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THE FURTHER CHARACTERIZATION OF AUTOANTIBODIES REACTIVE WITH EXTRA-ADRENAL STEROID-PRODUCING CELLS IN PATIENTS WITH ADRENAL DISORDERS

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SUMMARY

Antibodies reactive with the steroid-producing cells in the gonads are described in the sera of ten patients, the majority of whom were known to have idiopathic adrenal insufficiency (Addison's disease) associated with premature ovarian failure. The immunofluorescent staining pattern of these antibodies with steroid-producing cells in the ovary, testis, placenta and adrenal cortex are illustrated. The staining patterns and the results of absorption studies indicate that there are a multiplicity of antibodies reacting with different antigens in the ovary and to a lesser extent in the testis. Most of these antigens are also represented in the adrenal cortex, but are not evenly distributed throughout the cortex. Some of these antigens are not represented in the zona glomerulosa while others are not represented in the zona reticularis.

INTRODUCTION

Antibodies to steroid-producing cells in the gonads and placenta, cross-reacting with adrenal cortex, have been described in the sera of two patients by Anderson *et al.* (1968) and in the sera of six patients by Irvine *et al.* (1968). The latter observed a good correlation between the presence of these antibodies in the sera reactive with steroid-producing cells in the ovary and a clinical history of premature or complete failure of ovarian function. Five of the six patients had idiopathic adrenal insufficiency (Addison's disease) and one had Cushing's disease. The present paper describes in greater detail some of the characteristics of antibodies reactive with steroid-producing cells.

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MATERIALS AND METHODS

Ten sera (A–J) reacting with ovary, testis or placenta were included in the present study. Sera A–F have previously been referred to (Irvine *et al.*, 1968). Patients A–E had idiopathic Addison's disease with premature or complete ovarian failure. Patient F had Cushing's disease. Patient G was a 72-year-old female with idiopathic Addison's disease, pernicious anaemia, diabetes mellitus and idiopathic hypothyroidism. Patient H was a 16-year-old male who developed idiopathic Addison's disease at the age of 15 years and who was not known to have any associated endocrinopathy although his pubic hair was noted to be sparse. Patient I was a female aged 51 years with idiopathic Addison's disease and was not known to have any other endocrinopathy. Patient J was a 20-year-old girl with primary hypoparathyroidism at 2 years, idiopathic Addison's disease at 19 years and who had normal menstruation (Irvine, 1969; Irvine & Scarth, 1969).

The incidence of antibodies reactive with steroid-producing cells of the gonads in the sera of patients with Addison's disease has been indicated previously and also the rarity of this group of antibodies in the sera of patients with other diseases characterized by organ-specific autoimmunity and in the sera of control subjects (Irvine *et al.*, 1968).

The procedure used in the indirect immunofluorescent antibody technique was as described by Irvine, Chan & Williamson (1969) using horse anti-human IgG–FITC conjugate with a fluorescein–protein ratio of 0·67. The human tissues used in the fluorescent antibody technique were obtained as follows: adrenal from patients undergoing bilateral adrenalectomy for metastatic carcinoma, testis from a 61-year-old male undergoing orchidectomy for prostatic carcinoma, biopsy specimens from the ovary of a 42-year-old woman undergoing surgery for uterine fibroids and from the corpus luteum of a 32-year-old woman at termination of pregnancy at 12–14 weeks. The human placenta was obtained at a normal full term delivery. Ovaries were obtained from rabbits in oestrus and in psuedopregnancy. Rabbit testis was also used. All sera were also tested against rat kidney sections as controls to exclude the presence of 'M' antibodies (Doniach *et al.*, 1966).

Absorption studies on selected sera were done with veronal buffer extracts of human adrenal, human testis, rabbit ovary and human placenta and with serum E using extracts of human thyrotoxic thyroid tissue, mucosa of the body of human stomach and rat liver. Extracts of these tissues were prepared by homogenizing the tissue with 3 volumes of veronal buffer, centrifuging at 1750 *g* for 10 min. Absorption was done by incubating the supernatant with 1 volume of serum with continuous mixing for $3\frac{1}{2}$ hr at room temperature, followed by repeat centrifugation.

RESULTS

The results with sera A–J using adrenal, ovary, testis and placenta in the indirect immunofluorescent antibody test are summarized in Table 1.

Ovary

In the rabbit ovary it was noted that certain sera gave immunofluorescent staining of the theca interna and also of the outer layer of granulosa cells of the larger Graafian follicles (Figs. 1, 2 and 3). Whether or not the outer layer of the granulosa cells was stained as well as the theca cells seemed to depend upon the stage of development of the Graafian follicles. This aspect was not studied in detail. Staining of the granulosa and theca cells of human

TABLE 1. Results in the indirect immunofluorescent antibody technique with sera A–J tested against the various steroid-producing tissues

Patient	Sex	Age	Human ovary						Rabbit ovary			Testis				Placenta	Adrenal cortex		
			Corpus luteum				Follicular phase		Granulosa cells/ theca	Hyperplastic interstitial cells	Ova	Human		Rabbit		Human	Human		
			Clumpy	Confluent	Patchy	Theca	Granulosa cells/ theca	Ova				Interstitial	Spermatids	Interstitial	Spermatids		Glomerulosa	Fasciculata	Reticularis
A	F	17	++	neg	neg	++	++	++	++	++	++	++	+	++	++	++	+	++	+
C	F	35	++	neg	neg	++	+	neg	+	neg	neg	+	neg	+	neg	+	+	+	+
J	F	20	++	neg	neg	++	nt	++	++	++	+	++	nt	++	++	++	++	++	++
B	F	20	neg	neg	+	++	++	neg	++	neg	neg	+	neg	+	neg	+	neg	++	++
E	F	43	neg	neg	+	++	++	neg	++	neg	neg	++	neg	++	neg	neg	neg	++	++
H	M	16	neg	neg	++	++	nt	neg	++	neg	neg	++	neg	++	neg	neg	neg	++	++
D	F	34	neg	+	neg	++	+	neg	neg	neg	neg	neg	neg	neg	neg	neg	++	++	++
F	F	43	neg	++	neg	neg	+	neg	neg	neg	neg	neg		neg	neg	neg	++	++	++
G	F	72	neg	++	neg	neg	nt	+	++	++	neg	neg	neg	+	neg	++	++	++	neg
I	F	51	neg	neg	neg	++	nt	neg	neg	neg	neg	neg	nt	neg	neg	neg	neg	+	+

nt = Not tested.

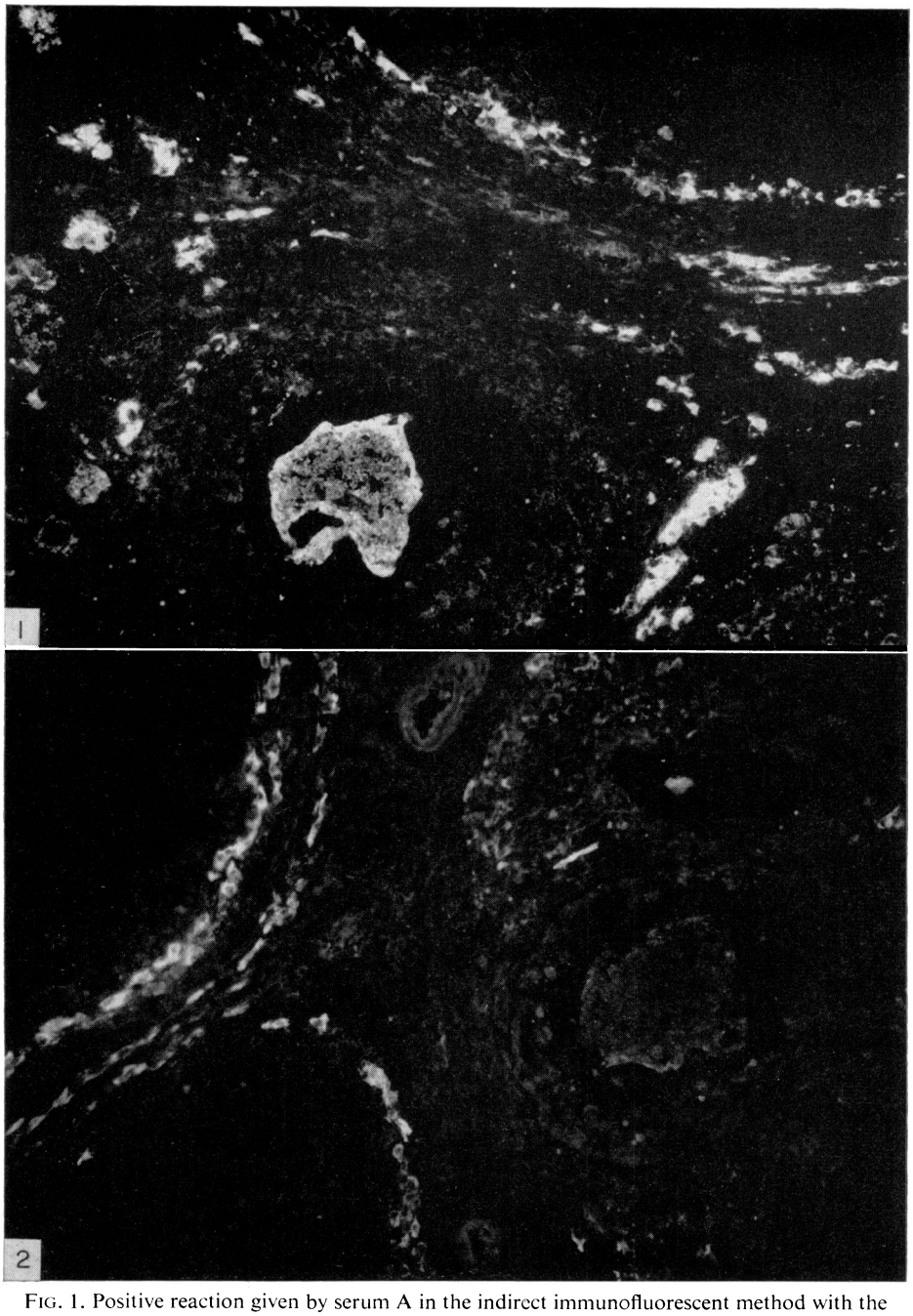
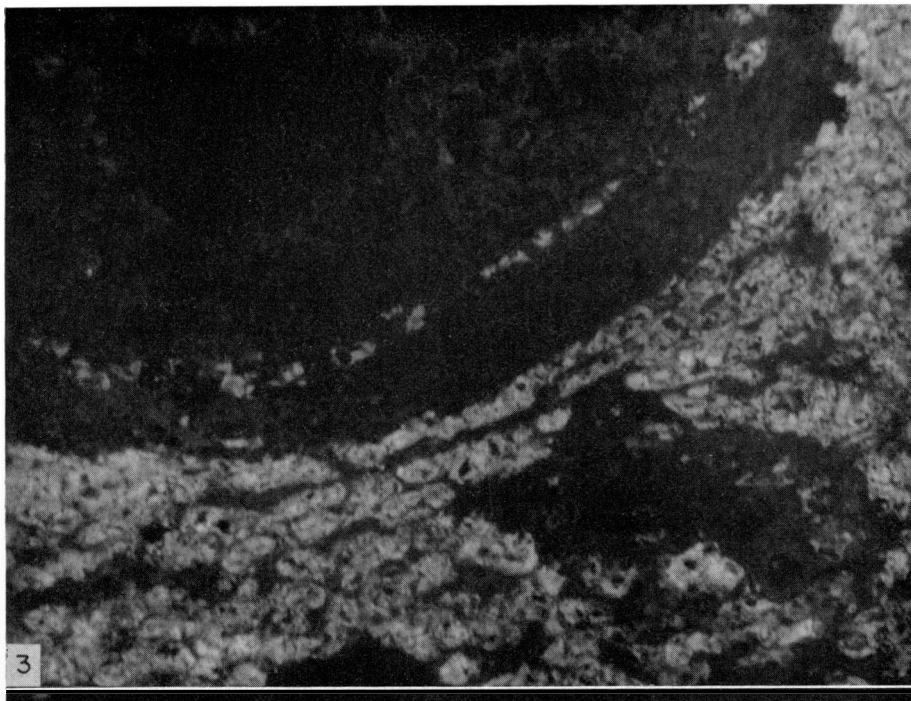


FIG. 1. Positive reaction given by serum A in the indirect immunofluorescent method with the outer layer of granulosa cells and with the theca cells of a fairly large Graafian follicle, with scattered interstitial cells, and with ovum. There was little or no reaction with the granulosa cells of the small follicle containing the ovum. Dark ground UV–blue light, × 225.

FIG. 2. Positive immunofluorescence with a layer of granulosa cells and a layer of theca cells in relation to a fairly large Graafian follicle in rabbit ovary using serum E, but negative reaction interstitial cells and with ovum. Dark ground UV–blue light, × 230.



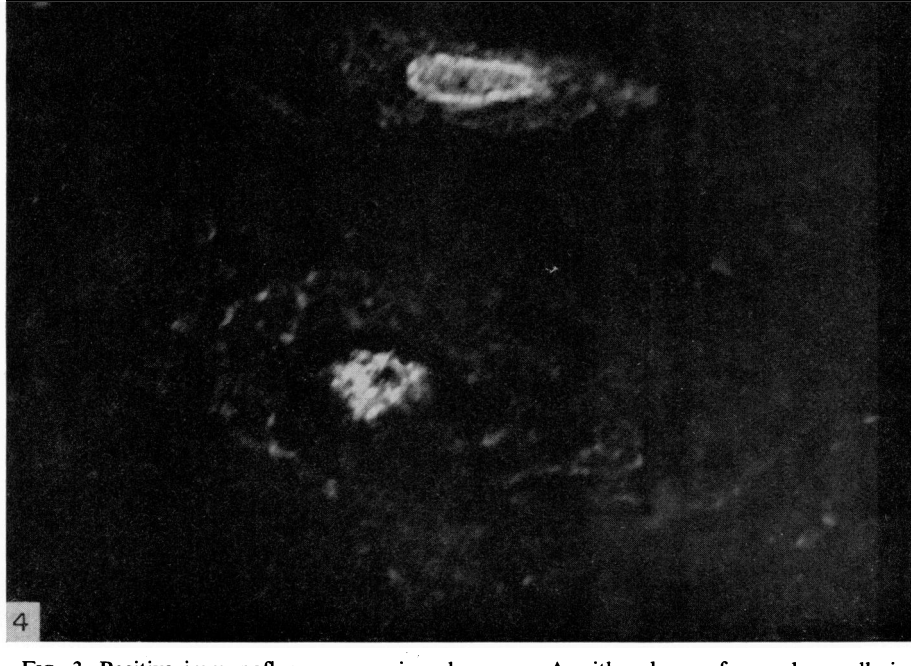


FIG. 3. Positive immunofluorescence given by serum A with a layer of granulosa cells in relation to a fairly large Graafian follicle, and with the hyperplastic interstitial cells characteristic of the rabbit ovary in pregnancy or pseudopregnancy. Dark ground UV–blue light, × 280.

FIG. 4. Positive immunofluorescence with ova and weakly with the granulosa cells of the primordial or early follicles in the ovary of patient E using the serum of patient A in the indirect fluorescent antibody technique. Dark ground UV–blue light, × 360.

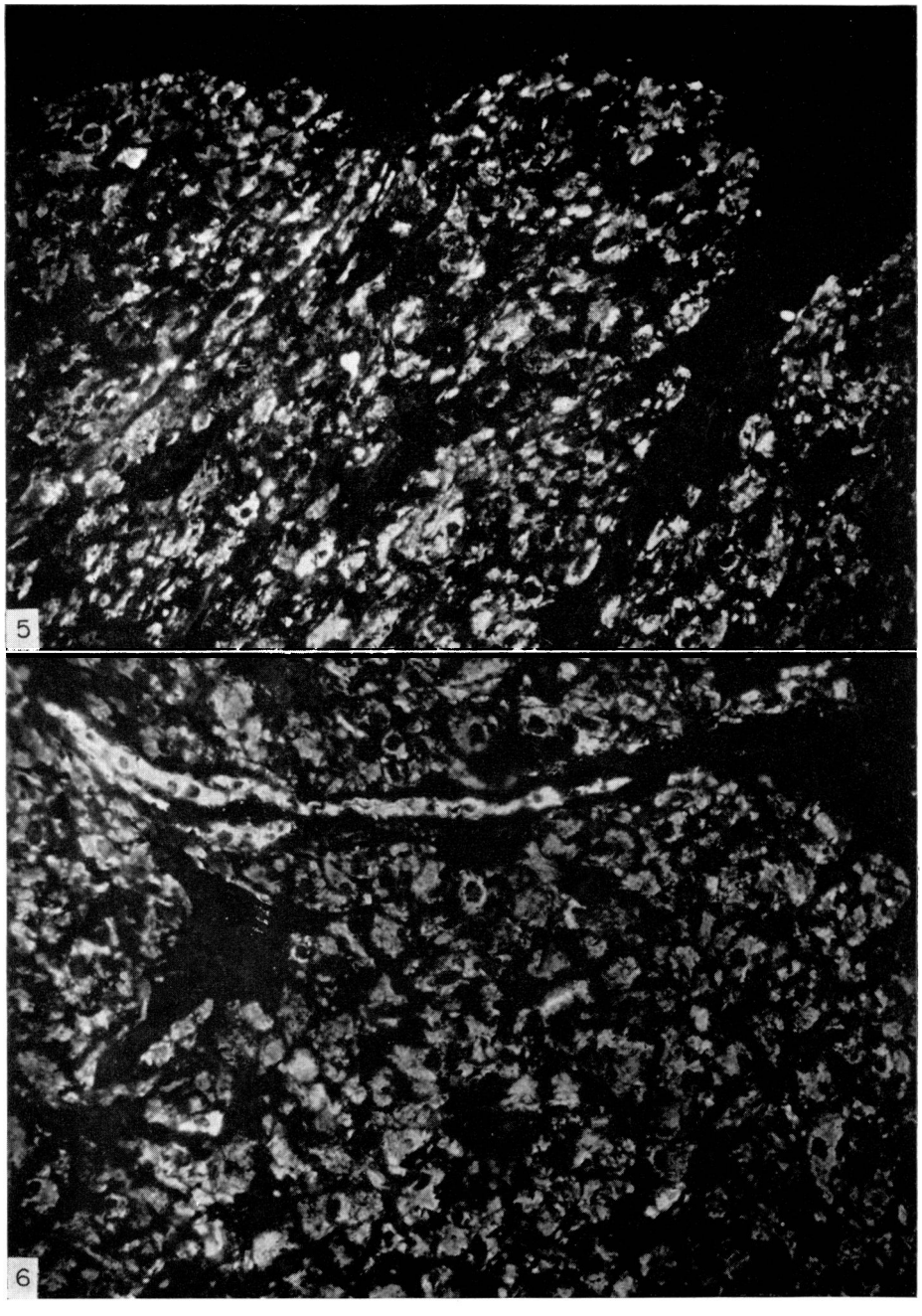
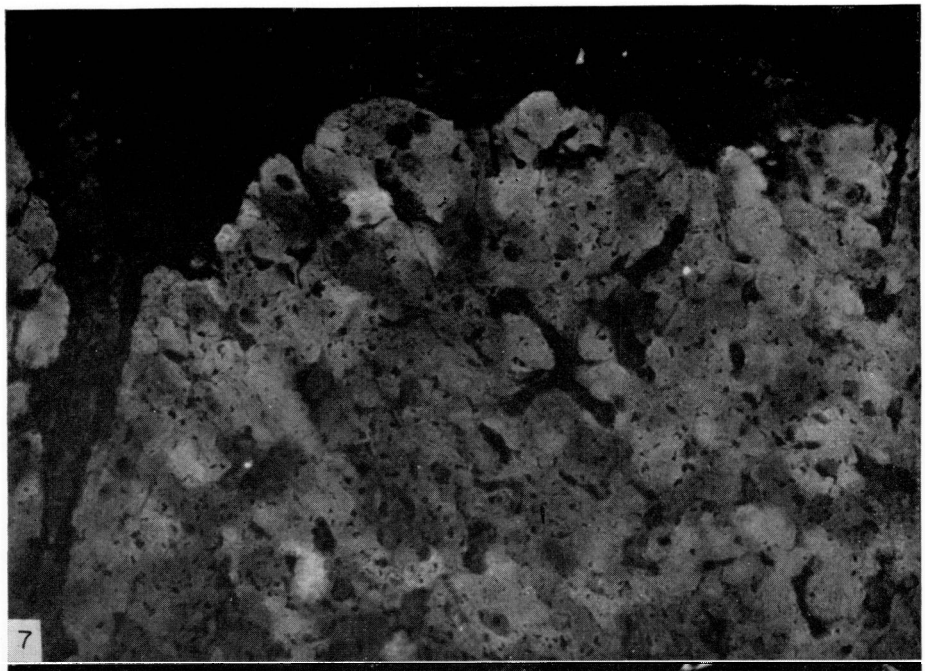


FIG. 5. Positive immunofluorescence staining of human corpus luteum given by serum A showing a clumpy pattern diffusely throughout the corpus luteum. Dark ground UV–blue light, × 225.

FIG. 6. Same as Fig. 5, but showing in addition brilliant immunofluorescent staining of the theca cells in the trabeculae of the human corpus luteum. Dark ground UV–blue light, × 250.



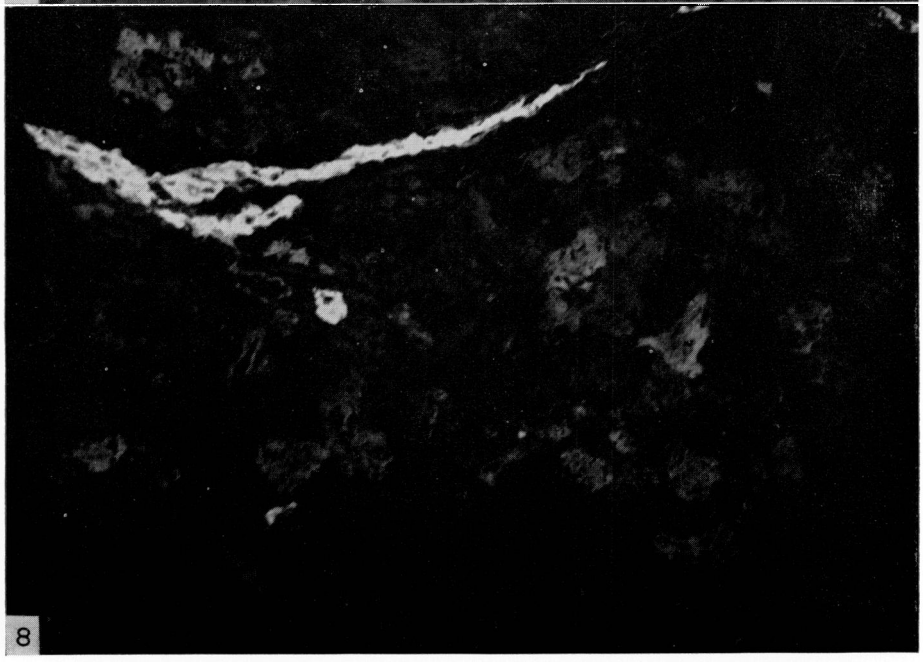


FIG. 7. Positive immunofluorescent staining of human corpus luteum given by serum F showing a confluent 'squamous-like' pattern throughout the corpus luteum. Dark ground UV–blue light, × 240.

FIG. 8. Patchy immunofluorescent staining pattern of human corpus luteum and intense immunofluorescent staining of the theca cells in the trabeculae of the corpus luteum given by serum E. Dark ground UV–blue light, × 240.

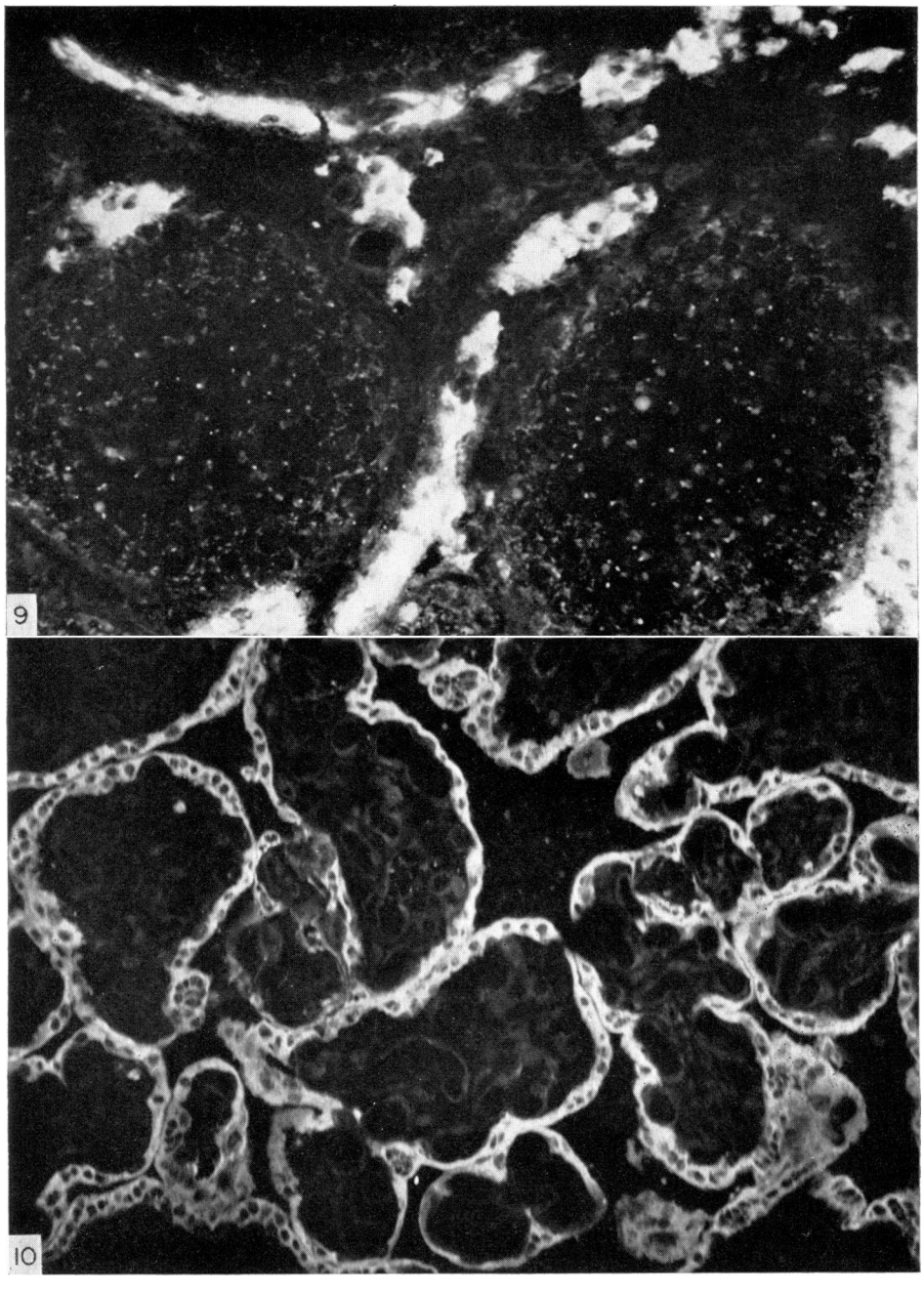


FIG. 9. Positive staining in the indirect immunofluorescent antibody technique of the interstitial cells and of the spermatids in rabbit testis given by serum A. Dark ground UV–blue light, × 230.

FIG. 10. Positive staining of human placental trophoblasts in the indirect immunofluorescent antibody technique given by serum E. Dark ground UV–blue light, × 240.

Graafian follicles has been illustrated elsewhere (Irvine *et al.*, 1968). Some sera reacted with the interstitial cells of the rabbit ovary in the oestrus phase (Fig. 1). Marked hyperplasia of the interstitium is characteristic of the ovary of the pregnant or pseudopregnant rabbit. Sera A, G and J gave immunofluorescent staining of the hyperpastic interstitial cells while sera A, B, E, H and J reacted with a layer of granulosa cells in the same sections (Fig. 3). Interstitial cells were not conspicuous in the human ovarian tissue that was available. Fig. 4 shows that serum A, for example, also reacted with human ova. The tissue used in Fig. 4 was an ovarian biopsy from patient E (Irvine *et al.*, 1968; Irvine & Hartog, 1969).

Four clearly distinct immunofluorescent staining patterns were observed when human ovary in the luteal stage was used. These may be described as:

(1) A clumpy staining pattern diffusely throughout the corpus luteum (Figs. 5 and 6).
(2) A confluent squamous-like staining throughout the corpus luteum (Fig. 7).
(3) A patchy staining of the corpus luteum involving scattered cells with varying intensity (Fig. 8).
(4) An intense staining of a rim of cells at the periphery of the corpus luteum and in its trabeculae (Figs. 6 and 8).

The cells mentioned under reaction (4) did not form a continuous rim round the corpus luteum and the ovarian sections had to be carefully searched for their presence. It was thought that these cells represented the theca interna.

The results in Table 1 have been presented so as to group together the immunofluorescent staining patterns noted in relation to the human corpus luteum and its surrounding cells.

Testis

Seven of the ten sera showed a positive immunofluorescent reaction with the interstitial cells of testis. Six of these reacted with testis of human or rabbit origin, while one (serum G) reacted only with rabbit testis. An illustration of this type of immunofluorescent reaction is included in Irvine *et al.* (1968). A further example is shown in Fig. 9. Fig. 9 also shows positive immunofluorescence given by serum A with the spermatids of rabbit testis. Serum J also reacted with rabbit spermatids and with the interstitial cells of human or rabbit testis in the same manner as serum A.

Placenta

Fig. 10 illustrates a positive reaction with human placental trophoblasts. Such a reaction was observed with five of the ten sera.

Adrenal

All ten sera reacted positively by immunofluorescence with human adrenal cortex. Sera A, C and I showed only a weak but definite reaction, while sera B, D, E, F, G, H and J reacted strongly. Five of the ten sera stained all three layers of the cortex. Sera B, E, H and I stained only the lower part of the zona fasciculata and the whole of the zona reticularis (Fig. 11); they did not stain the zona glomerulosa or the upper part of the zona fasciculata. Serum G stained only the zona glomerulosa and fasciculata, but not the zona reticularis (Fig. 12). This difference in staining pattern was quite distinct and was confirmed by using contiguous sections of the same human adrenal gland for testing the different sera. Consistent immunofluorescent staining patterns were also obtained using sections from the adrenals of other patients.

The results of complement fixation tests with adrenal placenta and testis have been described previously (Irvine *et al.*, 1968).

Absportion studies

The results of absorption studies with buffered saline extracts of human adrenal and of human testis on four of the sera with steroid-cell antibodies reactive with extra-adrenal tissue (as well as with adrenal cortex) and on one serum (B.1493) from a patient with idiopathic

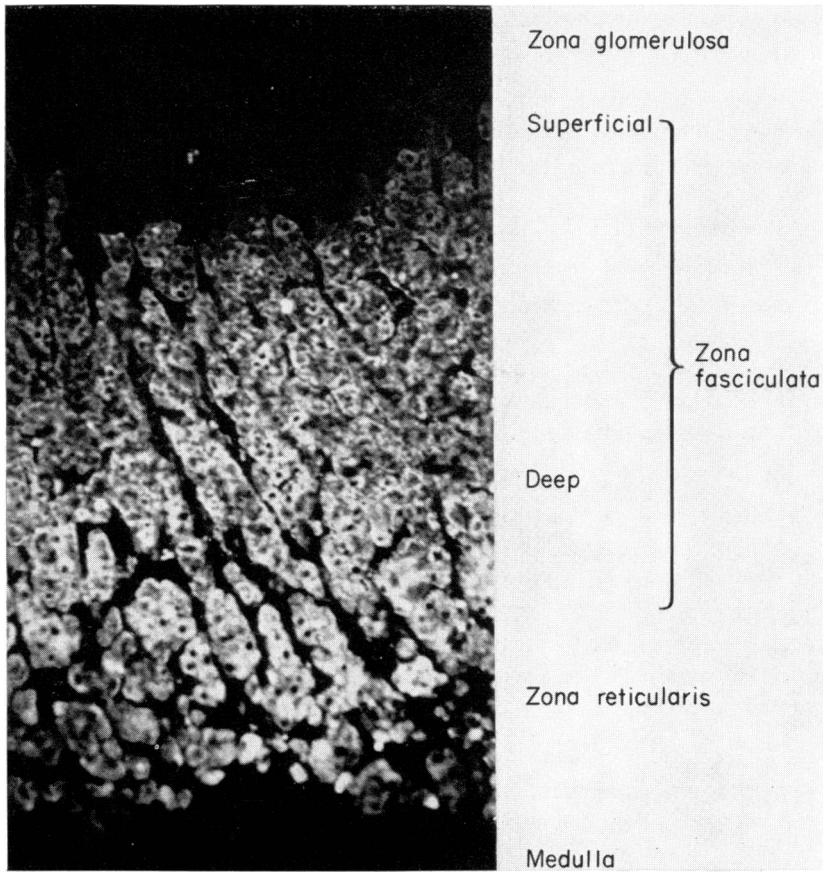


FIG. 11. Positive immunofluorescent staining of the human adrenal cortex confined to the lower part of the zona fasciculata and to the zona reticularis, but not involving the zona glomerulosa or the more superficial part of the zona fasciculata. Indirect immunofluorescent antibody method with serum E. Dark ground UV–blue light, × 220.

adrenocortical insufficiency whose serum reacted only with adrenal cortex are shown in Table 2. With sera from patients A, B, D and E, adrenal extract absorbed out or significantly reduced the titre of antibodies reactive with human adrenal cortex, with the interstitial cells of human or rabbit testis, with the granulosa or theca cells of the follicular phase of the human ovary and of the rabbit ovary in oestrus, and with human placental trophoblasts.

Human testis extract was similarly effective in absorbing antibodies reactive with these various tissues, indicating that there was a sharing of antigens between human adrenal, human testis and the other tissues studied. Extracts similarly prepared from human thyrotoxic thyroid tissue, from the mucosa of the body of human stomach and from rat liver had no significant effect on the immunofluorescent antibody titres shown by serum E with adrenal cortex, testis, ovary or placenta. Likewise, human testis extract had no effect on the

FIG. 12 Positive immunofluorescent staining of the zona fasciculata, but negative reaction with the zona reticularis of the human adrenal cortex given by serum G in the indirect immunofluorescent antibody technique. The clumpy staining of the zona reticularis is due to non-specific autofluorescence from lipid granules which fluoresce a reddish-brown as opposed to the specific green fluorescence in the zona fasciculata. Dark ground UV–blue light, × 260.

immunofluorescent titre of serum B.1493 with adrenal cortex, indicating that the adrenal antibody or antibodies in this patient with idiopathic Addison's disease were distinct in their specificity from at least some of these antibodies reactive with adrenal cortex (and other steroid-producing tissues) in sera A, B, D and E.

The results of absorption studies on the various immunofluorescent reactions with human

TABLE 2. Results of absorption studies using extracts of human adrenal and of the human testis as well as control studies using extracts of thyroid, gastric mucosa and liver

Test tissue	Serum	Immunofluorescent titres					
		Before absorption	After absorption with extracts of:				
			Adrenal (human)	Testis (human)	Thyroid (human)	Gastric mucosa (human)	Liver (rat)
Adrenal cortex (human)	A	8	neg	neg			
	B	4	neg	neg			
	D	8	2	2			
	E	32	8	8	32	32	32
	B.1493	16	4	16			
Testis (human) interstitial cells	A	8	neg	neg			
	B	4	neg	neg			
	D	4	neg	neg			
	E	32	4	8	32	32	32
	B.1493	neg	neg	neg			
Testis (rabbit) interstitial cells	A	8	neg	2			
	B	4	neg	neg			
	D	4	2	neg			
	E	32	4	8	32	32	32
	B.1493	neg	neg	neg			
Ovary (human follicular theca)	A	8	neg	2			
	B	8	neg	neg			
	D	8	2	2			
	E	128	16	32	128	128	128
	B.1493	neg	neg	neg			
Ovary (rabbit oestrus theca)	A	8	neg	2			
	B	2	neg	neg			
	D	2	neg	neg			
	E	32	8	16	32	32	32
	B.1493	neg	neg	neg			
Placenta (human)	A	4	neg	2			
	B	2	neg	neg			
	D	neg	neg	neg			
	E	neg	neg	neg	neg	neg	neg
	B.1493	neg	neg	neg			

ovary in the luteal phase are summarized in Tables 3 and 4. While the diffuse clumpy staining of the corpus luteum given by sera A and C could be absorbed by either human adrenal or by human testis extracts, only adrenal was effective in absorbing the antibodies that gave the patchy staining (serum E) or confluent staining (serum F) of the corpus luteum (Table 3). Human adrenal extract absorbed the antibodies in sera A, C, D and E reactive with the theca cells at the periphery and in the traberculae of the corpus luteum of

TABLE 3. Absorption studies on sera reactive by immunofluorescence with human corpus luteum

Patient	Pattern of immunofluorescence staining of corpus luteum	Reactivity absorbed by:	
		Human adrenal	Human testis
A	Clumpy: diffuse	Yes	Yes
C	Clumpy: diffuse	Yes	Yes
E	Patchy	Yes	No
F	Confluent	Yes	No

TABLE 4. Absorption studies on sera reactive by immunofluorescence with theca interna cells at the periphery of the corpus luteum in human ovary

Patient	Reactivity absorbed by:	
	Human adrenal	Human testis
A	Yes	Yes
C	Yes	Yes
D	Yes	No
E	Yes	No

human ovary but absorption with human testis extract was only effective in preventing this reaction given by sera A and C and was ineffective with regard to sera D and E (Table 4). These findings confirm that there must be a variety of steroid cell antigens only some of which are shared between the human testis and the ovary in the luteal phase, but that they may be all represented in one or more zones of the adrenal cortex. These findings also indicate that the theca cells surrounding the corpus luteum in human ovary contain more than one antigen.

DISCUSSION

This paper establishes that there must be a complex of IgG antibodies reactive with adrenal cortex and with steroid-producing cells in the ovary, testis and placenta. An attempt has been made to characterize these different antibodies by their different immunofluorescent

staining properties. It has been demonstrated that antibodies reacting with the corpus luteum of human ovary cross-react with antigens in the adrenal cortex. It is evident that at least some of the cross-reacting antigens may not be evenly distributed between the three layers of the adrenal cortex. The antigens in the corpus luteum, responsible for the patchy immunofluorescent staining pattern given by sera B, E and H, are not present in the zona glomerulosa or upper part of the zona fasciculata while the antigens responsible for the confluent immunofluorescent staining pattern would appear not to be present in the zona reticularis. That the theca interna cells of the luteal phase of human ovary contain more than one antigen may be inferred from the observation that IgG antibodies in the sera of four patient's reactive with this cell type could be absorbed by adrenal extract while an extract of human testis was only effective in absorbing this antibody or antibodies from the sera of two of these patients.

The analysis of the data in Table 1 is complicated by the fact that antibodies specific for components of the adrenal cortex, and not cross-reacting with other steroid-producing cells, are known to occur in some 50% of the sera of patients with idiopathic Addison's disease (Irvine *et al.*, 1968). Such antibodies have so far invariably reacted with all three layers of the adrenal cortex. These adrenal-specific antibodies are likely to be present in a proportion of the ten sera in the present study.

Although the number of sera available for study that reacted with steroid-producing cells in the gonads was small, there is a strong indication of three patterns of reactivity. Firstly, there is the pattern of immunofluorescent staining of all three layers of the adrenal cortex, diffuse clumpy staining of the human corpus luteum, positive reaction with theca interna cells surrounding the corpus luteum, reactivity with a layer of granulosa or theca cells in the rabbit Graafian follicles and a positive reaction with human placental trophoblasts. This contrasts with the second pattern which is characterized by patchy immunofluorescent staining of the human corpus luteum, positive reaction with theca cells surrounding the corpus luteum, positive reaction with granulosa or theca cells of the rabbit Graafian follicles and a reaction only with the deeper portion of the zona fasciculata and with the zona reticularis in the human adrenal cortex. The third pattern indicates that antigen at least in part responsible for the confluent staining pattern of human corpus luteum is not represented in the interstitial cells of the testis, or in the placental trophoblasts or in the zona reticularis of the human adrenal cortex.

From experience so far, it would appear that human corpus luteum is the most suitable tissue to use when screening sera for antibodies to steroid-producing cells in tissues other than adrenal. All ten sera in the present study were positive either in relation to the corpus luteum itself or the surrounding theca cells while only a proportion of the sera were positive with placenta, testis or ovary in the follicular phase. Human corpus luteum is simpler to obtain at termination of pregnancy than is human ovary in the follicular phase.

The remarkable correlation between the presence of gonadal antibodies and a clinical history of premature gonadal failure would suggest that immunological factors may have some pathogenic role in this condition. To date, with one exception (patient J), in female subjects it is only patients who have ovarian failure associated with adrenal autoimmunity that have been shown to have antibodies reactive with the steroid-producing cells of the gonads. The histological appearance of the ovaries on biopsy in patient E (Irvine *et al.*, 1968; Irvine & Hartog, 1969) was characterized by lymphocytic and plasma cell infiltration in the developing Graafian follicles. This would strongly suggest an autoimmune pathogenesis.

By using frozen sections from this patient's ovarian biopsy, it was shown that her gonadal antibodies were truly autoimmune. As in other autoimmune diseases, the mediator of immunological damage may be lymphocytes, as suggested by the adoptive transfer experiments of McMaster & Lerner (1967) in relation to experimental chronic thyroiditis and of Levine & Wenk (1968) in relation to experimental chronic adrenalitis. Nerup, Andersen & Bendixen (1969) have demonstrated using the leucocyte migration test the existence of a state of organ-specific, anti-adrenal hypersensitivity of the cellular type in nine out of fifteen patients with idiopathic Addison's disease. Alternatively, there may be synergism between circulating antibody and lymphocyte-mediated antibody.

The present findings provide the first demonstration that an associated pathology (namely premature gonadal failure) in relation to what has generally been considered to be an organ-specific autoimmune disorder (namely idiopathic Addison's disease) may be due to cross-reacting antibodies.

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