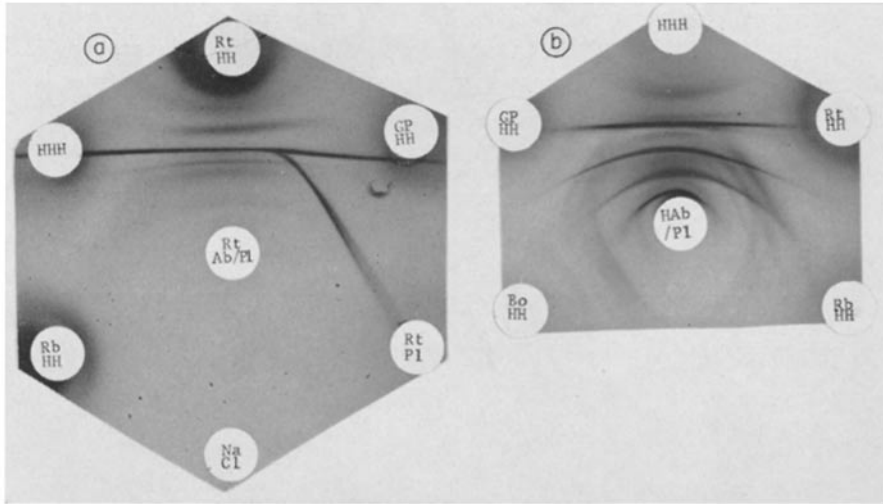


FIG. 4. Suggestive spurs of "partial identity" seen in some heterologous heart immunodiffusion reactions.

RtAb/Pl, rabbit antibody to rat heart, absorbed with lyophilized pooled normal rat plasma (60 mg/ml). HAb/Pl, rabbit antibody to human heart, absorbed with lyophilized pooled normal human plasma (60 mg/ml). RtHH, GPHH, RbHH, HHH: heart homogenates of rat, guinea pig, rabbit, and human respectively; all 600 mg wet weight/ml. RtPl, normal pooled rat plasma. NaCl, buffered physiological saline.

TABLE II

Mortality Rates During Immunization of Rabbits with Heart Tissue from Different Species

Immunizing antigen	No. dead / Total immunized	No. of doses given before death			
		1	2-3	4-5	>5
Rabbit heart	2/14	0	0	1	1
Human heart	4/11	0	1	0	3
Rat heart	3/11	0	1	0	2
Guinea pig heart	10/15	5	3	2	0

sources were used for each group. In the few rabbits which survived repeated immunizations with guinea pig hearts, the immune responses to guinea pig heart extracts were often as strong as those seen with the other heterologous heart immunizations. They also showed autoantibodies to rabbit heart in the

same proportion as those found after injection of rat or human heart. Animals receiving repeated injections of rat, human, or rabbit heart appeared to remain in relatively good health. The spontaneous mortalities which were encountered in these latter groups did not seem to be significantly different from those seen in the animal colony generally.

Autoantibodies to rabbit heart often first appeared only after several immunizations, and they sometimes showed a tendency to wane as the injection

TABLE III
Fluctuations in Cardiac Antibodies During Immunization

Rabbit No.	Immunized with heart from	Immunodiffusion test heart antigen	No. of precipitin systems* after booster dose							
			A	B	C	D	E	F	G	H
18	Rat	Rat	3	5	5	8	8	11	7	—
		Rabbit	0	1	0	1	2	0	0	—
15	Rat	Rat	4	5	4	7	7	7	4	—
		Rabbit	0	0	0	1	1	0	0	—
12	Human	Human	5	10	8	9	9	8	5	7
		Rabbit	0	1	1	2	2	0	0	0
11	Human	Human	5	13	10	13	14	12	11	11
		Rabbit	0	1	1	1	2	2	2	1
1	Guinea pig	Guinea pig	3	3	6	9	8	11	11	11
		Rabbit	0	0	2	1	1	3	2	3
25	Guinea pig	Guinea pig	5	8	9	6	10	9	—	—
		Rabbit	0	1	1	0	0	1	—	—
43	Rabbit	Guinea pig	2	2	3	2	3	2	—	—
		Rabbit	2	4	3	3	3	2	—	—

* Cardiac tissue reactions after complete absorption with normal plasma from the species supplying the immunizing antigen.

courses were prolonged. Examples of this are shown in Table III with rabbits 18 and 12, which were immunized with rat and human hearts respectively. There was a general tendency for the heart autoantibodies to be most numerous in those sera which demonstrated the largest number of reactions with the immunizing antigen, but many discrepancies were observed.

Tissue Specificity.—When the antisera were tested for their reactivities with noncardiac rabbit tissue extracts, considerable variability was observed, as seen in a few examples summarized in Table IV. In some instances, only

rabbit heart reacted with such antisera, while rabbit liver, kidney, pancreas, skeletal muscle, plasma, adrenals, testis, salivary gland, lung, and spleen failed to do so, (e.g., 11E, 15D, 19E, 25B, and 38C). Such restricted reactions with heterologous anti-heart sera and rabbit tissues are illustrated in Fig. 5. In other instances, reactive antigens were also detected in varying numbers of these tissues. Often, but not always, the precipitin bands found with rabbit heart extracts showed "reactions of identity" with those seen against other

TABLE IV
Rabbit Tissue Cross-Reactions of Rabbit Sera Containing Rabbit Heart Antibodies

Rabbit antiserum	Immunized with heart of	No. of precipitin systems with rabbit tissues*										
		Heart	Liver	Kidney	Pancreas	Skeletal Muscle	Plasma	Adrenal	Testis	Salivary gland	Lung	Spleen
11E	Human	2	0	0	0	0	0	0	0	0	0	0
28E	"	3	1	1	1	2	0	0	0	0	0	0
12E	"	2	0	0	1	0	0	0	0	0	0	0
10E	"	2	1	1	1	0	0	1	1	1	1	1
14E	"	3	1	0	0	0	0	0	0	0	0	0
16B	Rat	2	0	0	0	1	0	0	0	0	0	0
15D	"	2	0	0	0	0	0	0	0	0	0	0
19E	"	2	0	0	0	0	0	0	0	0	0	0
25B	Guinea pig	1	0	0	0	0	0	0	0	0	0	0
1D	" "	2	0	0	0	1	0	0	0	0	0	0
42B	Rabbit	3	1	0	1	1	1	0	1	2	0	0
43C	"	4	1	1	2	2	0	0	0	1	1	0
38C	"	1	0	0	0	0	0	0	0	0	0	0

* By two directional immunodiffusion against homogenates after absorption with normal plasma from the species supplying the immunizing antigen.

rabbit tissues. In all cases, however, the number of lines with rabbit heart were greater than those found with any other rabbit tissue.

Examples of the rabbit tissue specificity of cardiac autoantibodies induced homologously (rabbit anti-rabbit heart) are shown in Fig. 6. Antisera derived from rabbits immunized with homologous or with heterologous heart showed similar proportions of specimens with rabbit cardiac restricted specificities, compared to those possessing a portion of the antibodies reactive with other rabbit tissues.

In order to obtain conclusive evidence regarding the specificity of any of

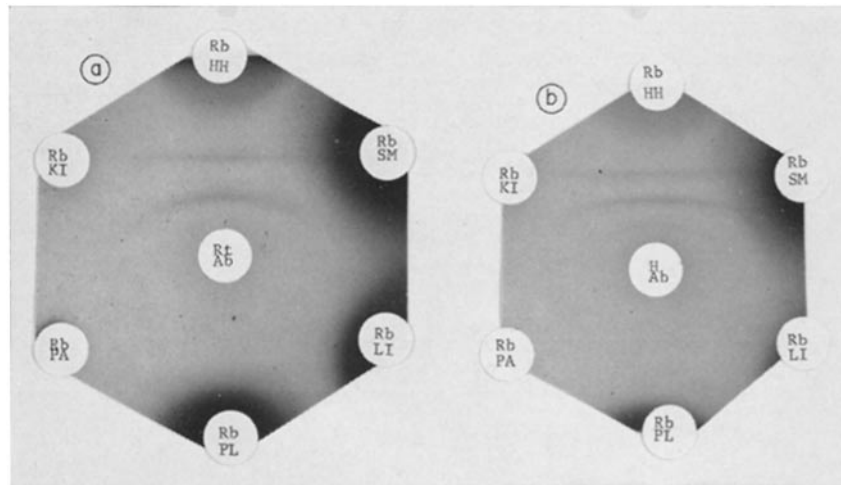


FIG. 5. Restricted rabbit cardiac specificity of rabbit anti-rat heart (*a*) and rabbit anti-human heart serum, (*b*) in reactions with several pooled rabbit tissue homogenates, all at 600 mg wet weight/ml.

RtAb, HAb; rabbit antisera to rat heart and human heart respectively. RbHH, rabbit heart; RbSM, rabbit skeletal muscle; RbLI, rabbit liver; RbPA, rabbit pancreas; RbKI, rabbit kidney; RbPL, rabbit plasma.

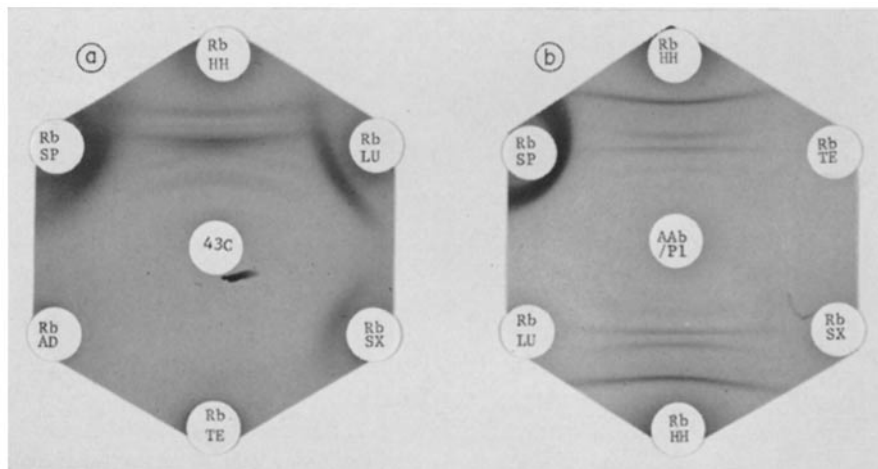


FIG. 6. Immunodiffusion reactions of rabbit anti-rabbit heart antibodies with other pooled rabbit tissue homogenates, all at 600 mg wet weight/ml.

43C, rabbit number, antiserum sample. AAb/Pl, rabbit anti-rabbit heart antibody concentrate absorbed with lyophilized pooled normal rabbit plasma (60 mg/ml). RbHH, rabbit heart; RbLU, rabbit lung; RbSX, rabbit submaxillary gland; RbTE, rabbit testis; RbAD, rabbit adrenal; RbSP, rabbit spleen.

these antibodies for components restricted to cardiac tissue, a series of absorption studies were carried out. Potent rabbit heart antisera or antibody concentrates were thoroughly absorbed with pooled lyophilized normal rabbit plasma, and then with homogenates of skeletal muscle, liver, and lung. These were subsequently tested against rabbit heart extracts, as well as other rabbit

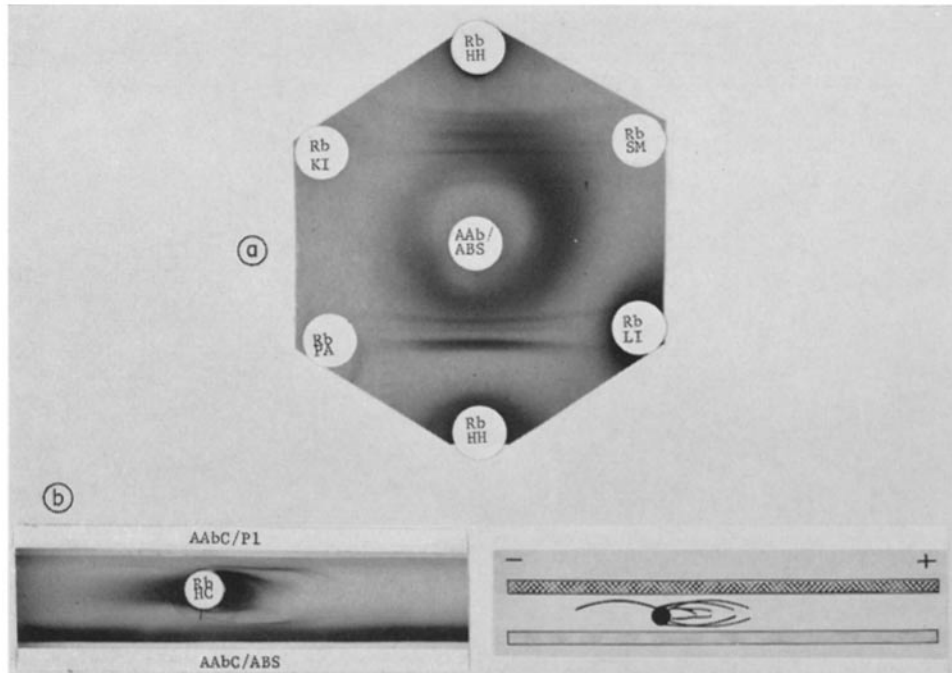


FIG. 7. Rabbit cardiac-restricted specificities of rabbit anti-rabbit heart antibodies demonstrated after thorough combined absorption with several rabbit tissues.

AAb/ABS, rabbit anti-rabbit heart antibody concentrate absorbed with rabbit plasma, skeletal muscle, liver, and lung. RbHH, rabbit heart homogenate; RbSM, rabbit skeletal muscle homogenate; RbLI, rabbit liver homogenate; RbPA, rabbit pancreas homogenate; RbKI, rabbit kidney homogenate; all 600 mg wet weight/ml. RbHC, rabbit heart extract, concentrated by ammonium sulfate precipitation. AAbC/Pl, rabbit anti-rabbit heart antibody concentrate absorbed with rabbit plasma.

tissues. In several instances, autoantibodies still clearly reactive with rabbit heart were found, as shown in Fig. 7. At least three cardiac specific autoimmune systems could be demonstrated, and they reacted with similar antigens in heart extracts from other mammalian species, as shown in Fig. 8. Such tests proved more satisfactory with antibody concentrates, but even with these, haloes of turbidity about the antiserum wells after multiple tissue absorptions proved troublesome.

Autoantibody Nature of the Reactions.—That the antibodies detected above represented true autoantibodies to heart was clearly indicated by tests with sera from five animals who died or were sacrificed during the course of immunization, using their own hearts as antigen in the immunodiffusion reactions. Some of these findings are illustrated in Fig. 9, demonstrating equivalent results with the heart of the antibody producer and with pooled rabbit heart. In addition, several tests were performed with a number of individual normal rabbit heart homogenates and potent antisera, all of which showed similar reactions with the cardiac autoantibodies, as seen in the examples of Fig. 10.

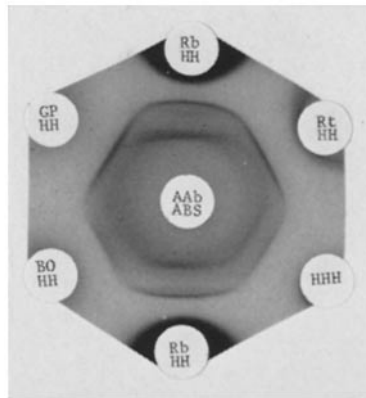


FIG. 8. Cross-reactivity of several mammalian heart extracts with cardiac restricted rabbit anti-rabbit heart antibodies. The latter had been thoroughly absorbed with rabbit plasma, skeletal muscle, and liver.

RbHH, rabbit heart homogenate; RHH, rat heart homogenate; HHH, human heart homogenate; BOHH, bovine heart homogenate; GP HH, guinea pig heart homogenate; all 600 mg wet weight/ml.

On the basis of these results, it seems probable that most, if not all, of the cardiac antibodies observed represented true autoimmune reactions.

In all instances, preimmune serum samples from each of these rabbits failed to demonstrate any reactivity with extracts of rabbit heart. In addition, a series of 20 potent rabbit antisera against a variety of antigens also failed to reveal any reactions with rabbit heart extracts. These antisera were obtained by an immunization procedure identical with that used here, with the same complete Freund's adjuvant mixture. They represented samples harvested after short or prolonged courses of immunizations, some up to 1 or 2 yr, using a variety of antigens. The latter included bovine serum albumin, human gamma globulin, tobacco mosaic virus, influenza virus, penicilloyl rabbit serum albumin, and penicilloyl bovine serum albumin.¹

¹ The authors are indebted to the Cordis Laboratories, Miami, Fla., for these specimens

Preliminary investigations have been performed on the salting out of the cardiac autoantigens found in rabbit heart. Either ammonium sulfate or potassium phosphate proved suitable for concentrating all of these substances. Details of these results, as well as progress in characterizing and purifying the cardiac autoantigens, will be presented in a subsequent publication.² Sufficient for the present report, these ammonium sulfate-precipitated autoantigen con-

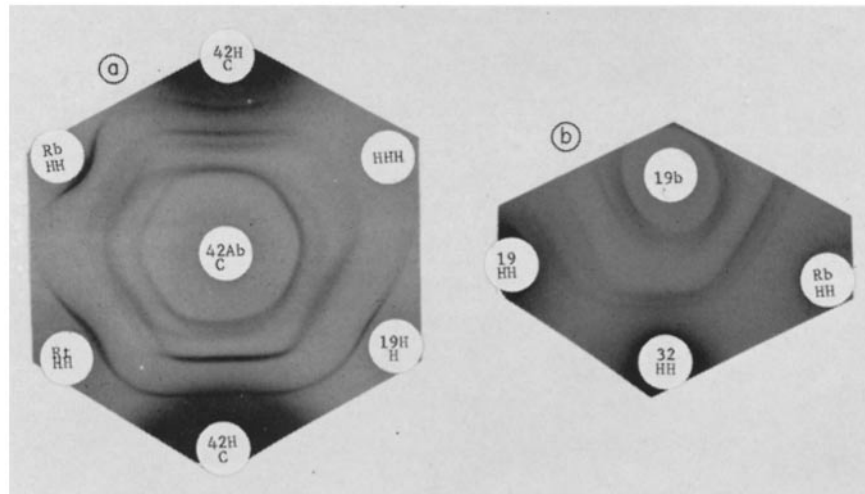


FIG. 9. Cardiac autoantibodies demonstrated by immunodiffusion between the hearts of two rabbits tested against their own sera. In (a), rabbit 42 had been immunized with rabbit heart (homologously), while in (b), rabbit 19 had been immunized with rat heart (heterologously). The "identity" of these reactions with other mammalian hearts is shown in the former (a), while the identity with pooled rabbit heart and another individual rabbit heart shown in both.

19b, rabbit number, antiserum sample. RbHH, pooled rabbit heart homogenate; 19HH, homogenate of heart from rabbit 19; 32HH, homogenate of heart from rabbit 32; HHH, human heart homogenate; RtHH, rat heart homogenate; all 600 mg wet weight/ml. 42AbC, antibody concentrate from sera of rabbit 42. 42HC, extract of heart from rabbit 42, concentrated by ammonium sulfate precipitation.

centrates usually yielded more satisfactory immunodiffusion patterns than did the simple extracts or homogenates, often with less halo effects surrounding the antigen wells.

All of these anti-heart sera were routinely tested for their reactivity with Group A streptococcal fractions derived from organisms grown on antigen-free medium. In a few instances, total extracellular and cellular harvests at high concentrations revealed one reaction by immunodiffusion. When they were

² Holm, S., S. P. Halbert, and S. Sobran. Data to be published.

encountered, comparative tests with the rabbit heart extracts showed that the autoimmune and the streptococcal cross-reacting systems appeared to be unrelated (see Fig. 11). All of the preimmune sera failed to show any detectable reaction with these streptococcal concentrates, nor did the control antisera described above.

DISCUSSION

These results demonstrated a surprising complexity of the autoimmune responses of rabbits to rabbit heart. Under certain conditions, most readily

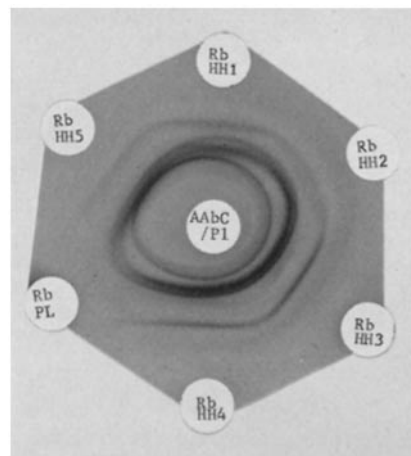


FIG. 10. Reactions of rabbit anti-rabbit heart autoantibody concentrate in tests against individual rabbit heart homogenates. AAbC/Pl, rabbit anti-rabbit heart antibody concentrates absorbed with lyophilized pooled normal rabbit plasma (60 mg/ml). RbHH1, etc, homogenates of individual rabbit hearts, 600 mg wet weight/ml. RbPl, pooled normal rabbit plasma.

with concentrates of the autoantibodies and the autoantigens, up to five reactions could be unequivocally detected with the most potent rabbit anti-rabbit heart sera. Although antibodies to plasma protein allotypes were occasionally encountered with these rabbit anti-rabbit heart sera, absorption with pooled rabbit plasma did not significantly alter the number of reactions found with rabbit heart antigens.

Many of the antisera showed reactions *only* with rabbit heart extracts, but certain of them revealed that some of the detected cardiac antigens were shared with other rabbit tissues. However, multiple absorptions with these latter tissues resulted in the persistence of at least three rabbit cardiac restricted reactions with the most potent rabbit heart autoantibodies, indicating the existence of several cardiac-specific autoantigens. Attempts are presently in progress to purify and characterize them biochemically. The same, or simi-

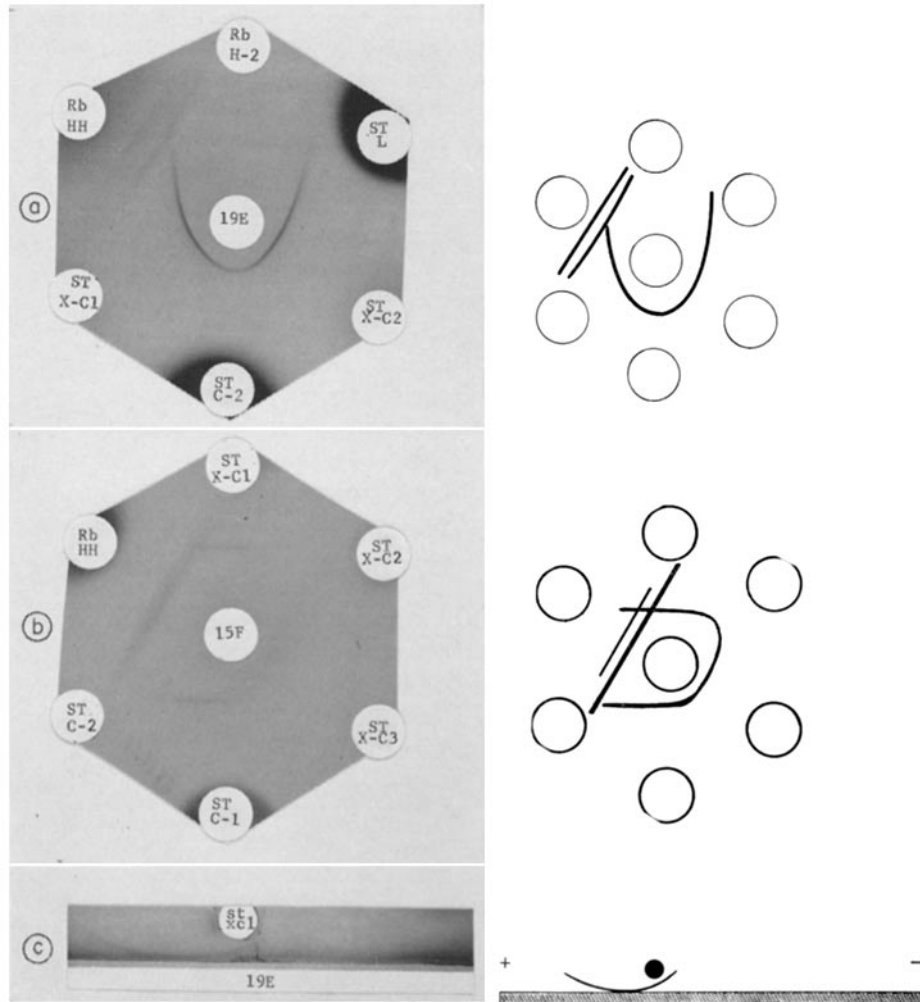


FIG. 11. Reactions of two rabbit anti-rat heart sera with Group A streptococcal concentrates. 19E, 15F: rabbit numbers, antiserum samples. STX-C1, STX-C2, STX-C3: concentrates of Group A extracellular products prepared from the C203S strain grown in antigen-free small molecular weight medium derived from soytone, proteose-peptone No. 3, and casitone respectively. STC-1, STC-2: extracts prepared from washed C203S streptococcal cells grown in the above media. Mickle disintegration with ballotini glass beads was used for disruption. ST-L, acid extract (pH2) of streptococcal cell residues. RbHH, rabbit heart homogenate. RbH-2, acid extract (pH2) of rabbit heart residue.

lar, autoantigens appear to be present in the other mammalian hearts tested, including that of man. In view of this fact, it will be of interest to study such purified preparations for their reactivity with the spontaneous cardiac auto-antibodies found in certain human diseases (7, 18-20).

The large number of autoimmune reactions seen do not appear to be due to artefacts caused by hydrolytic breakdown of the antigens into fragments. For instance, results of tests with freshly obtained unfrozen rabbit heart extracts proved indistinguishable from the reactions using frozen cardiac tissue. Had hydrolytic processes brought about an increase in the number of precipitin lines, it would have been anticipated that the fresh extracts would reveal fewer of these autoimmune precipitates. Furthermore, exposure of the cardiac extracts to a variety of proteinases for differing periods of time,² always revealed a decrease or complete disappearance of the reactions detectable with these cardiac autoantibodies. An increase in the number of immune systems observed due to antigen fragmentation was never observed. Further indication of this bona fide complexity of the cardiac autoantigen system was the finding of similar cross-reacting antigens in hearts derived from other species. It seems unlikely that these would reveal such parallel and uniform antigen fragmentation.

The autoantibodies to heart were also not due to artefacts caused by immunization of the animals with complete Freund's adjuvant. A variety of antisera developed in rabbits to several unrelated antigens, using exactly the same immunization program and the same adjuvant mixture, uniformly yielded negative results against rabbit heart extracts. The relative success in evoking cardiac autoantibodies in the experiments described here, as compared to other reports (7-11), may be due to a combination of effective and prolonged immunization schedules, and a highly sensitive immunodiffusion technique.

Although not tested in all instances, it seems likely that most, if not all, of the tissue autoantibodies encountered were true autoantibodies. The results obtained with the hearts of animals sacrificed or dying during the studies, in tests against their own serum, pointed in this direction. These latter reactions appeared to be "identical" with those noted when the same sera were tested against pools of other rabbit hearts. The similarity of the reactions of these antisera with a series of individual rabbit heart extracts further suggests the true autoantibody nature of the responses. Only in the case of the occasional rabbit anti-rabbit heart serum specimen, which revealed reactions with normal rabbit plasma pools, were indications of an isospecific response obtained. In these latter instances, the reactions due to plasma protein allotypes did not appear to contribute to the precipitin systems detected against rabbit heart.

Considerable variability was encountered in the rabbit tissue cross-reactivities found with different antiserum specimens. In some instances, only rabbit cardiac tissue reacted, while in others, some of the antigens inducing an autoimmune response to rabbit heart were found in either a small number, or a wide range, of rabbit tissues. It was also of some interest that in some animals, the rabbit heart autoantibodies would wane and disappear during the pro-

longed immunization schedule. It is conceivable that this represents the development of an immune unresponsiveness to the antigens involved. Whatever the mechanism, this fluctuation could contribute to some of the discrepancies reported in the literature.

The cause of the early and high mortality in the rabbits immunized with guinea pig hearts is not clear. Because of the possibility that infection may have played a role, preparations of fresh frozen guinea pig hearts were obtained from two different commercial sources, one in Arizona and the other in Illinois. The pattern of lethal outcome after the injections of guinea pig heart appeared to be the same with both samples. These observations raise the intriguing possibility that the deaths might represent an experimental auto-immune disease, possibly associated with cardiac lesions, but histological studies were not carried out. Those rabbits which survived repeated injections of guinea pig heart over a prolonged period of time appeared to be in good health whether or not they showed detectable autoantibodies to rabbit cardiac tissue. Although definitive tests have not yet been performed on the pathogenetic significance of the observed cardiac autoantibodies, it is unequivocally clear that rabbits can survive for prolonged periods of time with their abundant presence in the circulation.

It must be pointed out that the above investigations relate only to soluble components of rabbit heart which are not sedimentable at centrifugal forces as high as 140,000 *g*. It is conceivable that aqueous insoluble autoantigens may also have elicited immune responses, in view of the data reported by Kaplan (6). Attempts to solubilize additional detectable cardiac antigens from the saline insoluble residues by a variety of techniques have been unsuccessful thus far, however.

In those few instances where reactions were seen between the anti-heart sera and streptococcal concentrates, no relationship was observed between these immune systems and those directed against the rabbit heart autoantigens. Further work is needed to explore more conclusively any possible relationship between the cardiac autoantigens seen above and the heart components which are cross-reactive with streptococcal constituents (21-23).

SUMMARY

1. A high proportion of rabbits immunized with pooled rabbit heart homogenates in complete Freund's adjuvant responded with the production of multiple precipitating antibodies to soluble rabbit heart antigens. The most potent antisera revealed at least five distinct antigens.

2. Rabbit anti-rabbit heart antibodies reacted with similar antigens in other mammalian hearts, (human, rat, guinea pig, and bovine) with apparent "reactions of identity" by immunodiffusion.

3. Rabbits immunized with human, rat, or guinea pig heart homogenates

also responded with multiple precipitating antibodies directed against rabbit cardiac antigens, although somewhat less intensively than animals immunized homologously. The specificities of these antibodies appeared to be the same as those evoked homologously.

4. The autoantibody nature of the homologously and heterologously induced responses was unequivocally demonstrated in several instances by reactions between the sera from immunized rabbits and their own hearts.

5. Many of the autoantibodies appeared to be directed against antigens restricted to the heart, judging by comparative immunodiffusion tests with other rabbit tissue extracts. This was convincingly confirmed by multiple absorption of potent antisera with several rabbit tissues. The cardiac-restricted antigens were also present in heart extracts of other mammalian species. In those instances where some of the cardiac-evoked autoantibodies reacted with other rabbit tissues, the tissue cross-reactions were quite variable.

6. Rabbits immunized with guinea pig heart homogenate suffered a high early mortality of undetermined cause, compared to animals immunized with rabbit, human, or rat hearts.

7. A small proportion of the anti-heart sera revealed immunodiffusion reactions with Group A streptococcal products, derived from organisms grown in antigen-free media. In these few instances, the reactions appeared unrelated to cardiac autoantibody responses.

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