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Histoimmunochemical Localization of Three Guinea-pig Spermatozoal Autoantigens

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Summary. By the indirect immunofluorescence technique three distinct guinea-pig spermatozoal autoantigens named S, P and T have been localized. Autoantigen S is present on proacrosomal granules, acrosomal granules, acrosome and head cap of spermatids and spermatozoa. Autoantigen P is present on the same formations except the head cap. Autoantigen T is also localized on acrosomes and head caps but probably on their membranes as well as on the cytoplasmic membranes of spermatozoa and spermatids.

These three autoantigens are testis specific since they were not found in other organs in guinea-pigs; moreover S was detected on the acrosomal apparatus in rabbits, rats and mice.

The natural antispermatozoal antibodies found in guinea-pig serum do not combine with autoantigens S and P, but the possibility that they could react with autoantigen T is considered.

The cytological localization of S, P and T together with their previously described physicochemical and immunological properties allow a tentative explanation of their *in vitro* and *in vivo* immunopathological properties.

INTRODUCTION

It has been repeatedly demonstrated that guinea-pig autoimmune or alloimmune antibodies induced by guinea-pig testis or spermatozoal homogenate or extracts can bind to spermatozoal acrosomes and to seminiferous tubule central cells (Baum, 1959; Baum, Boughton, Mongar and Schild, 1961; Brown, Glynn and Holborow, 1963; Hekman and Shulman, 1971; Mancini, Davidson, Vilar, Nemirovsky and Bueno, 1962; Toullet, Voisin and Nemirovsky, 1970). Similar findings were reported in the mouse (Barth and Russell, 1964; Pokorna, Vojtiškova, Rychliková and Chutna, 1963), in the rat (Mancini *et al.*, 1964) and in man (Mancini, Andrada, Saraceni, Bachmann, Lavieri and Nemirovsky, 1965).

At least four different autoantigens have been identified in guinea-pig spermatozoa. Three of them have been studied for their physicochemical and immunological properties which have been demonstrated to be strikingly different. The three of them, however, when injected in Freund's complete adjuvant into guinea-pigs induce lesions of autoimmune aspermatogenic orchitis (AIAO) indistinguishable from each other when completed (Voisin and Toullet, 1968, 1969; Toullet and Voisin, 1969; Toullet *et al.*, 1970). Are these three autoantigens localized in spermatozoal acrosomes? When do they first appear during

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spermatogenesis? The indirect immunofluorescence technique was chosen in order to seek an answer to these two questions. Auto- or alloimmune guinea-pig sera specifically directed against each one of the three spermatozoal autoantigens were utilized followed by fluorescent immunoglobulin from rabbit anti-guinea-pig immunoglobulin sera. Preliminary results of this work have been previously presented (Toullet *et al.*, 1970).

MATERIAL AND METHODS

1. *The three spermatozoal autoantigens*

Their preparation and properties have already been described (Voisin and Toullet, 1968, 1969; Toullet and Voisin, 1969). The procedures of extraction and purification utilized in this study are summarized in Table 1 and the main physicochemical and immunological properties of these antigens are summarized in Table 2.

The quantification of the three antigens was made by reference to the intensity of a specific serological activity of the extracts as compared with standard reference preparations (whole spermatozoa or whole spermatozoa crude extract) when tested against standard reference sera. The serological tests utilized are haemagglutination inhibition for S; Mancini's immunodiffusion technique for P and complement fixation for T. The quantity of autoantigen present in a given preparation is therefore expressed as equivalent (≎) to the number of spermatozoa contained in the standard reference preparation giving the same serological reactivity.

2. *Preparation of guinea-pig immune sera against spermatozoal autoantigens*

Castrated male Hartley guinea-pigs were immunized with either their own spermatozoa or one of the three autoantigens (S, P or T) extracted from homologous spermatozoa. The first injection made in the hind foot-pads with complete adjuvant amounted to 10^8 spermatozoa or autoantigen equivalent. Three- to four-monthly injections i.d. or s.c., without adjuvants were made thereafter. Four types of immune sera were obtained:

(a) anti-autospermatozoa containing antibodies directed against several autoantigens (S, P, T and Z).

(b) anti-S (tested by passive haemagglutination).

(c) anti-P (tested by immune precipitation in gel).

(d) anti-T (tested by immune spermotoxicity).

3. *Preparation of fluorescent rabbit immunoglobulins against guinea-pig globulins*

These will be subsequently referred to as FRG. Guinea-pig serum globulin, precipitated five times with 40 per cent saturated $(NH_4)_2\,SO_4$, was used for immunization (two injections: the first one in foot-pads with complete adjuvant, the second one i.v. as a booster). Resulting sera were similarly precipitated and labelled with fluorescein isothiocyanate (Wood, Thompson and Goldstein, 1965). After the step of DEAE-cellulose chromatography, three pools of fluorescent globulins were formed, the mean fluorescein/protein ponderal ratios being respectively $3{\cdot}5 \times 10^{-3}$, 7×10^{-3} and 11×10^{-3}. The second pool was found the most satisfactory and was mainly used. It must however be mentioned that the third pool was found not to give interfering unspecific immunofluorescence. These three pools were active mainly against guinea-pig IgG (7S γ_1 and 7S γ_2) and slightly against an undetermined globulin of $\alpha 2$ electrophoretic mobility. In a few cases the fluorescent rabbit immune serum of the Pasteur Institute was utilized, after dilution.

TABLE 1

PREPARATION OF GUINEA-PIG SPERMATOZOAL AUTOANTIGENS S, P AND T

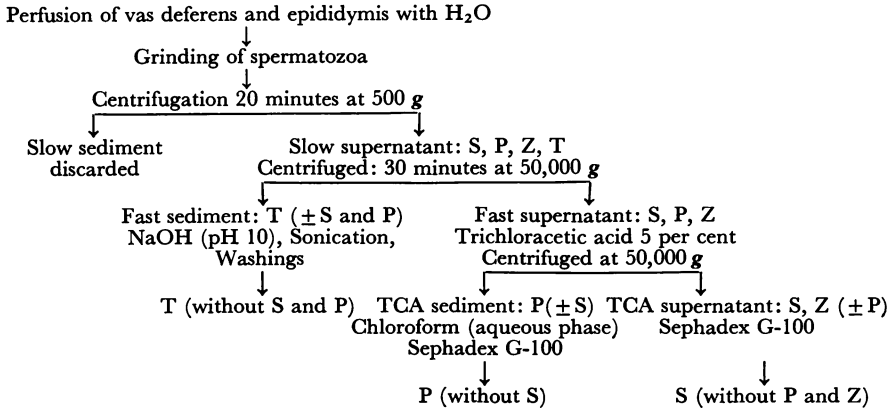

TABLE 2

MAIN PROPERTIES OF THREE GUINEA-PIG SPERMATOZOAL AUTOANTIGENS

Properties	Autoantigens		
	S	P	T
Physicochemical properties			
Solubility in water	+	+	−
Solubility in 5 per cent trichloracetic acid	+	−	Destroyed
Destruction by heating 56°	−	−	−
Destruction by heating 100°	−	−	−
Destruction by proteases	+	−	±
Destruction by lipase, ribonuclease	−	−	−
Destruction by periodate	+	−	+
Electrophoretic mobility	β_2	β_1	
Sephadex G-200–100 filtration	< 900,000	< 60,000	
properties: similar to proteins of mol. wt	> 160,000		
Content in proteins	+	++	+
Content in glucids	++	±	+
Immunological properties			
Cellular immunity (D.H.)	+	+	+
Serological manifestations			
passive cutaneous anaphylaxis	+	+	− ?
Passive haemagglutination (tannic acid)			
(and inhibition)	+	−	
Precipitation	−	+	
C fixation	−	+	+
Cytotoxicity	−	−	+
Spermotoxicity	−	−	+
Spermagglutination	−	−	+
AIAO induction (with complete adjuvants)	+	+	+

4. *Preparation of target cells*

Several types of preparation were studied by immunofluorescence: isolated spermatozoa, testicular cells and testis sections.

(a) *Spermatozoa.* These were collected from the epididymis (cauda) by retroperfusion through the vas deferens with PBS pH 7·4 and adjusted at 2×10^6 spermatozoa/ml. 0·75 ml of the suspension was dropped on a slide, allowed to sediment during 15 minutes at room temperature in moist atmosphere. The supernatant fluid was then removed and the slides dried in front of a fan 20–30 minutes.

(b) *Testis imprints.* These, made with testis sections, were utilized in most cases. They were air-dried with a fan for 30 minutes at room temperature.

(c) *Isolated cells from testis* were used in a few cases. Testis cells were dissociated in trypsinized (0·25 per cent) Parker 199, agitated 3 minutes at 37°, filtered, centrifuged 15 minutes at +4° at 100 *g*. Five millilitres of a suspension containing 10^6 cells/ml in Melnick–Hanks's medium were placed in a Leighton–Barski tube containing a cover slip. After 1 hour at 37° the cover slip was taken and flooded with specific serum immediately or after fixation.

(d) *Frozen sections* were also used in a few cases. After freezing in dry ice, sections of 4·5 μm were made, air dried 20–30 minutes (fan) and fixed.

(e) *Fixation procedures.* Ethanol at 95°, 2 minutes and formalin fumes at 2 per cent, 3 minutes were mostly utilized. Other procedures were: ethanol 95°, 10 minutes; methanol, 10 minutes; acetone, 2 or 10 minutes; trichloracetic acid (TCA) 1 per cent, 1 minute, then 2 per cent, 1 minute and 5 per cent, 2 minutes; formalin 2 per cent, 2 minutes.

5. *Immunofluorescent staining*

The indirect immunofluorescent method was used.

(a) *Specific antibody application.* The fixed preparations were incubated with the anti-spermatozoa specific sera usually diluted 1/5 (control sera pure or 1/2), for 25 minutes in moist atmosphere at 37° or at room temperature (no difference noticed). Two washings were made with PBS during 8 minutes (or three times 5 minutes).

(b) *Fluorescent immunoglobulin application.* Both incubation and washings were done in the same way as above described for specific immune sera.

(c) *Mounting.* After last washing, the slides were rapidly dipped into distilled water, dried, cleared in xylol and mounted with DPX (BDH or Gurr).

(d) *Fluorescent examination.* Was made on an Ortholux Leitz microscope adapted with a mercury super pressure lamp (HBO 200 W). Excitation filters have always been BG 38 plus BG 12 or UG 1. Barrier filter was K 490. Anscochrome was used throughout the study.

(e) *Histocytological preparations.* Orth's fixation and Hotchkiss's staining procedures were applied according to Leblond and Clermont (1952) on guinea-pig testis imprints and sections.

(f) *Control tests* included the following. 1. Absorption of guinea-pig immune sera with excess of spermatozoal autoantigen S, P or T or whole sperm preparations or ground testis for 1 hour at 37° plus overnight at +4°. 'Control absorptions' were made with the other spermatozoal antigens (S and P for anti-T immune sera and *vice versa*) as well as with spleen cells and kidney, liver and brain extracts. 2. Replacing specific guinea-pig immune sera by sera from an untreated guinea-pig or from a guinea-pig injected with complete adjuvant and saline or thyroid extracts. 3. Omitting guinea-pig serum. 4. Performing blocking tests in order to test the specificity of the FRG.

RESULTS

The experimental results bear mainly on the localization of the three guinea-pig spermatozoal autoantigens demonstrated by the fixation of specific guinea-pig anti-spermatozoal autoantigen-immune sera. Also studied were the organ and species specificity of these autoantigens *in situ* as well as the conceivable existence of 'natural' auto-antibodies against these (or other) spermatozoal autoantigens.

I. CYTOLOGICAL DATA

PAS staining of imprints and sections showed aspects similar to those described by Leblond and Clermont (1952). Golgi vesicles were pink in spermatocytes; more deeply coloured in spermatids were proacrosomal granules, acrosomal granules, head caps and acrosomes (Fig. 1). These aspects provide the cytological basis for the study of immuno-fluorescent data to be described.

II. LOCALIZATION OF 'S' AUTOANTIGEN (Fig. 2)

S autoantigen (soluble in water and TCA, sugar-rich β_2 glycoprotein, Sephadex G-100 excluded, giving rise to soluble immune complexes) was localized in spermatozoal acrosomes and head caps. The fluorescence was bright and the edges well defined but not especially sharp or highly regular (Fig. 2h). The same results were obtained with various preparations fixed with five different procedures. Spermatids observed at different stages of spermiogenesis (Golgi phase, cap phase, acrosome phase and maturation phase, according to Leblond and Clermont (1952), exhibited clear positive staining of proacrosomal granules (Fig. 2a), acrosomal granules (Fig. 2b), acrosomes and head caps (Fig. 2c, d, e and f) as soon as they could be distinguished by PAS stain. The structures that are stained (at various stages of their development) by anti-S immunofluorescence resemble closely and strikingly the ones that can be stained by PAS (Figs 1 and 2; see also Leblond and Clermont, 1952; Guraya, 1971).

In frozen sections of the testis, bright fluorescent crescent-like structures having the shape and dimensions of acrosomes were seen on the dark background. These were always at or close to the centre of the seminiferous tubules and never close to the tubular walls.

Procedures of fixation

Different procedures lead to minor variations: with formalin fumes, all the above described structures were stained, although the head caps were less intensely stained. With ethanol, acrosomal granules were never seen fluorescent and the fluorescence of acrosome granules and immature acrosomes was usually (but not always) limited to the periphery of the structure. Air drying, acetone and TCA, although found not satisfactory for the integrity of cellular structures, led to the same immunofluorescent localizations.

Active dilutions

With a good anti-S serum (titrating 1/1000 in passive haemagglutination), the immuno-fluorescent staining was already clearly less intense at 1/10 and became negative at 1/40 in our experimental conditions.

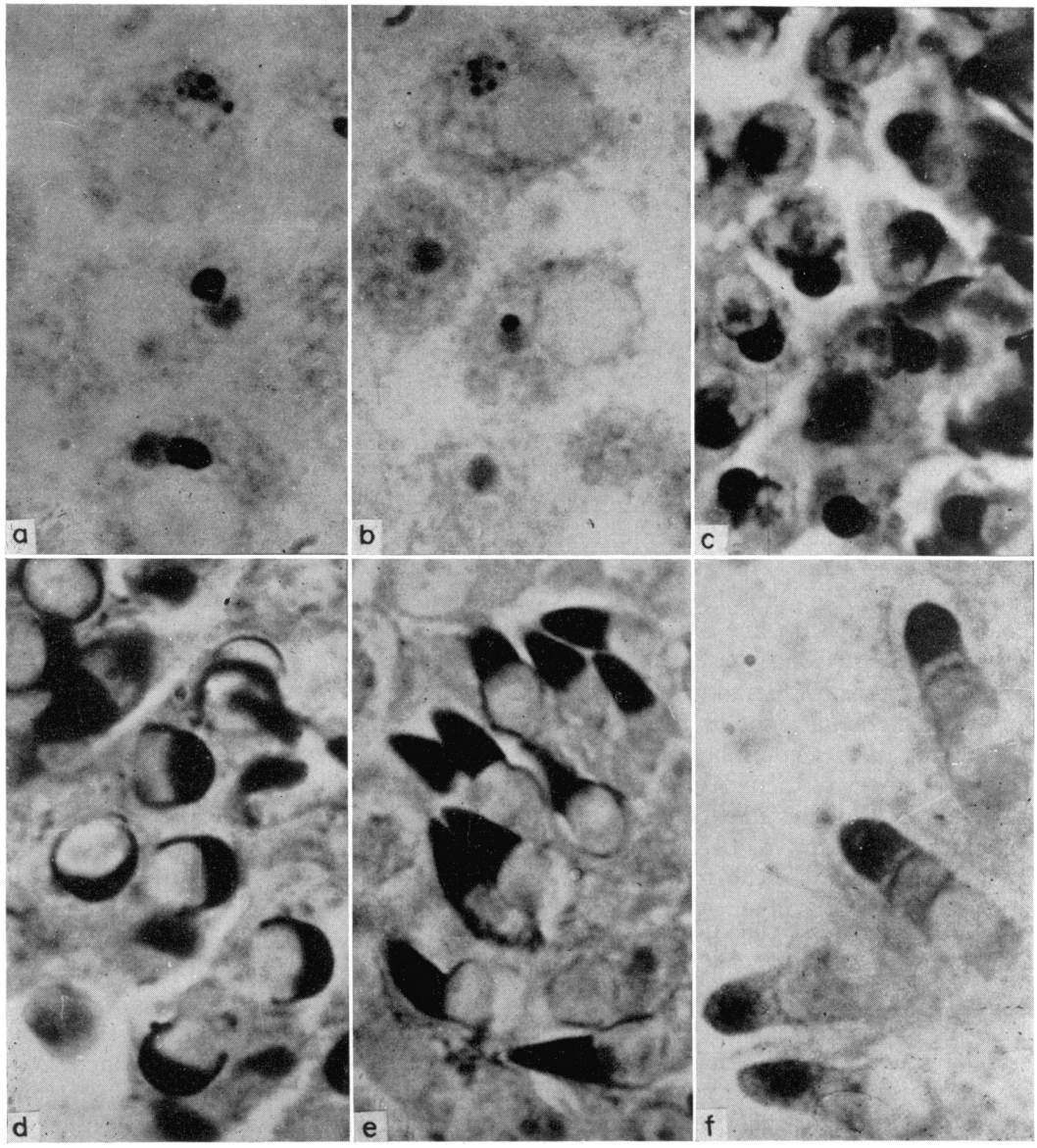

FIG. 1. Periodic acid–Schiff staining of imprints (a, b and f) and sections (c, d and e) of guinea-pig testis, showing the development of acrosomal apparatus at successive phases described by Leblond and Clermont (1952): proacrosomal granules (a and b, top), acrosomal granule (a and b, middle and bottom), acrosome and head cap during cap phase (c and d) and during acrosome phase (e and f). (Magnification ×600.)

Absorption of anti-S serum

Non-specific absorption procedures resulted in no decrease in the observed fluorescence. These were performed with excess of P or T autoantigens ($\simeq 10^8$–9×10^8 spermatozoa—as described in Materials and Methods—for 0·1 ml of serum). Actually Fig. 2b and c were obtained from a preparation where anti-S serum was absorbed with P and Fig. 2d, with T.

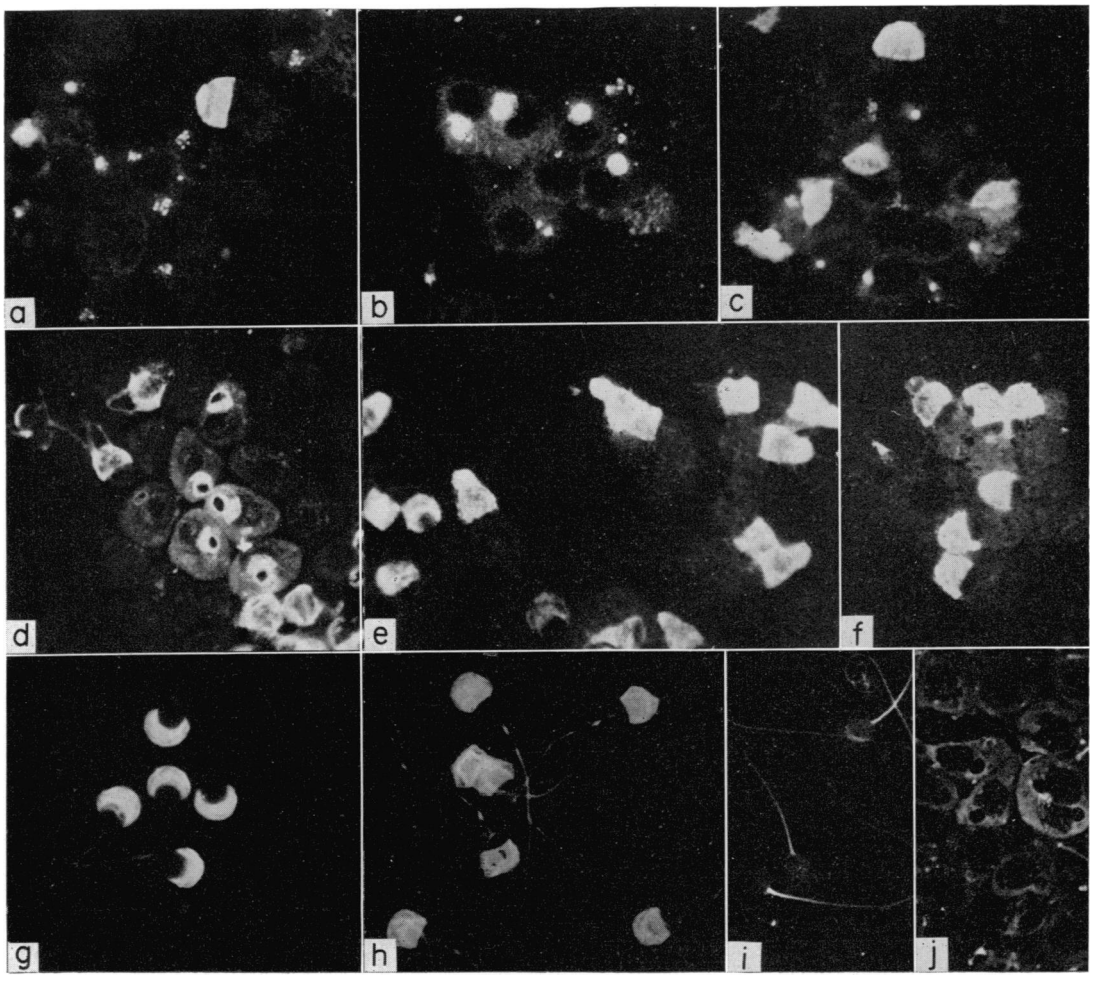

FIG. 2. Immunofluorescent localization of autoantigen S in spermatids and spermatozoa. Staining of proacrosomal granules: (a), acrosomal granule (b), acrosome and cap during the cap phase (c and d), during the acrosome phase (e and f) and on spermatozoa (g and h). After incubation of anti-S serum with autoantigen S the fluorescent pictures disappear (i and j). Testis imprints and spermatozoa smears fixed with formalin fume (a, b, c, e and g) or with alcohol (d, h, i and j) or only air dried (f). One may notice a striking difference between (d) (alcohol fixation) with 'holes' in the acrosomes centres and (c) and (e) (formalin fixation) where acrosomes are fully stained. (Magnification ×300.)

In contrast, absorption with S autoantigen ($\simeq 1{\cdot}5 \times 10^8$–$3 \times 10^9$ spermatozoa for 0·1 ml of serum) leads to disappearance of the fluorescent staining of the corresponding structures in spermatids at various stages (Fig. 2j). In mature spermatozoa, the fluorescence of the acrosomes completely disappeared, but a weak fluorescence persisted on the juxta-

nuclear part of the head caps of some of the spermatozoa (Fig. 2i). This was similar to the faint staining that was observed at the same site with 31/67 normal guinea-pig sera. In both cases this weak fluorescence is completely removed by absorption of the immune serum with crude soluble extract of guinea-pig spermatozoa and with ground testis.

III. LOCALIZATION OF 'P' AUTOANTIGEN (Fig. 3)

P auto-antigen (precipitated by TCA, a protein of β_1 electrophoretic mobility, giving rise to precipitating antibodies) was localized in spermatozoal acrosomes at the exclusion of head caps. The fluorescent staining was exceedingly bright with sharp and highly regular edges (Fig. 3g and h). The same aspect was observed with every anti-P serum utilized. It was also observed in immature sperm cells where P was localized in the pro-acrosomal granules, acrosomal granule and spermatid acrosome and never in the cap (Fig. 3a, b, c, d, e and f).

P auto-antigen was detected in the testis cells in every case after fixation with formalin (fumes or liquid), ethanol-formalin or TCA; sometimes after ethanol fixation, but never if the cells were only air dried.

Absorption of anti-P serum

Non-specific absorption procedures performed with S or T autoantigens in excess (≃ $1{\cdot}5 \times 10^8$ spermatozoa for 0·1 ml of serum) were without effect on the intensity of P fluorescent staining. Actually Fig. 3b and d resulted from a preparation absorbed with S and Fig. 3c and f from a preparation absorbed with T. In contradistinction, absorption with P autoantigen (≃10^8 or $4{\cdot}5 \times 10^8$ spermatozoa for 0·1 ml of serum) resulted in a complete disappearance of fluorescent staining (except for the faint, trivial staining observable with FRG alone) (Fig. 3i and j).

IV. LOCALIZATION OF 'T' AUTOANTIGEN (Fig. 4)

T autoantigen (TCA destroyed, giving rise to toxic, C-fixing antibodies) gives the following localization results.

Spermatozoa preparations

After ethanol or formalin fixation, acrosome and head cap are fluorescent, the edges being brighter than the centre. Post-nuclear cap is also slightly fluorescent, separated from the fluorescent acrosome by a dark band (Fig. 4g, h and i).

Testicular cells (imprints)

Spermatid staining varies with the developmental stage. At the end of the Golgi phase, a bright cytoplasmic structure is seen against the nucleus: either an open ring (ethanol fixation, Fig. 4b) or a disk, brighter at its periphery (formalin fixation, Fig. 4a).

During the cap phase, a dome-like structure on the nucleus is stained with brighter edges and less bright centre (with either ethanol or formalin fixation). The acrosome and the head cap cannot be distinguished as they can with anti-S antibodies (Fig. 4c and d).

During the acrosomal phase, the acrosome and the head cap are stained (edges more than centre) and are seen distinctly (Fig. 4e). Some unidentified immature cells are entirely stained and rather uniformly but for a stronger staining at the periphery suggest-

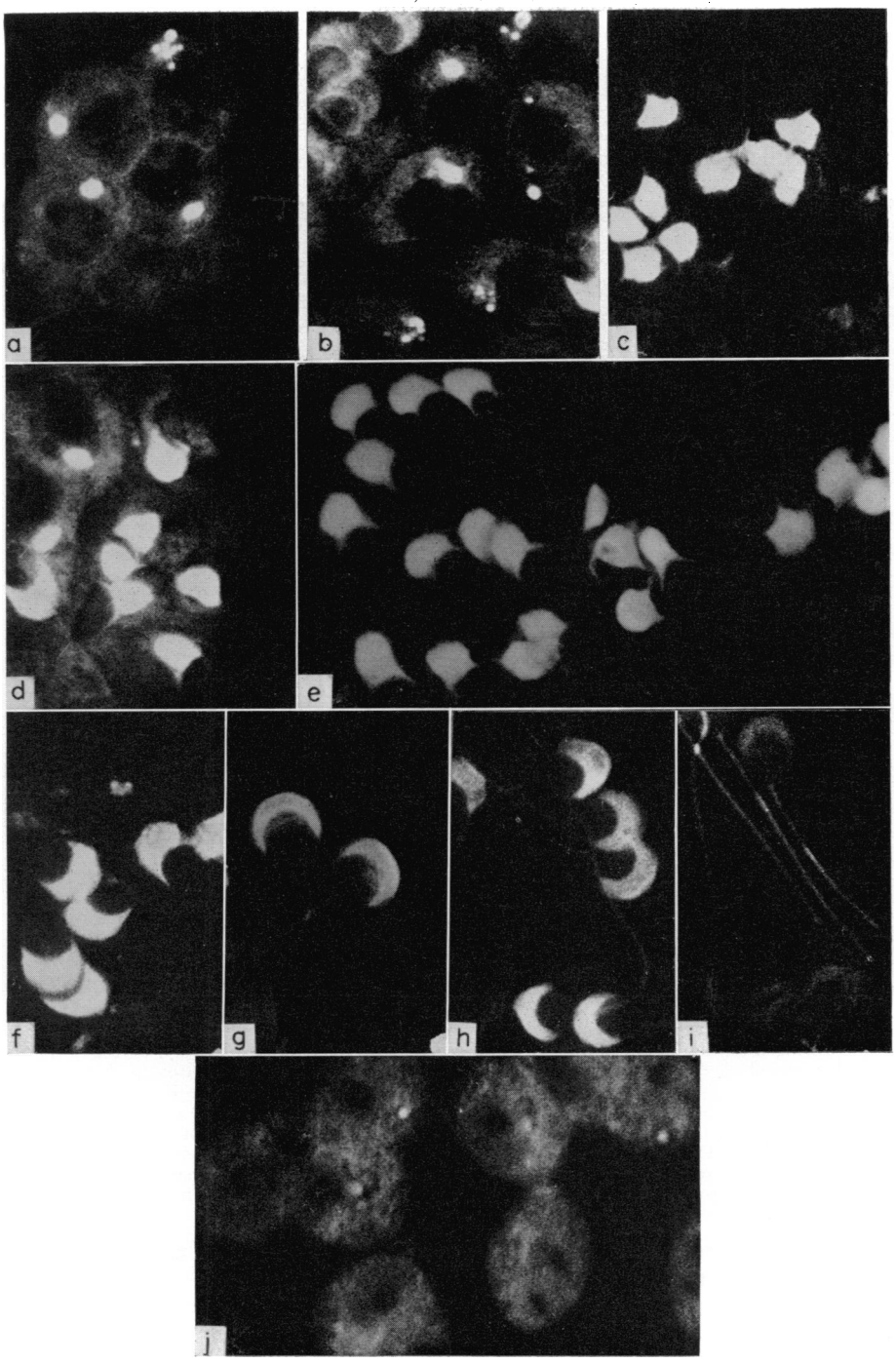

FIG. 3. Immunofluorescent localization of autoantigen P. P is present in proacrosomal granules and acrosomal granules (a and b), in spermatid acrosomes during cap phase (c and d), acrosome phase (e) and maturation phase (f) and in spermatozoal acrosomes (g and h). Incubation of anti-P sera with autoantigen P makes the staining disappear (i and j). Testis imprints and spermatozoal smears fixed with formalin fumes (a–e, and j) or trichloracetic acid (h and i). (Magnification ×550.)

ing cytoplasmic membrane. These are seen after the following fixations: acetone, methanol, ethanol and air drying. A few of these cells resemble highly evoluted spermatids, others are much larger (Fig. 4e and f).

Frozen-sections of testis

All types of cells in the seminiferous tubules are stained with the exception of the most

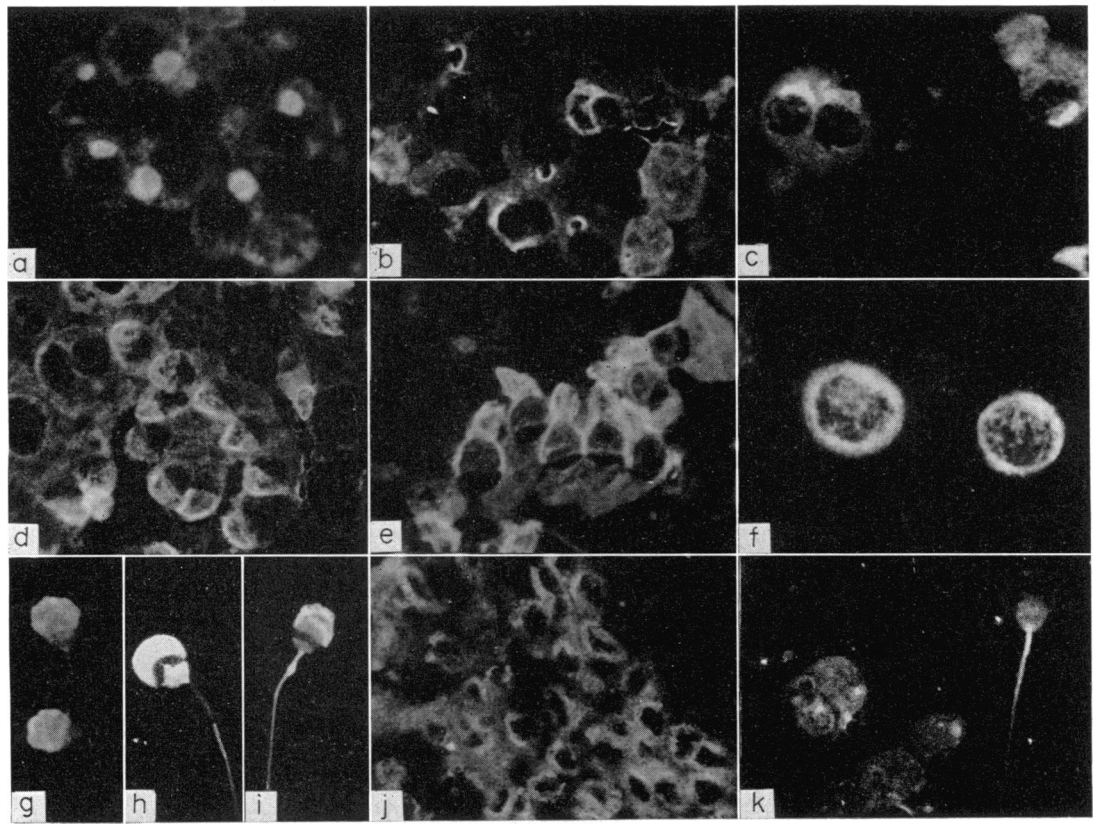

FIG. 4. Immunofluorescent localization of autoantigen T. Fluorescence was seen in: the acrosomal granule during the Golgi phase (a and b) and the beginning of the cap phase (c); acrosome and cap during the cap phase (d), during the acrosome phase (e) and in the spermatozoa (g, h and i). The post-nuclear cap of spermatozoa is stained (compare with Figs 2g and 3f) as the cytoplasm of some spermatids (see b and e) and presumed secondary spermatocytes (f). In (k) the anti-T serum has been absorbed with T autoantigen. (j) Is a picture of a non-fixed cryostat section of a seminiferous tubule incubated with anti-T serum: spermatids are fluorescent with brighter crescents or rings (probably acrosomes) but primary spermatocytes and spermatogonia (right upper corner) are dark. Fixation with formalin fumes (a and h), alcohol (b,d,e, g and k), acetone (c and f). (Magnification $\times 420$ except for d $\times 266$ and j $\times 315$.)

peripheral (two or three outer rows). At the centres of the tubules the staining is more ring-shaped and brighter (Fig. 4j).

Absorption of anti-serum

Non-specific absorption procedures performed with water soluble extracts of guinea-pig spermatozoa containing S, P and Z auto-antigens in excess ($\simeq 9 \times 10^8$ spermatozoa for 0·1 ml of serum) did not modify the fluorescent staining. Actually the spermatozoon in Fig. 4i

was incubated with an anti-T serum absorbed with a mixture of autoantigens S and P. Absorption with large quantities of T autoantigen or with whole spermatozoa (2×10^9 for 0·1 ml of serum) totally inhibits the fluorescent staining in all cases. Absorption with smaller quantities ($T \simeq 2 \times 10^8$ or spermatozoa 10^9 for 0·1 ml of serum) abolishes all fluorescence of the cytoplasm, post-nuclear cap and often acrosomes, but the latter may sometime retain some degree of staining.

V. IMMUNOLOGICAL SPECIFICITY OF THE THREE SPERMATOZOAL AUTOANTIGENS

This specificity has been studied at several levels.

(a) *Independence from each other.* The strikingly opposite results obtained with absorption procedures of specific immune sera by the relevant autoantigen (extinction of fluorescence) as opposed to the two irrelevant ones (no modification of fluorescence) suffice to show this independence, which is also demonstrated by other means (Voisin and Toullet, 1968).

(b) *Organ specificity.* There was no fluorescent staining when anti-S, anti-P or anti-T guinea-pig sera were applied followed by FRG to frozen sections of the following guinea-pig organs fixed with ethanol or formalin (the two main fixation procedures used with sperm and testis): adrenal, ovary, kidney, liver, spleen, brain and thyroid. Conversely, absorption procedures of anti-S, anti-P and anti-T sera with preparations from the guinea-pig organs: spleen (10^9 cell for 0·1 ml of serum), kidney or liver homogenate (1 g for 0·1 ml of serum) led to either no decrease of the seminiferous cell fluorescence or to a slight decrease, presumably non-specific.

(c) *Species-specificity.* The presence of autoantigens S, P and T was sought in spermatozoa of other species. Spermatozoal smears or testis imprints of rabbit, rat and mouse were fixed with ethanol and with formalin. They were studied for immunofluorescence with anti-S, anti-P and anti-T sera. Anti-P gave no staining. Anti-T gave a doubtful one; but anti-S gave a bright fluorescence of spermatozoa and spermatid acrosomes in the three animal species (Fig. 5). Absorption of anti-S serum with S autoantigen completely abolishes the staining of rat acrosomes. S autoantigen is therefore organ-specific and not species-specific within the species studied.

VI. CONTROL EXPERIMENTS

The following controls were studied:

(a) *Without serum* and with several fixation procedures, no spontaneous fluorescence was observed (except a faint fluorescence of the middle piece of the tail).

(b) *With rabbit fluorescent immunoglobulin (anti-guinea-pig immunoglobulin)-RFG—alone.* No more fluorescence was observed than without serum, except a faint staining of the acrosome after TCA fixation.

(c) *With sera from guinea-pigs immunized against various guinea-pig tissues.* In no instance was fluorescence comparable to the three types described in this article observed. However observations were made identical to the ones made during experiments with normal guinea-pig sera as described in the following paragraph.

VII. RESULTS WITH NORMAL SERA (Fig. 6)

Here again it must first be clearly stated, that, in no case, was any fluorescence seen that could be compared in intensity to that seen and described with immune sera. However,

in view of the reported presence of natural anti-sperm antibodies in normal guinea-pig sera (Chang, 1947; Johnson, 1968; Monastirsky and Fernandez-Collazo, 1968; Spooner, 1964; Swanson Beck, Edwards and Young, 1962) it was felt necessary to examine this possibility with great care. Ninety-five sera were tested on testis imprints and spermatozoal smears fixed by different methods. These sera came from either untreated male or female guinea-pigs or from males injected with complete adjuvants alone or mixed with thyroid

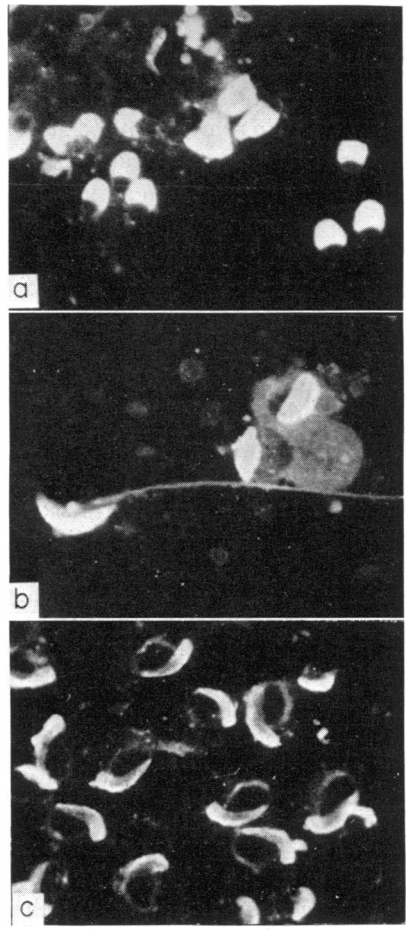

FIG. 5 Staining of spermatid acrosomal apparatus of rabbit (a), rat (b) and mouse (c) after incubation with a guinea-pig immune serum directed against guinea-pig autoantigens S. (Magnification ×420.)

extracts. Eighty-four per cent of these sera (that is 28/31 untreated female, 29/37 untreated males and 23/27 males treated with complete adjuvants) gave some type of weak immunofluorescence. Although this was of the kind that is considered as negative by many investigators, it has been taken into consideration and is described below.

Spermatid and spermatozoal acrosomes were most frequently observed (80/95 sera positive after formalin fixation and 53/98 after ethanol fixation). However the acrosomal crescents were thin and faintly stained (Fig. 6c, d and e). With TCA fixation a few sera gave a bright fluorescence of a non-specific nature thus leading us to discontinue TCA as a fixative.

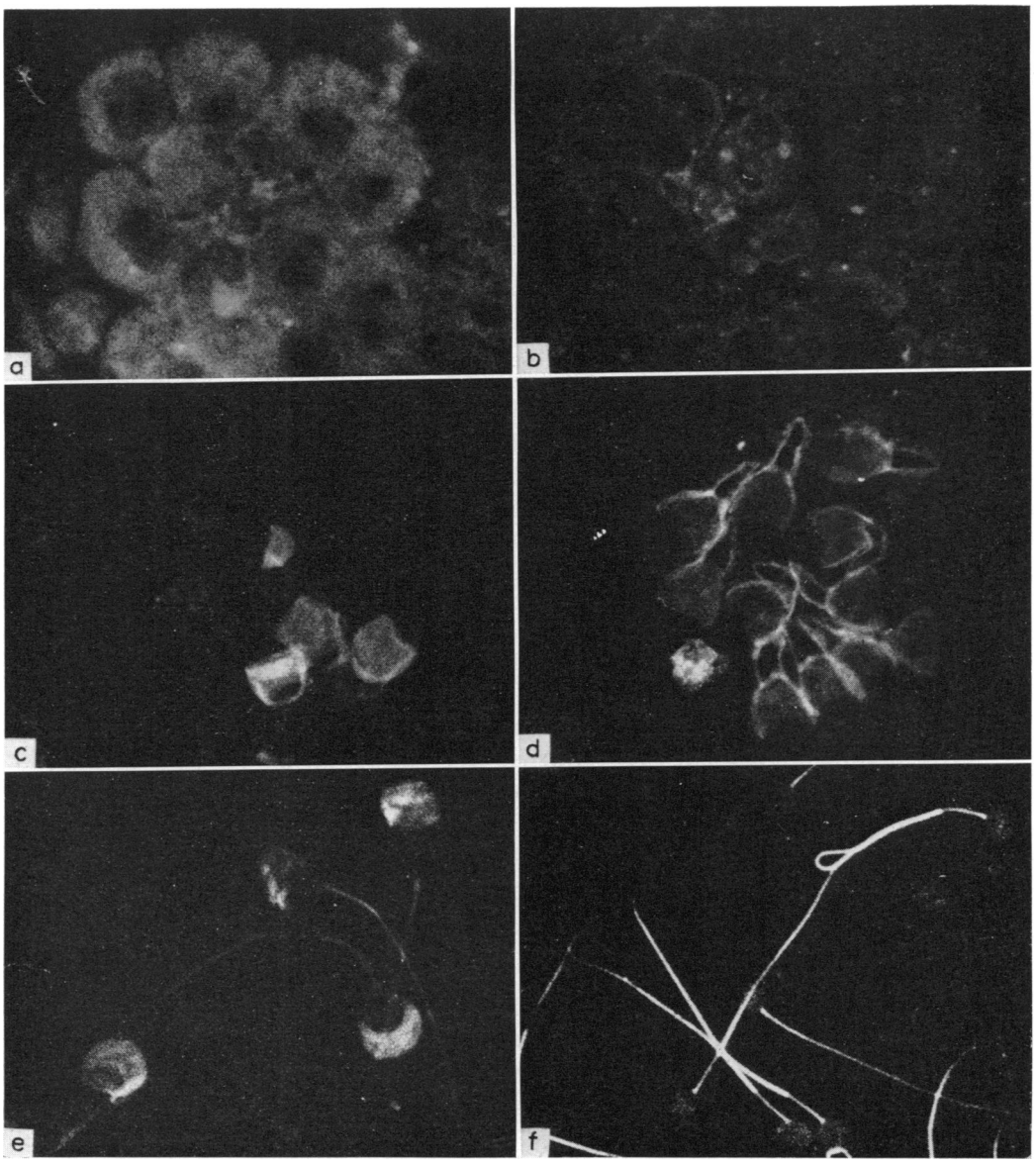

FIG. 6 Immunofluorescent staining after incubation with control sera. (a) and (b): Pool of guinea-pig anti-homologous thyroid extract sera on testis imprints fixed with alcohol (a) or formaline (b). The following pictures (c to f) are the brightest that we observed in the study of normal half diluted sera: staining of spermatids and spermatozoa cap fixed with alcohol (c and d), and spermatozoal acrosome fixed with formalin (e); fluorescence of the principal piece of spermatozoa tail (f, no fixation). Staining is lost after incubation of normal serum with spermatozoal crude soluble extract but not after incubation with soluble purified S and P autoantigens. (Magnification $\times 540$ except for f $\times 340$.)

Acrosomal granules were often visible after formalin fixation (Fig. 6b).

Head caps of spermatids and spermatozoa were weakly stained by 7/31 female sera and 31/67 male sera after ethanol fixation (Fig. 6a, c and d) and only in 4/95 sera (two females, four males) after formalin fixation.

The principal piece of the tail was clearly fluorescent (Fig. 6f) with 12/92 sera and doubtfully with many other sera. This was observed with either one of the two fixation procedures (ethanol or formalin). The fluorescence was mat but rather strong and formed two parallel lines at the edges of the tail, separated by a dark central line. The same type of staining was observed with an anti-P immune serum but it did not disappear after absorption with P autoantigen.

On *the middle piece of the tail* a fluorescence was observed with several normal and immune sera but not in each experiment. It concerned bulgings suggesting cytoplasmic remnants of the middle piece (see Figs. 2h and 4i).

Absorption experiments. Although the preceding immunofluorescent aspects obtained with normal guinea-pig sera (and sera from guinea-pigs immunized against non-spermatozoal constituents) were very weak, the very fact that all sera do not provoke them suggested the possible action of natural antibodies. Absorption experiments were therefore done, making use of a particularly strong 'normal' serum (still giving some detectable degree of fluorescence at 1/4 (Fig. 6c, d, and e). Whole spermatozoa and crude extracts were able completely to absorb the immunofluorescent staining capacity of this normal serum (acrosome and tail, after ethanol or formalin fixation). On the contrary purified S and P preparations were not able to absorb either staining after fixation (Fig. 6c and d represent cells incubated with normal serum absorbed with autoantigen S) but T preparations (not purified), had some limited capacity, preventing only acrosome fluorescence and only after ethanol fixation.

Eight other normal (or control) sera from male and female guinea-pigs, that gave the usual weak immunofluorescence (less intense than the preceding normal serum) were incubated with testis, kidney, liver and spleen homogenates. This procedure resulted, but inconsistently and sometimes doubtfully, in a reduced fluorescence of alcohol-fixed smears but not of formalin-fixed ones. The results appeared to depend more on the sera than on the absorbing tissue.

DISCUSSION

The purpose of this study was to localize, by indirect immunofluorescence, the three guinea-pig spermatozoal autoantigens S, P, and T previously described and analysed for physicochemical, immunological and immunopathological properties (Voisin and Toullet, 1968, 1969, 1971; Toullet and Voisin, 1969, 1971). The three of them have been localized in the acrosomal apparatus and one (T) also on the external membrane. Their appearance at the various steps of their formation during spermatogenesis has been described.

The main problems to be discussed concern not only the cellular localization of the spermatozoal autoantigens but also the interspecies specificity of some of them, the question of 'natural' autoantibodies, the role of histological fixation and, finally, the possible implications of the preceding results.

Cellular localization of the spermatozoal autoantigens

Although largely localized at the same sites, S P and T autoantigens are completely

independent. This was demonstrated in previous experiments (Voisin and Toullet, 1968; Toullet and Voisin, 1969) and is reconfirmed here since no cross-absorption of the specific sera was possible. Several immunofluorescence pictures that are presented were actually obtained with monospecific sera incubated with one or two of the other antigens (without modification of the results: see Figs 2b, c, d; 3b, c, d, f and 4i).

S autoantigen is present in or on the acrosomal crescent and head cap. It appears at the same time as proacrosomal granules and other components of the acrosomal vesicle that will form the outer part of the acrosome. From the fact that S—in contrast to P—is PAS positive and rich in glucides (Voisin and Toullet, 1968) and from the similar localization of anti-S immunofluorescence and PAS positive areas in spermatozoa, spermatids and, possibly, in secondary spermatocytes, it would seem that the same material is responsible for both. It is nevertheless difficult precisely to localize S on or inside the acrosomal membranes by simple immunofluorescence. From the staining intensity, it would appear that S exists inside the acrosome (especially acrosomal crescent), but the intense staining of the head cap and the irregular limits of the acrosomal fluorescence (like spermatozoal membranes after fixation) suggest that S is fixed on the acrosomal membrane—or about it.

There are, however three reasons for thinking that it is not *on* the cytoplasmic membrane but inside it: (1) Only the acrosomal crescent and head cap are stained (and not the whole spermatozoal membrane). (2) Anti-S antibodies are not spermotoxic, even the scanty part of them that is able to fix complement (most anti-S antibodies are IgG1; isolated and concentrated IgG2 from anti-S sera are able to fix complement (Toullet and Maillard, unpublished)). (3) Living spermatozoa are not stained for immunofluorescence by anti-S sera. All the preceding data seem compatible with S being condensed on or about the inner membrane of the acrosome.

As for P autoantigen only proacrosomal granules, acrosomal granule and crescent are stained by anti-P antibodies (not the whole head cap). Like S autoantigen and for the same reasons P autoantigen is certainly not on the surface of spermatozoa, in as much as anti-P antibodies are strongly able to fix complement and are not spermotoxic. Furthermore, the clearcut and regular limits of the fluorescence suggest that P (a protein compound: Voisin and Toullet, 1968) is not linked to the membranes which are less regular.

T autoantigen is localized on the acrosome, but probably only on the membrane as on the cytoplasmic membrane of spermatids and immature cells (spermatocytes). The evidence for this is: (1) the immunofluorescent staining is bright at the edges of the acrosomal apparatus of spermatozoa and spermatids with staining of the post-nuclear cap (normally very dark) and staining of immature cells; (2) its water insolubility (Voisin and Toullet, 1968), while it is found present in the membranous fraction in rapid centrifugation; (3) the spermotoxic (Voisin and Toullet, 1968), cytotoxic and spermagglutinating (Toullet and Voisin, 1971) activity of anti-T antibodies. Finally the fact that, when incubated with living spermatozoa, anti-T are the only antibodies able to become fixed on the spermatozoa as revealed by immunofluorescence (the whole spermatozoa head being coloured with a brighter staining of the acrosomal superior edge). Tung, Unanue and Dixon (1970) by immunofluorescence technique detected an antigen at the surface of living testicular cells and spermatozoa; the sera came from guinea-pigs immunized against an ammonium-sulphate-precipitated testicular material; this antigen is probably different from T, because T is absent from this material. It seems interesting to note that the properties of T resemble those of transplantations antigens; however they do not have an allogenic specificity and anti-T antibodies cannot be absorbed by spleen cells from the

same guinea-pigs that were used to make the T preparations. The possibility arose of two distinct T autoantigens being present: T_1 localized on the acrosome and T_2 localized on external membranes, but it has not been substantiated so far.

Organ- and interspecies-specificity of the spermatozoal autoantigens

S, P and T have been detected only in spermatozoa and precursor cells and are therefore considered as being organ-specific. S has even been shown to possess an interspecies organ specificity since it has been detected by guinea-pig anti-S immune sera in spermatozoa and spermatids from rats, mice and rabbits, showing at least an antigenic determinant community. It had indeed been previously observed (Voisin, Toullet and Maurer, 1958) that guinea-pigs immunized with spermatozoa from rabbits, rats and mice produce anaphylactic antibodies able to react (in PCA) with guinea-pig spermatozoal extracts, including the spermatozoa from the antibody producer. Conversely, sera from autoimmunized or alloimmunized guinea-pigs were able to react (in PCA) with rabbit spermatozoal extracts. S antigen was also probably at the origin of the interspecies acrosomal immunofluorescent staining obtained in mouse, rat and guinea-pig spermatozoa with mouse antimouse testis immune sera (Barth and Russell, 1964).

Normal sera and 'natural' autoantibodies

Since the work of Chang (1947), thermolabile, spermotoxic antibodies against autologous, homologous and heterologous spermatozoa have been repeatedly described with a titre variable according to the authors.

We have observed some rather weak fluorescence (acrosome mainly, but also tail) obtained with normal sera. It was only observed with undiluted or weakly diluted sera. Heating them 30 minutes at 56° did not modify the results. These sera had been kept a few weeks at −20° and we have observed that the same procedure inhibited the spermotoxic properties of natural autoantibodies. This conceivably might be responsible for the absence of any strong fluorescence observed in this study with normal sera, although three fresh sera gave similar results. In spite of being less easy with weak reactions, the interpretation of absorption experiments (especially after alcohol fixation) is that natural autoantibody properties are specific. The anti-acrosomal fluorescence is not due to anti-S or anti-P antibodies; it seems to be due, at least partly, to anti-T activity. The anti-tail (middle-piece and principal piece) activities are directed against antigens independent of the three described autoantigens S, P and T.

Role of histological fixations

Some difference was observed according to the fixation (alcohol and formalin fumes mainly). Trichloracetic acid, which had been initially chosen in view of its precipitating activity (without denaturation) on P antigen, was discontinued because of non-immunologically specific immunofluorescence. P antigen is possibly solubilized or not fixed (rather than denatured or destroyed) by alcohol while it is fixed (and not denatured) by formalin probably because of its chemical nature. As for S and T antigens, the effect of alcohol fixation (no staining of the immature spermatid acrosome centres) seems to be due to a solubilization of the constituent material. In mature spermatids and spermatozoa this effect is not observed, probably masked by the intense fluorescence of elements around the acrosomal granule. Another difference is a retraction of spermatozoal head caps and a decreased fluorescence of spermatid head caps after formalin fixation.

Formalin appears to be more responsible than alcohol for non-specific stainings as can be seen by using normal sera that have been incubated with various organ extracts or homogenates. These stainings however cannot be compared in any way to the bright fluorescence induced by autoantibodies and cannot bring any modification to our conclusions concerning the localization of spermatozoal autoantigens, in particular P.

Implications of these findings

They are apparently concerned with the appearance of autoantigens and the immunopathological mechanisms of epididymal and testicular lesions in autoimmune aspermatogenic orchitis.

Appearance of autoantigens. Katsh (1960) showed that antispermatogenic factor begins to appear in the testis when secondary spermatocytes (and the idiosomic-acrosomal apparatus) begin to appear in large number during spermatogenesis. It is remarkable and probably meaningful that three different autoantigenic substances are localized in the same cells (spermatozoa) and their precursors (spermatids and probably secondary spermatocytes), are detected at the same place (acrosomal apparatus) and arise at the same stage (after meiosis, during spermatogenesis). This may suggest the possibility of some derepression of the haploid genome leading to the synthesis of new molecules unless (less likely) haploidy leads to the formation of incomplete molecules with chemical determinants not apparent in diploid cell metabolism.

Immunopathological mechanisms of autoimmune aspermatogenic orchitis (AIAO)

The three spermatozoal autoantigens S, P and T are able, even when purified and freed from each other, to induce lesions of AIAO indistinguishable from each other when observed at their mature stage (about 3 weeks). At the beginning, however, differences have been described (Toullet *et al.*, 1970; Voisin et Toullet, 1971). The differences in properties and localization of these autoantigens inside or on the sperm cells, as well as the biological properties of the corresponding immune agents as well as the accessibility of the latter to the various parts of the epididymo-testicular apparatus and the possibility of a passive transfer of lesions by antibodies support the hypothesis of the following three mechanisms being at the origin of AIAO.

T autoantigen being linked to the cell surface is accessible to anti-T antibodies which are able to agglutinate sperm-cells or to kill them in the presence of complement (Toullet and Voisin, 1971). This may happen *in vivo* in the rete testis, vasa efferentes and epididymis known to be permeable to immunoglobulins (Kormano and Koskimies, 1972), and probably complement. This will give rise to an inflammatory reaction with increased vascular and tissular permeability leading to a progressive injury of close seminiferous tubules and so on. A possible backward flow in the seminiferous tubules might play a role. These mechanisms might explain the fact that in T-induced early AIAO lesions, some degree of aspermatogenesis is seen in the seminiferous tubules before any significant peritubular cell infiltration. Also that, in 20 per cent of the cases, lesions can be transferred by anti-T antibodies (Toullet and Voisin, 1972).

S and P autoantigens being inside the acrosomal apparatus are not accessible in living spermatozoa to specific antibodies. Being highly soluble and diffusible, S and P antigens are able to diffuse from a few dead spermatozoa into interstitial tissue of the epididymis and to meet corresponding antibodies or sensitized cells at the vascular wall level. Anti-P

antibodies, able (in conjunction with anaphylactic ones) to give strong Arthus reactions would do so in the epididymis leading to a strong polymorphonuclear infiltration which is often actually observed. This possibility is corroborated by the passive transfer of lesions by anti-P antibodies in half of the cases (Toullet and Voisin, 1972). It might initiate the events leading to the classical aspect of AIAO through the release of inflammatory mediators.

As for S, able to induce a state of strong delayed hypersensitivity as well as anaphylactic antibodies, it would act first at the epididymis level by a combination of these two types of hypersensitivity since anti-S antibodies are not able passively to transfer the lesions and delayed hypersensitivity alone is not well correlated with the lesions while the association of both is effective (Brown, Glynn and Holborow, 1967; Toullet and Voisin, 1969; Voisin and Toullet, 1969).

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