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Induction of Autoimmunity to Adrenal Gland

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Summary. Rabbits that were immunized with homologous and heterologous adrenal homogenates in complete adjuvant responded with complement-fixing heat-stable autoantibody primarily directed against adrenal. Rabbits responded with autoantibody production against cytoplasm of adrenal cortex and of ovarian and testicular cells as detected by immunofluorescence. Antibody, with sedimentation similar to 7S γ globulin, was directed against a heat-labile antigen in rabbit, guinea-pig and rat adrenal. Antibody responsible for reactions with the antigens in the complement fixation and immunofluorescent tests was absorbed by an ultra-centrifugal sediment of adrenal or ovary.

Histological evidence of adrenalitis was present only in those rabbits immunized with heterologous adrenal.

Guinea-pigs immunized with heterologous adrenal homogenates developed more extensive adrenal infiltrates than did guinea-pigs immunized with homologous adrenal. Antibody from guinea-pigs immunized with heterologous adrenal reacted with autologous organ antigens as well as with rabbit and rat adrenal. Skin tests with homologous adrenal revealed no evidence for delayed-type hypersensitivity.

The detection of autoantigens in foetal rabbit adrenal and ovary suggests that rabbits should be tolerant to such antigens. The greater efficacy of immunization with heterologous adrenal in eliciting autoantibody and autoimmune adrenalitis may be a feature of experimental autoimmunity induced by those organ-specific antigens to which the animal is tolerant. The presence of autoantibody in the serum, bound γ globulin at the site of adrenal lesions and the absence of delayed-type hypersensitivity to homologous adrenal suggest that the humoral immune mechanisms play a role in experimental autoimmune adrenalitis.

INTRODUCTION

The production of adrenalitis in guinea-pigs by immunization with guinea-pig adrenal homogenate in Freund's complete adjuvant, first described by Colover and Glynn (1958), has been repeated by Steiner, Langer, Schatz and Volpe (1960). Milgrom and Witebsky (1962) have reported organ-specific heat-stable antigen in bovine adrenal which on immunization of rabbits elicited an antibody specific for this foreign antigen but not reactive with autologous adrenal. In a second paper (Witebsky and Milgrom, 1962) they reported the production of autoantibody in rabbits and guinea-pigs following immunization with homologous or autologous adrenal. Neither Steiner nor Witebsky and Milgrom

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were able to demonstrate autoimmune adrenalitis in rabbits and the latter authors were unable to confirm the earlier observations made in guinea-pigs.

The present work investigates the relative efficacy in rabbits and guinea-pigs of homologous and heterologous adrenal immunization in inducing autoimmunity. The serological manifestations of autoimmunity were measured by complement fixation and immunofluorescence. The distribution of the autoantigens in the various tissues was investigated.

It was found that immunization with heterologous adrenal was more effective than homologous adrenal in inducing autoimmunity to adrenal by both serological and histopathological criteria.

MATERIALS AND METHODS

Immunization of Animals

Fresh whole adrenal glands, from rabbits, rats and guinea-pigs, were homogenized in cold 0·25 M sucrose to a suspension containing 1 g. of adrenal (wet weight) per ml. of homogenate. For immunization, these suspensions were emulsified with equal volumes of Freund's complete adjuvant (Difco) and administered by injection into all four footpads.

Twenty-five rabbits were arranged in four experimental groups. The first group (of eight animals) was immunized with rabbit adrenal in Freund's complete adjuvant by footpad injection, each animal receiving a total of 1 g. (wet weight) of adrenal tissue. A second group comprised nine rabbits, each of which was similarly immunized with footpad injections containing 1 g. of guinea-pig adrenal. Each of four rabbits in a third group received similar injections of 1 g. of rat adrenal, and in a fourth group of rabbits (four animals) each was given 1·0 ml. of Freund's complete adjuvant emulsified with 1·0 ml. of 0·25 M sucrose.

Two or four rabbits (see Table 7) from each of these four groups were given booster injections 10 weeks after primary immunization.

Thirty-two guinea-pigs were similarly arranged in four experimental groups. Eight guinea-pigs were immunized with 250 mg. guinea-pig adrenal in complete adjuvant given in all four footpads, eight were immunized with rabbit adrenal homogenate in complete adjuvant, eight with rat adrenal and eight with the 1 ml. Freund's complete adjuvant emulsified with 1 ml. of 0·25 M sucrose. None of the guinea-pigs in these four groups was given booster injections.

Twelve guinea-pigs were immunized with 60 mg. guinea-pig adrenal in complete adjuvant in all four footpads and intracutaneously in the nuccal regions every 14 days until four sets of injections had been given. Three guinea-pigs were similarly immunized with 60 mg. guinea-pig liver, three with guinea-pig kidney, and three with guinea-pig testis in complete adjuvant.

Six guinea-pigs were immunized with 2 ml. of 50 per cent complete adjuvant in 0·25 M sucrose every 14 days until four sets of injections had been given.

Sera

Rabbits were bled immediately prior to immunization. Following primary immunization the sera were obtained daily from the 3rd to the 7th day, the animals being subsequently bled between 10 and 14 days, and then weekly until the 10th week. Following booster immunization at the 10th week the twelve boosted rabbits were bled daily for 14 days.

Guinea-pigs were bled prior to immunization and 14 days following the last immunization.

Aliquots of sera at a dilution of 1 : 4 were heated at 56° for 30 minutes to inactivate complement and at 65° for 30 minutes to inactivate the complement-fixing antibodies similar to those described by Kidd and Friedewalde (1942).

Antigens

Homogenates of freshly removed organs were made 1/10 in cold 0·25 M sucrose and used on the same day or after storage at −18° for not longer than 2 weeks. It was found that this period of storage did not affect the antigenic activity; however, once thawed, antigen suspensions were not refrozen for subsequent use. Antigen was diluted to the desired strength on the day of use with cold calcium magnesium saline buffered with barbiturate, the diluted antigens being kept in ice-water until required.

In this way antigens for complement fixation were prepared from rabbit, guinea-pig and rat adrenals; suspensions of other organs were similarly made from brain, liver, kidney, ovary and testis of normal adult rabbits and guinea-pigs.

Autoantigens were obtained from members of each group of immunized rabbits and guinea-pigs at autopsy.

Foetal rabbit antigens were prepared from the pooled organs of three litters obtained 23 days after mating, and from the separately pooled organs of two litters obtained at full term. The protein concentration of adult and foetal organ homogenates ranged from 9 to 12 mg./ml. when diluted 1 : 10.

Skin Tests

Evidence of delayed hypersensitivity to the tissue antigens was sought in immunized guinea-pigs and rabbits. The antigens used were whole sucrose homogenates of adrenals and other organs diluted in saline so that each intradermal skin test dose (0·1 ml.) contained 10 mg., 1 mg. or 0·1 mg., of diluted antigen referred to wet weight of original tissue. Adrenal homogenate was found to contain 0·6–1·3 mg. protein per 10 mg. wet weight.

Complement Fixation

Complement fixation was undertaken in M.R.C. pattern Perspex plates following the method of Donnelley (1951). One hundred ml. of 3 per cent thrice-washed sheep red cells were sensitized with 0·075 ml. of Burroughs Wellcome rabbit anti-sheep haemolysin. Pooled guinea-pig serum was used as a source of complement; usually four 100 per cent haemolytic units were used in the test. Fixation was carried out at 37° for 40 minutes and lysis for 45 minutes.

The time course of the antibody responses to primary and booster immunization was tested using antigen dilutions of 1/160. The distribution in various organs of homologous and autoantigens reactive with the sera was studied by block titration, i.e. dilution of both antigen and antibody.

The results are expressed as the reciprocal of the highest serial dilution of antigen (or antibody) giving 2+ (50 per cent) complement fixation estimated visually. Two-volume antigen and antibody controls were always included.

Absorption of Sera

Antigens for absorption were prepared as follows. A volume of 10 ml. of a 1/10 homogenate of fresh tissue in 0·25 M sucrose was centrifuged at 100,000 *g* for 30 minutes; the

sediment was suspended in 10 ml. of cold buffered saline diluent and the suspension recentrifuged. This sediment was rewashed with a further 10 ml. of saline diluent and centrifuged again. The resulting sediment was resuspended in 7 ml. of saline diluent, and 1 ml. of the serum to be absorbed was added. After 30 minutes at 37° and 30 minutes at 4° the mixture was centrifuged at 100,000 *g* for 60 minutes; the supernatant, which contained the absorbed serum at a dilution of 1/8 was stored at −18°. The absorbed sera were inactivated immediately prior to complement fixation and precipitin studies. Rabbit serum was mixed with an equal volume of thrice-washed and packed sheep red blood cells, incubated at 37° for 30 minutes and then centrifuged at 500 *g* for 15 minutes. The supernatant-absorbed serum was removed and absorbed a second time with packed sheep red blood cells.

Immunofluorescence

Goat anti-rabbit γ-globulin serum and rabbit anti-guinea-pig γ-globulin serum were each prepared by the method of Milgrom, Dubiski and Wozniczko (1956); the γ-globulin fractions of these sera were conjugated with fluorescein isothiocyanate (Marshall, Eveland and Smith, 1958). Immunoelectrophoresis of these antisera disclosed only anti-γ and anti-β globulins—but no antibody against α globulin or albumin. Before use, both conjugates were absorbed twice with an equal volume of an acetone-dried powder of guinea-pig liver and spleen or rabbit liver for 10 minutes at 4°. Non-specific adsorption of antisera was minimized by adding bovine serum albumin conjugated to rhodamine, one part to nine parts of the goat anti-rabbit fluorescein conjugate (Smith, Marshall and Eveland, 1959). The processing of tissue and the staining procedures followed that of Coons and Kaplan (1950), and the final washing of the conjugate-treated preparations was carried out at 37°.

The fluorescence microscope used was equipped with a Cooke dark ground condenser. The light source was a Cooke u/v mercury vapour lamp, 250 W., with a Chance 18A filter to remove visible light; a Wratten 2B filter was incorporated into the eyepiece to remove the ultraviolet light. Photographs were taken with Kodak R55 recording film.

Processing of Autopsy Material

Rabbits and guinea-pigs were killed at the intervals shown in 'Results' by cardiac puncture under Nembutal or ether anaesthesia. Small pieces of a wide range of organs were quickly removed and rapidly frozen to −70° in thin-walled glass tubes. This rapid freezing allowed excellent preservation of tissue architecture for immunofluorescence studies. Histology of the organs was obtained from formalin-fixed material. From each animal, both adrenals and both gonads were examined; in addition to the usual viscera, sections were also taken of midbrain, cerebellum and cerebral cortex. All sections were examined for histopathology by one of us (L. E. Glynn) without prior knowledge of the source of the tissue section.

Homogenates of the organs of these animals were made, as previously described, in cold 0·25 M sucrose for use as autoantigens.

Zone Centrifugation

Fractionation was achieved by centrifuging 1 ml. samples in buffered sucrose gradients in 'Lusteroid' tubes at 34,000 rev./min. in a Spinco SW 39L rotor for 16 hours (Edelman, Kunkel and Franklin, 1958). In each centrifugation one 'Lusteroid' tube contained a

TABLE 1

GEOMETRIC MEAN TITRE OF COMPLEMENT-FIXING ANTIBODIES AGAINST RABBIT ADRENAL (1/160) FOLLOWING PRIMARY AND BOOSTER IMMUNIZATION OF RABBITS WITH GUINEA-PIG, RABBIT AND RAT ADRENAL IN FREUND'S COMPLETE ADJUVANT (SERA INACTIVATED AT 56° AND 65°)

Immunizing antigen	*Temperature sera inactivated (°C.)*	*Pre-immunization titre*	*Time after first injection*					*Time after booster injection (days)*							
			3 days	*5 days*	*7 days*	*4 weeks*	*10 weeks*	1	2	3	4	5	7	10	14
Guinea-pig adrenal (9)*	56	8+	16	22	82	64	24	24	16	32	128	256	512	512	256
	65	2	4	8	16	16	8	8	8	16	94	190	512	512	256
Rabbit adrenal (8)	56	4	8	16	16	8	4	4	4	4	6	24	24	16	24
	65	3	3	4	5	3	2	2	2	2	4	12	24	16	16
Rat adrenal (4)	56	8	NT	NT	48	24	6	6	3	4	8	24	24	24	24
	65	3	NT	NT	4	5	2	2	2	2	2	16	16	12	8
Complete adjuvant only (4)	56	3	NT	NT	7	5	4	3	3	3	4	8	4	6	8
	65	3	NT	NT	2	2	2	2	2	2	2	2	2	3	3

* Figures in brackets refer to number of animals in each group.
NT = Not tested.

TABLE 2

GEOMETRIC MEAN TITRE OF COMPLEMENT-FIXING ANTIBODIES FROM RABBITS IMMUNIZED AND BOOSTED WITH RABBIT OR GUINEA-PIG ADRENAL, TESTED AGAINST HOMOLOGOUS AND HETEROLOGOUS ANTIGENS (ALL AT 1/160 DILUTION)

Test antigen	*Temperature sera inactivated (°C.)*	*Immunized and boosted with guinea-pig adrenal*			*Immunized and boosted with rabbit adrenal*		
		*Pre-I† titre (5)**	*2W† primary (5)*	*2W† secondary (2)*	*Pre-I titre (4)*	*2W primary (4)*	*2W secondary (2)*
Rabbit adrenal	56	5	58	256	5	24	24
	65	< 4	29	256	< 4	14	16
Rabbit ovary	56	4	25	190	4	4	16
	65	< 4	< 4	190	< 4	< 4	8
Rabbit testis	56	4	< 4	95	< 4	< 4	12
	65	< 4	< 4	64	< 4	< 4	6
Rabbit brain	56	⩽14	⩽14	⩽32	7	10	8
	65	< 4	5	⩽32	< 4	< 4	4
Rabbit liver	56	6	5	48	6	10	16
	65	< 4	4	16	< 4	< 4	4
Rabbit kidney	56	14	12	32	6	28	32
	65	< 4	< 4	16	< 4	< 4	4
Guinea-pig adrenal	56	< 4	128	1024	< 4	30	20
	65	< 4	32	512	< 4	14	16
Rat adrenal	56	NT	NT	NT	< 4	32	24
	65	NT	NT	NT	< 4	16	16

* Figures in parentheses refer to number of animals in each group.
† Sera taken before immunization (pre-I), 2 weeks after first injection (2W primary) and 2 weeks after second injection (2W secondary).
NT = Not tested.

control serum with rheumatoid factor and incomplete anti-D antibody as markers for macroglobulin and 7S globulins respectively.

RESULTS

THE ANTIBODIES AND ANTIGENS IN RABBITS

Kidd and Friedewalde (1942), described naturally occurring antibodies in rabbits which fixed complement in the presence of rabbit antigens. Such antibody was inactivated by heating at 65° for 30 minutes. In the present experiments the results reported with sera heated at 65° for 30 minutes are a measure of heat-stable antibody while those with sera heated at 56° for 30 minutes measured both heat-stable and the heat-labile, Kidd-Friedewalde type, antibody.

In Table 1 is shown the antibody response, as measured by complement fixation, of rabbits immunized with homologous or heterologous adrenal compared to adjuvant control rabbits. Immunization with guinea-pig adrenal resulted in a primary and booster antibody response greater than that obtained with rabbit or rat adrenal. It may be noted

TABLE 3

BLOCK TITRATION OF HYPERIMMUNE RABBIT SERUM

Minimum strengths of a range of autologous and homologous rabbit antigens which react with serum taken 2 weeks after booster immunization of rabbit 9/10. Rabbit immunized and boosted with guinea-pig adrenal in complete adjuvant. Serum diluted 1/32–1/1024.

Rabbit test antigen	*Temperature sera inactivated* (°C.)	*Antigen titre for serum diluted*					
		1/32	1/64	1/128	1/256	1/512	1/1024
Autologous adrenal	56	2560	2560	1280	1280	40	<20
	65	2560	1280	640	320	<20	<20
Homologous adrenal	56	2560	1280	640	320	<20	<20
	65	1280	1280	640	20	<20	<20
Autologous ovary	56	320	320	160	160	40	40
	65	640	320	320	80	<20	<20
Homologous ovary	56	640	1280	640	320	<20	<20
	65	640	2560	1280	640	<20	<20
Autologous liver	56	1280	160	20	<20	<20	<20
	65	<20	<20	<20	<20	<20	<20
Homologous liver	56	1280	160	<20	<20	<20	<20
	65	20	<20	<20	<20	<20	<20
Autologous kidney	56	320	160	40	⩽20	⩽20	⩽20
	65	40	⩽20	⩽20	⩽20	⩽20	⩽20
Homologous kidney	56	160	80	40	⩽20	⩽20	⩽20
	65	80	40	⩽20	⩽20	⩽20	⩽20
Autologous brain	56	160	80	⩽40	⩽40	⩽40	⩽40
	65	80	⩽40	⩽40	⩽40	⩽40	⩽40
Homologous brain	56	160	80	⩽40	⩽40	⩽40	⩽40
	65	80	⩽40	⩽40	⩽40	⩽40	⩽40

that the heat-labile antibody (56° heated sera) generally preceded the heat-stable response. Further, the adjuvant control rabbits had a definite increase only of heat-labile antibody.

Table 2 shows that the antibodies produced during the secondary response reacted with a wider range of homologous rabbit antigens than did sera taken during the primary response. Two weeks after the primary immunization with guinea-pig adrenal there was high-titre heat-stable antibody directed primarily against guinea-pig adrenal which cross-reacted only with rabbit adrenal. After the second injection, the heat stable antibody elicited was in higher titre and cross-reacted with rabbit adrenal, ovary and testis and in lower titre with rabbit brain, liver and kidney. Sera from rabbits immunized with one or two injections of rabbit adrenal had antibody to rat and guinea-pig adrenal which equalled in titre that to homologous rabbit adrenal. The cross-reactions with other rabbit organs were all low in titre but greater with rabbit ovary and testis than with brain, liver or kidney.

Chess-board titrations to demonstrate correspondence in the behaviour of autologous and homologous antigens with a given antiserum are shown in Table 3. Since the antiserum reacted as well with autologous organ antigens as with homologous antigens it may be called an autoantibody even though the immunizing antigen was from a heterologous species.

No statements can be made regarding the existence of isoantigenic groups of rabbit adrenal since homologous adrenal used for both testing and immunization was a homogenate pooled from many rabbits.

Table 4 shows that the sera of immunized rabbits also reacted with antigens present in foetal rabbit organs. The strongest reactions were given by sera taken from rabbits

TABLE 4

REACTION OF SERUM FROM RABBIT 9/10 WITH ADULT, NEWBORN AND FOETAL RABBIT ANTIGENS AFTER PRIMARY AND BOOSTER IMMUNIZATION WITH GUINEA-PIG ADRENAL

Rabbit test antigen		*Serum taken 2 weeks after first injection* *Serum dilution 1 : 8* *Heated 56°*	*Serum taken 2 weeks after second injection* *Serum dilution 1 : 32* *Heated 56°*	*Heated 65°*
Adrenal	Adult*	2560†	2560	2560
	Newborn	640	2560	2560
	23 day foetal	640	1280	1280
Ovary	Adult	408	1280	1280
Testis	Adult‡	80	320	320
	23 day foetal gonad	<20	160	160
Liver	Adult	<20	40	
	Newborn	<20	20	
	23 day foetal	<20	20	
Kidney	Adult	20	160	
	Newborn	<20	40	
	23 day foetal	<20	20	
Brain	Adult	≤20	80	
	Newborn	<20	<20	
	23 day foetal	<20	<20	

* Adult antigens obtained from pooled maternal organs.
† Titre of antigen giving 2+ complement fixation with serum dilution shown.
‡ Homologous adult rabbit testis.

that had received two injections of guinea-pig adrenal. In these sera both heat-labile and heat-stable antibodies were found which reacted with foetal, newborn and adult adrenal and gonadal tissues. Heat-labile antibodies reactive in low titre with newborn, foetal and adult liver and kidney were also found. Two weeks after the first injection heat-labile antibodies reacted only with adrenal and gonadal antigens. No complement fixation was obtained with these antisera and the cardio-lipid Wasserman antigen.

The nature of the cross-reactions was evaluated by absorption studies and the results tabulated in Table 5. In the serum of a rabbit immunized with guinea-pig adrenal,

TABLE 5

ABSORPTIONS OF ANTIBODY FROM SERUM OF RABBIT 9/10 (IMMUNIZED WITH GUINEA-PIG ADRENAL)

Test antigen	65° *test serum dilution*	*Unabsorbed*	*Absorbed with*			
			Guinea-pig adrenal	*Rabbit adrenal*	*Rabbit ovary*	*Sheep red cells*
Rabbit adrenal	1 : 32	512*	16	<16	32	512
	1 : 128	256	<16	<16	16	256
	1 : 512	<16	<16	<16	16	16
Rabbit ovary	1 : 32	512	<16	16	16	1024
	1 : 128	512	<16	<16	16	512
	1 : 512	16	<16	<16	<16	8
Guinea-pig adrenal	1 : 32	1024	16	1024	1024	1024
	1 : 128	1024	<16	1024	512	512
	1 : 512	256	<16	128	<16	512

* Titre of antigen giving 2+ complement fixation with serum dilution shown.

absorption with guinea-pig adrenal removed all antibody while absorption with rabbit adrenal or ovary removed only the antibodies cross-reactive with these homologous organs. Control absorptions with sheep red blood cells revealed no loss of antibody.

As pointed out by Dixon and Maurer (1955) a second injection of a heterologous protein antigen in rabbits sensitized by a closely related antigen will result in a booster response to both antigens. In two rabbits sensitized by an injection of guinea-pig adrenal in complete adjuvant and boosted with rabbit adrenal in complete adjuvant there was less than a four-fold increase in titre in antibody to rabbit or guinea-pig adrenal. In seven of eight rabbits given two successive injections of the same species of adrenal antigen (as in Table 2) there was a classical booster response.

Further information regarding the nature of the antigens was obtained by heating the antigen for 30 minutes at 56° before use in complement-fixation tests. Using an antiserum of low titre, e.g. from rabbit (No. 11/6) immunized with rabbit adrenal, the reactivity of all antigens used was found to be destroyed after heating at 56°. High-titre antiserum, e.g. from rabbit 9/10 immunized with guinea-pig adrenal, could demonstrate less well the heat lability of these antigens. Zone ultracentrifugation of serum from rabbits 9/3 and 9/10 obtained after two injections of rabbit and guinea-pig adrenal respectively showed that all complement-fixing antibody against adrenal, whether heat labile or stable, in these sera sedimented as 7S globulins.

Immunofluorescence

Table 6 gives the result of immunofluorescent staining of homologous and autologous rabbit tissue with the rabbit serum in a sandwich technique with a goat anti-rabbit globulin fluorescent conjugate. Sera positive at 1 : 4 dilution were also positive when diluted 1 : 40 and 1 : 160. Specific staining of cytoplasm in all three layers of adrenal

TABLE 6

IMMUNOFLUORESCENT STAINING OF HOMOLOGOUS OR AUTOLOGOUS RABBIT TISSUE BY 1 : 4 DILUTION OF SERUM SHOWN

No change noted whether serum heated at 56° or 65 ° for 30 minutes

Rabbit No.	*No. of immuniza-tions*	*Immunized with*	*Rabbit tissue substrate*		
			Adrenal	*Ovary*	*Testis*
9/2	1	Rabbit adrenal	+	Negative	Negative
11/16	1	Rabbit adrenal	+	Negative	Negative
9/3	2	Rabbit adrenal	+	+	+ Leydig, sperm
9/4	2	Rabbit adrenal	+	+	+ Leydig, sperm
11/3	1	Guinea-pig adrenal	+	+	Negative
11/5	1	Guinea-pig adrenal	+	Negative	+ Leydig
9/10	2	Guinea-pig adrenal	+	+	+ Leydig
9/11	2	Guinea-pig adrenal	+	+	Negative
9/16	1	Rat adrenal	+	Negative	Negative
9/13	2	Rat adrenal	+	Negative	Negative
9/14	2	Rat adrenal	+	N.T.	Negative
9/5	2	Adjuvant alone	Negative	Negative	Negative
9/6	2	Adjuvant alone	Negative	Negative	Negative

+ Specific staining detected.
N.T. = Not tested.

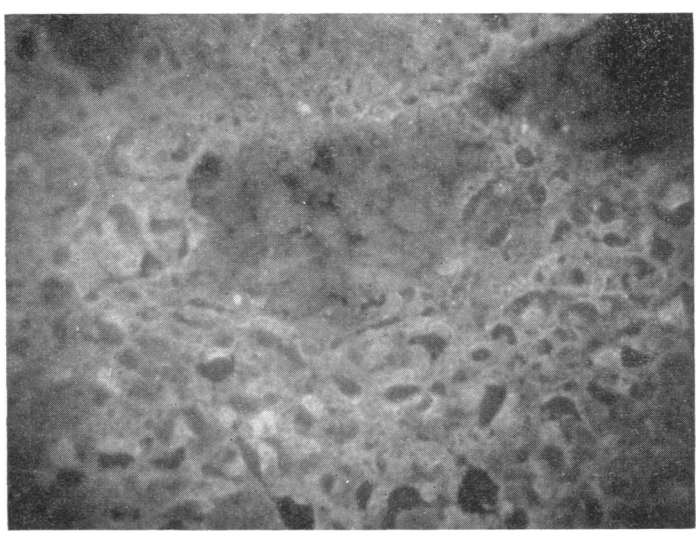

FIG. 1. Specific staining of zona reticularis of homologous rabbit adrenal but not of adrenal medulla by serum 9/10 obtained after two injections of guinea-pig adrenal in complete adjuvant. × 192.

cortex was obtained with antisera from rabbits immunized with homologous or heterologous adrenal. As shown in Fig. 1, the adrenal medulla did not give specific staining. Interstitial cells of rabbit ovary (Fig. 2) gave specific fluorescence with serum from rabbits immunized with rabbit or guinea-pig adrenal but not rat adrenal. Leydig cells and developing sperm (Fig. 3) as well as mature sperm in the epididymis gave specific staining with serum from rabbits immunized with homologous adrenal, but only Leydig cells were

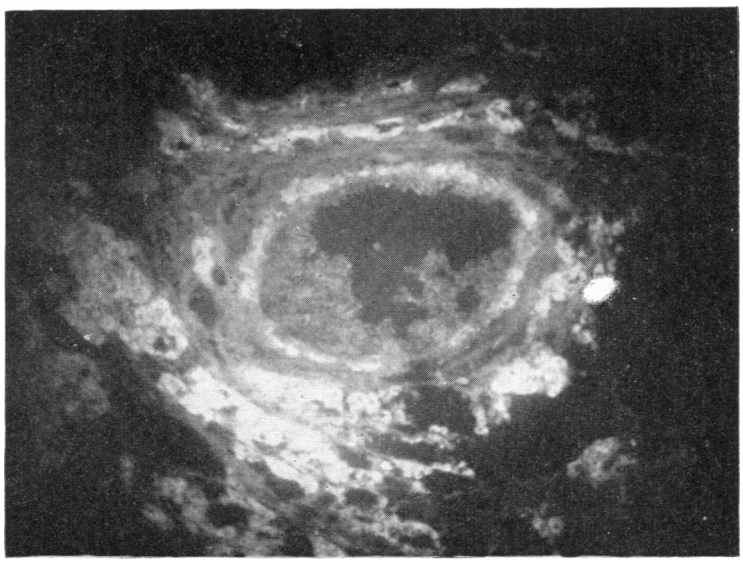

FIG. 2. Specific staining of interstitial cells of autologous rabbit ovary by serum 11/5 obtained after one injection of guinea-pig adrenal in complete adjuvant. × 80.

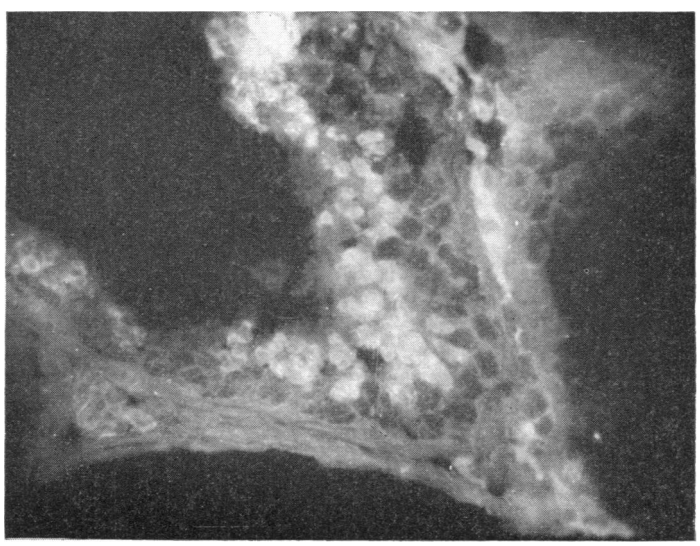

FIG. 3. Specific staining of homologous Leydig cells and spermatids with serum from rabbit No. 9/4 after two injections of homologous rabbit adrenal in complete adjuvant. × 192.

stained (Fig. 4) with serum from rabbits immunized with guinea-pig adrenal. All sera shown in Table 6 failed to give positive staining with homologous or autologous liver, kidney, spleen or brain. There was no specific staining of any sections by 1 : 4 dilutions of preimmunization sera from these rabbits. Absorption with sheep red cells did not affect

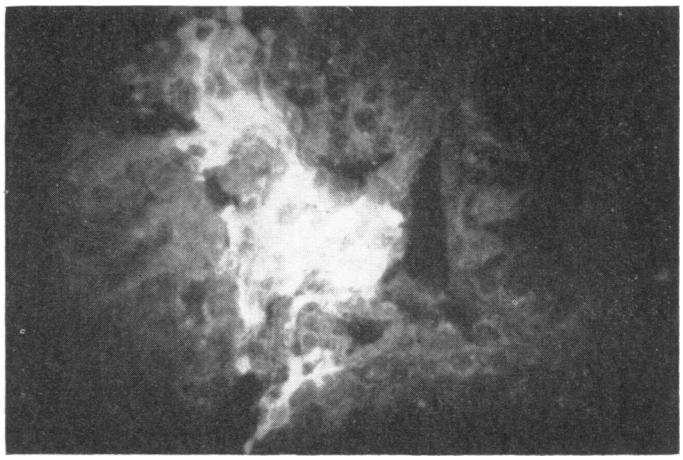

FIG. 4. Specific staining of homologous Leydig cells but not spermatids with serum from rabbit No. 9/10 after two injections of guinea-pig adrenal in complete adjuvant. × 192.

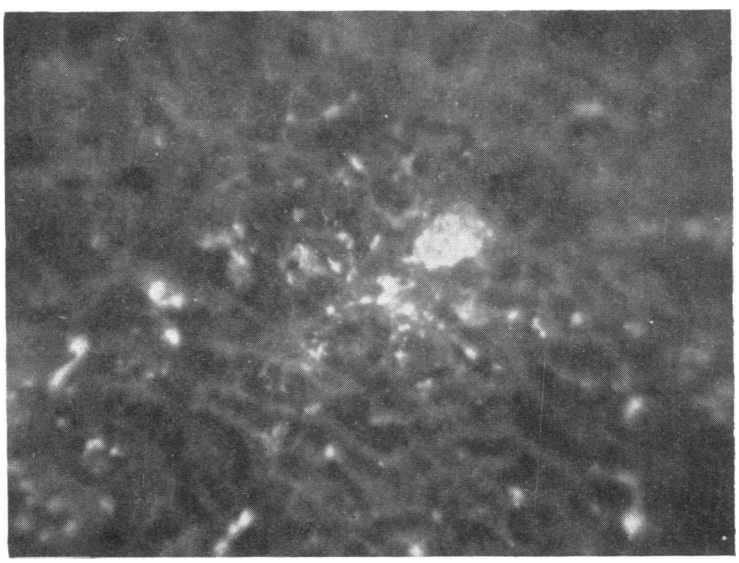

FIG. 5. Zona fasciculata of rabbit adrenal No. 11/5 in area of cellular infiltrate shown in Fig. 8. × 192.

the specific staining obtained with positive sera—but sera absorbed with guinea-pig adrenal, rabbit adrenal or ovary, failed, to stain with any rabbit-tissue sections.

Fig. 5 demonstrates bound γ globulin detected by direct Coons' technique in an area of cellular infiltration of adrenal gland from a rabbit immunized with heterologous adrenal.

Skin Tests

Seven of the twenty-five rabbits were skin tested with dilutions of homologous and heterologous adrenal homogenates. The results were inconstant and the number of rabbits too small, but none of the adrenal-immunized animals had greater delayed reactivity to rabbit adrenal than did the adjuvant controls.

TABLE 7

HISTOPATHOLOGY OF RABBIT ADRENAL GLANDS

Rabbit No.	*Species of immunizing adrenal antigen*		*Day sacrificed*	*65° auto-antibody titre*	*Pathology*
	First injection	*Second injection*			
9/1	Rabbit	—	150	2	A
9/2	Rabbit	—	150	2	A
9/3	Rabbit	Rabbit	84	32	A
9/4	Rabbit	Rabbit	(14 days after second injection)	8	None
11/6	Rabbit	—	28	16	A
11/7	Rabbit	—	28	8	A
11/8	Rabbit	Rabbit	84	32	None
11/9	Rabbit	Rabbit	84	16	None
9/9	Guinea-pig	—	14	16	C
9/10	Guinea-pig	Guinea-pig	84	256	None
9/11	Guinea-pig	Guinea-pig	84	256	None
9/12	Guinea-pig	—	150	8	A, B, C
11/1	Guinea-pig	Rabbit	150	32	A
11/2	Guinea-pig	Rabbit	150	64	A, C,
11/3	Guinea-pig	—	28	32	None
11/4	Guinea-pig	—	28	32	A
11/5	Guinea-pig	—	28	64	B
9/13	Rat	Rat	84	16	B, C
9/14	Rat	Rat	84	4	C
9/15	Rat	Rat	150	4	C
9/16	Rat	—	150	8	A
9/5	Complete adjuvant	Complete adjuvant	84	4	None
9/6	Complete adjuvant	Complete adjuvant	84	2	None
9/7	Complete adjuvant	Complete adjuvant	150	4	None
9/8	Complete adjuvant	Complete adjuvant	150	2	A

A—? lesion
B—Polymorphonuclear cells predominate in infiltrates.
C—Lymphocytes and plasma cells.

Histopathology

The histopathology of rabbit adrenals following immunization with homologous or heterologous adrenal homogenate or complete adjuvant alone is shown in Table 7. Loss of cell outline in the zona fasciculata and clustering of nuclei (lesion A, Fig. 6) was found in one of four adjuvant-immunized rabbits and eight of twenty-one rabbits immunized with adrenal homogenate and is most probably an artifact of fixation. Lesion B consisted of many large and small foci of polymorphonuclear cells, lymphocytes and plasma cells with the polymorphonuclear cells predominating. These were found in all layers of the

adrenal cortex (Fig. 7). Lesion C consisted almost exclusively of plasma cells and lymphocytes (Figs. 8 and 9) scattered throughout the adrenal cortex and the margins of the medulla. The adrenal cortical cells in the areas of such infiltrates were frequently abnormal with loss of nuclear definition and ballooning and poor staining of cytoplasm. Cellular

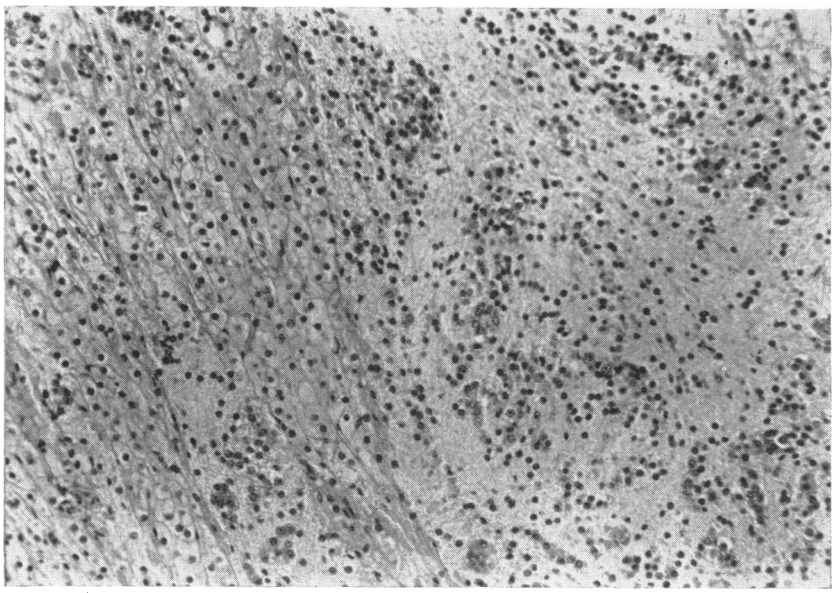

FIG. 6. Zona fasciculata or rabbit adrenal No. 9/3. Haematoxylin and eosin. × 130.

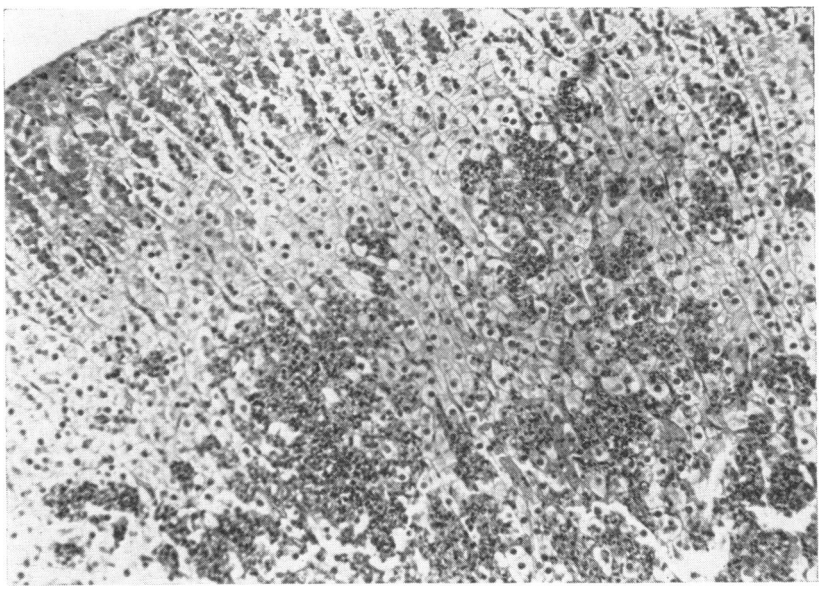

FIG. 7. Zona fasciculata and reticularis of rabbit adrenal No. 9/9 14 days after one injection of guinea-pig adrenal in complete adjuvant. Haematoxylin and eosin. × 130.

infiltrates, B and C lesions, were found in seven of thirteen rabbits immunized with foreign adrenal but in none of twelve immunized with homologous adrenal or complete adjuvant alone ($P < 0{\cdot}01$). Other rabbit organs showed only those changes described following immunization with Freund's adjuvant alone (Steiner, Langer and Schatz, 1960) and there were no differences noted between the experimental and control animals.

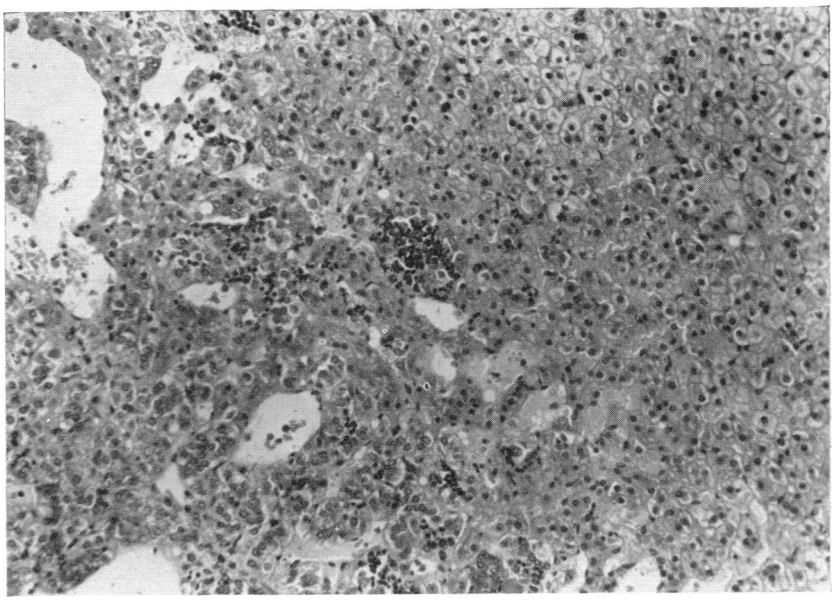

FIG. 8. Zona fasciculata of rabbit adrenal No. 11/5 28 days after one injection of guinea-pig adrenal in complete adjuvant. Haematoxylin and eosin. × 130.

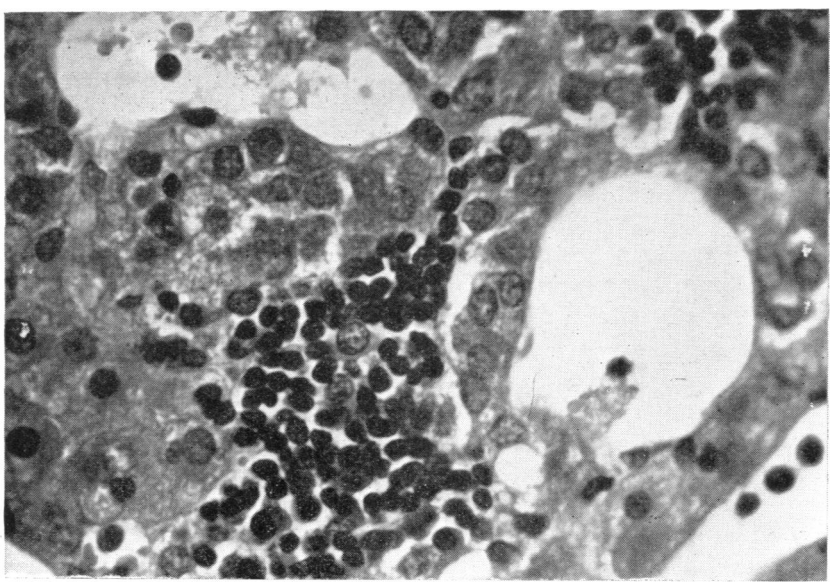

FIG. 9. As in Fig. 8. × 595.

Sera from guinea-pigs immunized with a single injection of homologous adrenal in complete adjuvant was anti-complementary to a titre of 8. There was no change in titre following a single injection of rat adrenal or complete adjuvant alone, while a single injection of rabbit adrenal in complete adjuvant gave a rise to a mean titre of anti-adrenal antibody of 12 with the mean titre of anti-complementary activity of 4.

Four injections of guinea-pig adrenal or other guinea-pig organ homogenates resulted in anti-complement activity in high titre. This anti-complementary effect was reduced by heating at 65° but the titre of serum giving 2+ complement fixation in the presence of antigen never exceeded the titre of anti-complementary activity.

A study of the distribution of autologous tissue antigens capable of reacting with antibody induced by immunization with rabbit adrenal is shown in Table 8. Antigen was

TABLE 8

ANTIGEN TITRE OF ORGAN HOMOGENATES GIVING 2+ COMPLEMENT FIXATION WITH SERUM OF GUINEA-PIG 12/9 AFTER ONE INJECTION OF RABBIT ADRENAL IN COMPLETE ADJUVANT

Test antigen	*Serum* 1 : 8	
	56°	65°
Autologous adrenal	5120	2480
Autologous testes	640	320
Autologous liver	640	320
Autologous spleen	1280	<20
Autologous kidney	160	<20
Autologous brain	80	<20
Rabbit adrenal	5120	1280
Rat adrenal	5120	320

detected by heat-stable antibody in autologous and homologous adrenal, testis and liver as well as rabbit and rat adrenal. Heat-labile antibody reacted with all autologous antigens tested.

No precipitins against guinea-pig adrenal were found in any of the sera tested. No antibody was detected by immunofluorescence in any of the sera tested using guinea-pig adrenal as substrate.

Skin Tests

The diameter of the skin test obtained with guinea-pig adrenal homogenates was no greater in guinea-pigs immunized with adrenal than in control animals immunized with adjuvant alone (Table 9). Guinea-pigs immunized with heterologous adrenal had specific reactivity greatest with the heterologous adrenal. Skin-test evaluations were difficult because of non-specific irritative response to tissue homogenate.

Histopathology

Only guinea-pigs immunized with homologous or heterologous adrenal in complete adjuvant were found to have cellular infiltrations of the adrenal (Table 10). These were composed almost entirely of round cells as previously described (Colover and Glynn, 1958; Steiner *et al.*, 1960), save in one animal immunized with four injections of homologous adrenal where there was haemorrhage and polymorphonuclear infiltration. Gram's stain of

TABLE 9

DELAYED SKIN TESTS IN GUINEA-PIGS IMMUNIZED WITH HOMOLOGOUS OR HETEROLOGOUS ADRENAL HOMOGENATES WITH COMPLETE ADJUVANT OR COMPLETE ADJUVANT ALONE

Test antigen homogenates	*Amount (mg.)*	*Guinea-pigs immunized with*															
		Guinea-pig adrenal				*Rat adrenal*				*Rabbit adrenal*				*Adjuvant alone*			
		1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Guinea-pig adrenal	10·0	8	5	9	10	8	8	7	7	—	—	2	3	10	11	9	6
	1·0	7	6	5	4	5	3	4	2	2	—	—	—	6	5	5	6
	0·1	2	4	2	2	—	—	—	—	—	—	—	—	1	2	0	2
Rabbit adrenal	10·0	9	8	9	9	14	9	8	9	25	25	18	16	10	9	12	10
	1·0	6	5	6	6	8	6	8	7	10N	11N	11N	9	7	6	4	6
	0·1	2	2	1	2	6	4	4	5	9	8	6	3	2	1	2	2
Rat adrenal	10·0					20N	18N	20N	22N	7	6	10	6	9	9	7	9
	1·0					8	10	11	10	6	4	5	4	4	5	5	4
	0·1					6	6	7	6	—	—	—	—	2	1	2	2
Heterologous testes	10·0					—	14	11	12	4	—	3	—				
	1·0					—	—	—	—	—	—	—	—				
	0·1					—	—	—	—	—	—	—	—				
Heterologous spleen	10·0					12	11	10	14	10	10	8	10				
	1·0					4	9	10	9	8	8	5	7				
	0·1					3	2	2	2	2	2	0	2				

Fourteen days after primary immunization, nine skin sites were tested with 0·1 ml. volumes containing 10, 1 or 0·1 mg. of organ homogenates and diameter of the areas of induration measured at 24 hours.
N = Necrosis.

TABLE 10

CELLULAR INFILTRATION OF GUINEA-PIG ADRENALS FOLLOWING IMMUNIZATION WITH ADRENAL HOMOGENATES IN COMPLETE ADJUVANT OR WITH CONTROL ANTIGENS

Immunizing antigen	*Dose (mg.)*	*Day sacrificed*	*No. with pathology/ Total No. immunized*
Guinea-pig-adrenal	250 × 1	21	6/8
	60 × 4	64	9/12
Rabbit adrenal	250 × 1	21	4/4
		35	1/4
Rat adrenal	250 × 1	21	4/6
		35	0/2
Guinea-pig liver	60 × 4	64	0/3
Guinea-pig kidney	60 × 4	64	0/3
Guinea-pig testes	60 × 4	64	0/3
Adjuvant alone	× 1	14	0/8
	× 4	64	0/6

these sections, as in the case of rabbit adrenals, disclosed no bacteria. The size and confluence of the infiltrates was not as great as previously described and this may be due to the difference in dosage schedule and the absence of human tubercle bacilli in the adjuvant.

Guinea-pigs immunized with rabbit or rat adrenal frequently had infiltrates in the zona glomerulosa (Fig. 10) as well as zona fasciculata and reticularis, but not the medulla.

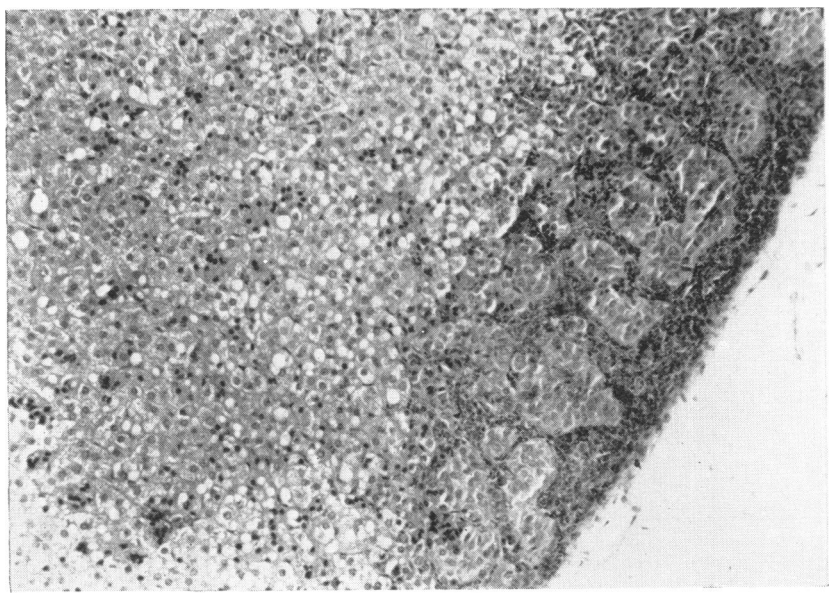

FIG. 10. Zona glomerulosa and fasciculata of guinea-pig adrenal 22/4 14 days after one injection of rat adrenal in complete adjuvant. Haematoxylin and eosin. × 140.

The infiltrates in guinea-pigs receiving homologous adrenal immunization were most prominent in the zona reticularis, as previously described. Two guinea-pigs immunized with rat adrenal had testicular atrophy, as did all three immunized with guinea-pig testis (Freund, Lipton and Thompson, 1953). The other organs showed no specific pathology.

DISCUSSION

ANTIGEN

The production of antibody reactive with homologous or autologous adrenal after immunization with homologous or heterologous adrenal homogenate in complete adjuvant would suggest the presence of an organ-specific antigen in the adrenal of rabbits, guinea-pigs and rats. This is supported by the results of antigen dilution and block titrations with serum of rabbits immunized with homologous adrenal where cross-reacting antigen in approximately equal titre was found in the adrenals of all three species. The titre of cross-reacting complement-fixing antigen was high in rabbit ovary but low in rabbit testis, liver, kidney and brain. Absorption of the complement-fixing and the fluorescent antibody directed against rabbit adrenal and ovary by the sediments of these organs as well as by sediment of guinea-pig adrenal also support this hypothesis.

The injection of rabbit adrenal into two rabbits previously sensitized with guinea-pig adrenal did not result in a booster response to either antigen. As pointed out by Dixon and Maurer (1955), the heterologous protein antigens must be closely related to demonstrate such an *in vivo* antigenic cross-reactivity. A negative finding in only two experimental animals is not conclusive but this finding does suggest that the adrenal antigens of these two species may be only distantly related, i.e. have only a few determinants or haptens in common.

The antigens responsible for complement fixation are relatively heat labile at 56° for 30 minutes. The antigens detected by complement fixation and immunofluorescence may not be identical. The contrast of the low complement-fixing titre of the cross-reacting antigen in testis with the marked immunofluorescence obtained with Leydig cells and sperm suggest that at least these two tests deal with different antigens. There is an immunofluorescent reaction between both Leydig cells and sperm with a serum from rabbits after immunization with homologous adrenal while serum from two rabbits immunized with guinea-pig adrenal reacts only with Leydig cells. This would suggest that in the rabbit there is a common antigen in sperm and adrenal cortex not present in guinea-pig adrenal. Yet other antigens present in rabbit adrenal cortex, ovarian interstitial cells and Leydig cells are present in guinea-pig adrenal. The situation is further complicated by the observation that immunization of guinea-pigs with their homologous testes results in antibody detected by immunofluorescent technique against sperm only (Brown, Glynn and Holborow, 1963). This suggests that there are at least two antigens in sperm, only one of which is present in adrenal cortex of the same species.

Witebsky and Milgrom (1962), on immunization of guinea-pigs with homologous adrenal, elicited a complement-fixing antibody which was directed against guinea-pig adrenal, but not other species of adrenal with only some cross-reaction with guinea-pig liver. In our hands sera from guinea-pigs immunized repeatedly with homologous adrenal were anti-complementary. Sera heated at 65° from guinea-pigs immunized with rabbit adrenal detected cross-reacting antigen in autologous guinea-pig adrenal, testis, and liver as well as in rabbit and rat adrenal. If common antigens are present in mammalian adrenals as detected by cross-reacting antisera raised in rabbits, similar cross-reacting antibody would be expected on immunization of guinea-pigs. The poorer antibody response in guinea-pigs than in rabbits may explain these minor contradictions.

ANTIBODY

With serum heated at 56° for 30 minutes there was complement fixation to high titre promptly following immunization with adrenal, a measurable titre prior to immunization, and some rise in titre in rabbits immunized with adjuvant alone. Sera heated at only 56° gave more cross-reactions with rabbit-organ antigens, i.e. brain, kidney, liver, than did sera heated at 65°. This was particularly noticeable with high-titre sera obtained after two injections of foreign adrenal. Heating at 56° for 30 minutes does not inactivate the natural antibody of Kidd and Friedewalde (1942), and tests done with serum heated at this temperature reflect the titre of Kidd and Friedewalde natural antibody plus whatever heat-stable antibody is present. Immunization with organ antigens—especially if from a foreign species—caused a rise in titre of this natural antibody.

A classical time course of heat-stable antibody production followed immunization with adrenal-organ homogenates. There was a modest rise following primary injection and an

accelerated, greater rise after the second injection. This antibody may be termed an autoantibody because of its equal reactivity with autologous and homologous adrenal, and indeed Witebsky and Milgrom (1962) were able to detect such antibody in hemi-adrenalectomized rabbits after immunization with autologous adrenal. This autoantibody was reactive with foetal adrenal and foetal ova-testis. The presence of these antigens in foetal life would suggest that the animal should be tolerant and fail to make an immunologic response. Alternatively, immunization with a foreign-organ antigen in Freund's adjuvant may provide a greater adjuvant effect for weak tissue antigens to which the animals were never tolerant. However, certain aspects of the serologic response to adrenal in rabbits suggests that they are indeed tolerant to adrenal. Smith (1961) was able only rarely to break tolerance to serum proteins by immunization with the homologous protein in complete adjuvant while Weigle (1961) was able to demonstrate the breaking of tolerance to serum albumin by immunization with distantly related, heterologous albumin in complete adjuvant. With liver microsomes (Asherson and Dumonde, 1962) and with uvea (Wacker and Dodd, 1961), kidney (Heyman, Kmetec and Cuppage, 1962) or heart (Gery and Davies, 1961; Kaplan and Meyerserian, 1962), autoantibody is more effectively elicited by immunization with heterologous-organ preparations in adjuvant than with homologous organs. Experimental allergic encephalomyelitis was first produced by injections of heterologous brain while immunization with homologous or heterologous brain in Freund's adjuvant produces disease (Kabat, Wolf and Bezer, 1947). However, with thyroid (Witebsky and Rose, 1959) and testis (Freund *et al.*, 1953), immunization with homologous or autologous antigen is optimal or essential for eliciting autoimmune disease. The greater efficacy of immunization with heterologous-organ antigen in the induction of autoimmunity in some systems but not in others is at present unexplained. Thyroid colloid which is not widely accessible to the immunologic mechanism and sperm which develops late may not confer tolerance upon the host. Those organs, i.e. adrenal, brain, uvea, kidney, heart and liver, with an abundant circulation are more accessible to the immunologically competent cells and would, therefore, be expected to confer tolerance.

Others (Broberger and Perlman, 1959; Asherson and Dumonde, 1962) have suggested that the presence of common haptens on heterologous-organ antigens could explain the induction of autoimmunity following immunization with foreign antigen. Indeed, Gell and Benacerraf (1961) have shown that antibody to haptens is elicited more effectively when the hapten is conjugated to a heterologous serum protein rather than to a homologous protein. Alternatively, the determinants need not be regarded as haptenic, but as an integral part of the antigen, since Weigle's (1961) experiments suggest that tolerance to a complex protein, e.g. BSA, can be broken, at least as far as some of its determinants are concerned, by another protein with these determinants in common.

SKIN TESTS

There was no evidence of delayed hypersensitivity to homologous adrenal in the skin of rabbits or guinea-pigs. Delayed hypersensitivity directed against the heterologous adrenal used for immunization was demonstrated in the guinea-pig.

The degree of delayed hypersensitivity to homologous antigens has been correlated with the presence of autoimmune disease, with brain (Waksman and Morrison, 1951), peripheral nerve (Waksman and Adams, 1955) and thyroid (Miesher, Gorstein, Benacerraf and Gell, 1961; McMaster, Lerner and Euxim, 1961). The absence of such cutaneous

hypersensitivity with adrenal, uvea (Wacker and Dodd, 1961) and testis (Freund, Thompson and Lipton, 1955), however, does not exclude cellular mechanisms of the tuberculin type in these disorders.

The use for skin testing and immunization of adrenal homogenates pooled from many animals precludes interpretations regarding isoantigenicity of adrenal. The use of pure antigenic preparations obtained from individual animals for skin testing might provide such information.

HISTOPATHOLOGY

Immunization of rabbits with heterologous adrenal resulted in an adrenalitis with predominantly polymorphonuclear cells in some glands and round cells in others. In these experiments as well as in others (Steiner *et al.*, 1960; Witebsky and Milgrom, 1962) immunization of rabbits with homologous adrenal in complete adjuvant did not result in adrenalitis.

The histopathologic picture of adrenalitis in those rabbits immunized with heterologous adrenal resembles adrenal cortical necrosis in man with or without haemorrhage (Tedeschi and Peabody, 1962). Antibodies against human adrenal cortex have been detected by complement fixation (Anderson, 1957), and by Coons' technique in cases of Addison's disease (Blizzard, Chandler, Kyle and Hung, 1962; Mead, 1962). Adrenal injury of whatever etiology may result in denatured (foreign) adrenal antigens capable of eliciting antibody. Such antibody may contribute to further damage of adrenal cortical cells.

The histopathologic picture of adrenalitis of guinea-pigs, where nearly all lesions are composed of round cells, has been compared with the rare human disease, cytotoxic contraction of the adrenal (references cited by Steiner *et al.*, 1960). Immunization with homologous, as well as heterologous adrenal homogenate in adjuvant resulted in adrenalitis. Guinea-pigs immunized with heterologous adrenal had more extensive infiltrates and these were more often located in the peripheral portions of the adrenal cortex. Since none of these guinea-pigs had delayed cutaneous hypersensitivity to homologous adrenal it is difficult to suggest delayed hypersensitivity as the significant mechanism although the round-cell infiltration has prompted others to this view (Waksman, 1959). The low titre of circulating antibody and the absence of a positive correlation between titre and disease in either rabbits or guinea-pigs do not suggest a humoral mechanism. Yet, as a group, animals receiving heterologous adrenal immunization had a higher titre and more evidence of disease.

The titre of antibody only measures that amount not bound to antigen and does not reflect the clinical situation even in serum sickness (Dixon, Vazquez, Weigle and Cochrane, 1958), where all evidence supports a humoral mechanism. The detection of γ globulin in the lesions of rabbit adrenals in sections washed to remove non-specifically absorbed proteins, suggests that an antigen–antibody reaction occurred at the site of these lesions.

In autoimmune adrenalitis as well as the other experimental autoimmune diseases there has been no evidence presented to exclude a role for circulating antibody or delayed-type hypersensitivity. Both mechanisms may play a role in autoimmunity and/or there may be other immune mechanisms as yet undefined.

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