# IMMUNE COMPLEX ORCHITIS IN VASECTOMIZED RABBITS*

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Vasectomy has rapidly become one of the most popular contraceptive methods in many countries. While only a few thousand vasectomies were performed annually during the early 1960's, the figure has now reached nearly one million per year in the United States alone (1). Because vasectomy involves surgical intervention in a healthy individual, the possibility that it may produce long-term local or systemic diseases, even in a small portion of the population, has been the subject of consideration (2, 3). There is an obvious need for a thorough investigation on the safety of this procedure.

The present study shows that vasectomized rabbits making an active antibody response to sperm antigens develop an orchitis associated with deposition of rabbit IgG, C3, and sperm antigens—presumably antigen-antibody complexes—in the basement membrane of the seminiferous tubules. These lesions are frequently accompanied by mononuclear cell infiltration and destruction of germinal epithelium. A minority of these rabbits also develop a mild glomerulonephritis accompanied by deposition of immunoglobulin and complement in glomerular structures.

## Materials and Methods

*Surgical Procedures.* 24 New Zealand White rabbits (Beckens Research Animal Farms, Sanborn, N. Y.) were bilaterally vasectomized, carefully avoiding trauma to the testes or their vessels. Rabbits weighing 3.1–3.6 kg were trial bled, tranquilized with paraldehyde (Elkin-Sinn Inc., Cherry Hill, N. J.), and anesthesized with sodium thiamylal (Surital; Parke-Davis, Baltimore, Md.). After surgical preparation a midventral vertical incision, about 2.5 cm long, was made in the skin of the suprapubic area and with blunt dissection both spermatic cords were located. The components of each cord were reached through a small cut in the cord wall, the vas deferens was isolated, ligated in two sites using silk sutures (Silk 4-0; Ethicon Inc., Somerville, N. J.), and the part between the sutures, approximately 1 cm long, was cut out. The other components of the cord were gently pushed back in place, the small cut in the cord wall and the incision in the skin were sutured using silk. Sham-vasectomies were performed on 20 rabbits weighing 3.1–3.6 kg, which were anesthesized and treated as described above, with the exception that the vas deferens, once

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isolated, was not ligated and cut out but gently pushed back into the cord with the other components.

Rabbits were bled regularly every 15–30 days from the central artery of the ear and sera were stored at −20°C. At different intervals after vasectomy, animals were sacrificed by exsanguination and their tissues stored at −70°C.

*Procedures for Demonstration of Circulating Antibodies to Testicular Antigens (4)*

TANNED CELL HEMAGGLUTINATION [TCH].[1] Human group O red cells treated with tannic acid were coated with testis extract diluted in phosphate-buffered saline (PBS). The extract had been prepared by homogenizing the tissue in the cold with a VirTis homogenizer (Model 45; Virtis Co., Gardiner, N. Y.) at approximately 5,000 rpm for 1 min, and centrifuging for 30 min at 70,000 *g* in a Beckman Ultracentrifuge (Model L; Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The protein content of the resulting supernates varied from 1.0 to 3.0 *g* % as determined by the biuret procedure. Inactivated rabbit sera were tested in twofold serial dilutions from 1:2 to 1:10,000 against the coated red cells using microtiter "U" bottom plates (Cooke Laboratory Products, Cooke Engineering Co., Alexandria, Va.) with 0.025 ml of each reagent. Plates were incubated for 2 h at room temperature and then overnight in the cold; results were read after 2 and 24 h.

COMPLEMENT FIXATION [CF]. Twofold serial dilutions of inactivated rabbit sera were prepared using triethanolamine-buffered saline (0.15 M, pH 7.3) in disposable "V" bottom plates (Linbro Chemical Co., New Haven, Conn.). Testis extract, prepared as described above, was diluted 1:10 and 0.025 ml was added, followed by 0.025 ml of guinea pig complement (Grand Island Biological Co., Grand Island, N. Y.) diluted 1:18. The plates were first incubated overnight at 4°C and then for 1 h at 37°C. This was followed by the addition of 0.2 ml of 2% sheep red cells sensitized with antisheep hemolysin (Baltimore Biological Laboratories, Div. of BioQuest, Cockeysville, Md.). The plates were again incubated at 37°C for 30 min, centrifuged at 5°C and 500 *g* in an IEC centrifuge (Model PR-600; Damon/IEC Div., Damon Corp., Needham Heights, Mass.) for 5 min, and read.

INDIRECT IMMUNOFLUORESCENCE [IF]. Sera from experimental and control rabbits were tested for the presence of antibodies to testicular antigens using the indirect IF technique with testis, adrenal, and liver from normal animals. 4-μm cryostat sections of such tissues were first incubated with the rabbit serum in twofold serial dilutions from 1:10 to 1:640. After washing, the sections were incubated with fluorescein-conjugated goat antirabbit IgG, washed, and mounted in buffered glycerol for examination by UV microscope.

AGAR GEL DOUBLE-DIFFUSION TEST [GD]. Extracts of rabbit testis, adrenal, liver, lung, and kidney, prepared as described above, were tested for reaction in agarose plates (Marine Colloids, Inc., Biomedical Systems, Rockland, Maine) against serial dilutions of sera from experimental and control rabbits.

*IF*

DIRECT IF ON TISSUE SECTIONS. The globulin fractions of antisera to rabbit IgG, C3, albumin, and fibrinogen (Cappell Laboratories, Inc., Dowingtown, Pa.) were conjugated with fluorescein (5). The specificity of the conjugated and unconjugated antisera was tested by immunoelectrophoresis. Small fragments of testis and kidney obtained from rabbits were snap-frozen in liquid nitrogen and stored at −70°C. 4-μm sections were cut in a cryostat (AO Cryocut; American Optical Corp., Scientific Instrument Div., Buffalo, N. Y.) and air dried. The staining of specimens was performed as described in a previous publication (6). The sections were viewed with a Leitz Dialux Microscope (E. Leitz, Inc., Rockleigh, N. J.) equipped with a Ploem fluorescence illuminator. The intensity and extent of fluorescence was arbitrarily graded as 0, negative; ±, minimal; 1+, slight; 2+, moderate; and 3+, marked. Photographs were taken using a Leitz Camera with Tri X Pan Kodak film.

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[1] *Abbreviations used in this paper:* CF, complement fixation; GD, gel double-diffusion test; H & E, hematoxylin and eosin; IF, immunofluorescence; PBS, phosphate-buffered saline; TCH, tanned cell hemagglutination.

INDIRECT IF FOR SPERM ANTIBODIES. Spermatozoa were obtained from the epididymis of normal rabbits after unilateral or bilateral orchiectomy and washed in medium RPMI 1640 (Associated Biomedic Systems, Inc., Buffalo, N. Y.). Smears were prepared on microscope slides using a cytocentrifuge (Cytospin; Shandon-Elliott, Shandon Instruments Inc., Sewickley, Pa.) at 1,500 rpm for 10 min. The slides were air dried, fixed in absolute methanol for 30 min at room temperature, washed in PBS, and incubated for 30 min with twofold serial dilutions of tested rabbit sera starting from a dilution of 1:10 to 1:160. The slides were washed in PBS, incubated for 30 min with fluorescein-conjugated goat antirabbit IgG at room temperature, washed again, and then mounted in buffered glycerol for reading. Sperm smears incubated with sera obtained from vasectomized rabbits (including a prevasectomy bleeding), sham-vasectomized rabbits, as well as normal rabbits at approximate monthly intervals were included in each experiment. As an additional control, the fluorescein-conjugated antiserum was used on sperm smears without previous incubation with sera.

The same indirect IF procedure was used on suspensions of spermatozoa washed in medium RPMI 1640, fixed for 10 min in methanol, washed several times with PBS, first incubated with the rabbit serum, as described above, and then stained with fluorescein-conjugated goat antirabbit IgG.

*Immunoelectron Microscopy for Localization of Sperm Antibodies.* Spermatozoa obtained from the epididymis were suspended for 2 min in the fixative of Stefanini et al. (7) diluted 1:2 with PBS, quickly washed in PBS, incubated for 2 min with the serum containing antisperm antibodies or with control sera, washed again, and then incubated for 2 min with ferritin-conjugated goat antirabbit IgG (8). After centrifugation the sediment was washed again, postfixed in 2% glutaraldehyde and in 1% osmium tetroxide, and embedded in Epon 812.

*Elution of Immunoglobulin Deposits from Tissues.* For elution of immunoglobulins from cryostat sections, the following methods were employed. 4-$\mu$m sections of testis were incubated, with either of the following reagents: (*a*) 0.02 M citrate buffer (pH 3.2) (9); (*b*) 2 M NaSCN (pH 4.5) (10); (*c*) 3 M KI (pH 6.8) (11); (*d*) PBS at 56°C (12); or (*e*) PBS at room temperature. Periods of incubations varied from 10 to 60 min, all with constant agitation. The sections were then tested with fluorescein-conjugated antibody to rabbit IgG in order to evaluate the amount of IgG removed by these reagents.

The elution of immunoglobulins from homogenates of testis or kidney tissues was performed as described by Lerner et al. (13). The eluates were concentrated 10 times with Amicon Filters (Minicon—A25; Amicon Corp., Scientific Sys. Div., Lexington, Mass.). The protein concentration was determined by the biuret procedure and the immunoglobulin concentration was established by gel radial diffusion (14).

*IF Localization of Sperm Antigens in Tubular Deposits of Testis.* The gamma globulin fractions obtained from the serum of rabbit 37-07 20 mo after vasectomy (Table I) were conjugated with fluorescein as previously described (5). As control, the gamma globulin fraction from the serum of rabbit 38-19, which did not have antibodies to sperm antigens, was similarly conjugated. In order to increase the staining for sperm antigens in tubular deposits, partial dissociation of immune complexes was attempted (9). Elution with acid buffers or with chaotropic ion-containing buffers for 60 min resulted in a complete removal of IgG and damage of testicular structure. As shown in Table II, incubation in PBS at 56°C for 60 min provided an optimal partial elution as well as good preservation of tubular structure. The eluted sections were stained with fluorescein-conjugated gamma globulin from rabbit 37-07 and from rabbit 38-19 and examined with the fluorescence microscope for comparison.

*Preparation of Tissues for Histological Studies.* Testis and kidney tissues were fixed in 10% buffered formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin (H & E), periodic-acid-Schiff (PAS) reagent, silver-methenamine (Jones), and Masson trichrome. Another portion of the specimen was fixed in a mixture of paraformaldehyde and glutaraldehyde (15), postfixed in osmium tetroxide, and embedded in Epon 818. Sections cut at 1 $\mu$m were stained

with toluidine blue and examined by light microscopy. Sections for electron microscopy were cut with an LKB Ultratome (LKB Instruments, Inc., Rockville, Md.) and examined with a Siemens 101 Electron Microscope (Siemens Corp., Medical/Industrial Groups, Iselin, N. J.)

## Results

*Serological Findings.* 11, from a total of 24 vasectomized rabbits (46%), had circulating antibodies to acrosomal antigens as detected by indirect IF. When the same sera were tested by TCH, CF, and GD against extracts of rabbit testis or by indirect IF on cryostat sections of normal rabbit testis, 14 were positive by at least one of the four methods. A total of nine rabbits (37%) were positive both for acrosomal antibodies and antibodies to testis antigens.

On the basis of these findings, the experimental rabbits were divided into three groups (Table I). Group I is comprised of four rabbits which showed positive indirect IF for acrosomal antibodies and were also positive for antibodies to testicular antigens. Rabbit 37-07 was positive for acrosomal antibodies,

TABLE I

*Circulating Antibodies and Immunopathological Findings in the Testes of Vasectomized Rabbits*

| Group | Rabbit | Months after vasectomy | Circulating antibodies to: | | | | | Immunopathological findings in testicular tissue | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Acrosomal Ags* | Testicular Ags | | | | IF localization of rabbit | | | Morphological findings | |
| | | | IIFS | TCH | CF | IIFT | GD | IgG | C3 | Fib | Light microscopy | Electron microscopy |
| I | 40-19 | 10 | ++ | + | 0 | 0 | 0 | + | + | 0 | + | ++ |
| | 38-12 | 14 | + | 0 | ++ | + | + | + | + | 0 | + | ++ |
| | 37-10 | 20 | + | +++ | ++ | + | + | +++ | ++ | 0 | ++ | ++++ |
| | 37-07 | 20 | +++ | +++ | +++ | +++ | + | +++ | +++ | 0 | ++ | +++ |
| II | 40-38 | 7 | 0 | 0 | + | 0 | 0 | 0 | 0 | 0 | 0 | + |
| | 40-36 | 8 | ± | 0 | 0 | 0 | 0 | + | 0 | 0 | 0 | 0 |
| | 40-09 | 10 | + | 0 | + | 0 | + | + | + | 0 | 0 | ± |
| | 38-13 | 11 | 0 | 0 | + | 0 | 0 | 0 | + | 0 | 0 | 0 |
| | 38-11 | 14 | + | 0 | + | + | 0 | 0 | 0 | 0 | 0 | + |
| | 38-17 | 14 | 0 | 0 | + | 0 | 0 | 0 | 0 | 0 | ± | + |
| | 38-18 | 14 | + | 0 | + | + | + | 0 | 0 | 0 | 0 | + |
| | 38-68 | 18 | + | 0 | + | 0 | 0 | + | ++ | 0 | 0 | + |
| | 38-64 | 20 | + | 0 | + | + | 0 | + | + | 0 | 0 | + |
| | 38-63 | 20 | 0 | 0 | + | 0 | 0 | + | + | 0 | 0 | 0 |
| | 38-67 | 21 | + | 0 | 0 | 0 | 0 | 0 | + | 0 | 0 | + |
| | 38-69 | 21 | ± | 0 | ++ | 0 | 0 | + | + | 0 | 0 | 0 |
| | 38-65 | 21 | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | + |
| III | 40-35 | 8 | ± | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 40-05 | 10 | ± | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 40-06 | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 40-18 | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 40-20 | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 40-21 | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 38-19 | 12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

* *Abbreviations:* Ags, antigens; Fib, fibrinogen; IIFS, indirect IF for sperm antibodies; IIFT, indirect IF on cryostat sections of normal rabbit testis; 0, negative; ±, minimal amount; +, a slight amount; ++, moderate amount; and +++, marked amount.

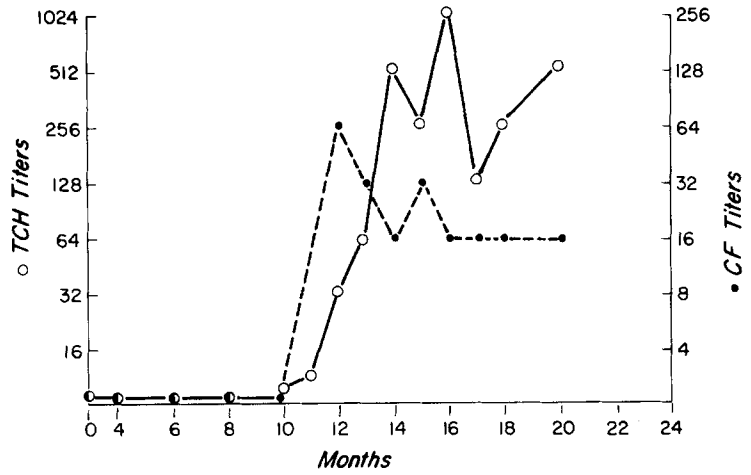


FIG. 1. Titers of antibodies to testicular antigens in vasectomized rabbit 37-07 as determined by tanned cell hemagglutination (TCH) and complement fixation (CF).

with a titer of 160. It was also positive by all four serological methods. The titers of the antibodies detected by TCH and CF in samples of sera obtained over a period of 22 mo are shown in Fig. 1; it can be seen that the antibodies to testis antigens were present in high titers for about 8 mo. Rabbits 37-10 and 38-12 had lower titers of acrosomal antibodies but were similarly positive by the serological procedures employed to detect antibodies to testicular antigens. Rabbit 40-19 had high titers of acrosomal antibodies and was also positive by the TCH.

Group II is comprised of 13 rabbits, 7 of which had low-titered antibodies to acrosomal antigens of spermatozoa and 10 had low-titered antibodies to testicular antigens, detectable only by IF or CF. Only five of these rabbits were positive for antibodies to both acrosomal and testicular antigens.

Group III is comprised of 7 rabbits, whose sera were consistently negative. A fourth group, not shown in Table I, is comprised of 20 sham-vasectomized rabbits and 6 normal rabbits which were equally negative in all serological tests.

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FIGS. 2–22. Figs. 2–22 (with the exception of Fig. 11) illustrate pathologic aspects of testes from rabbits of group I.

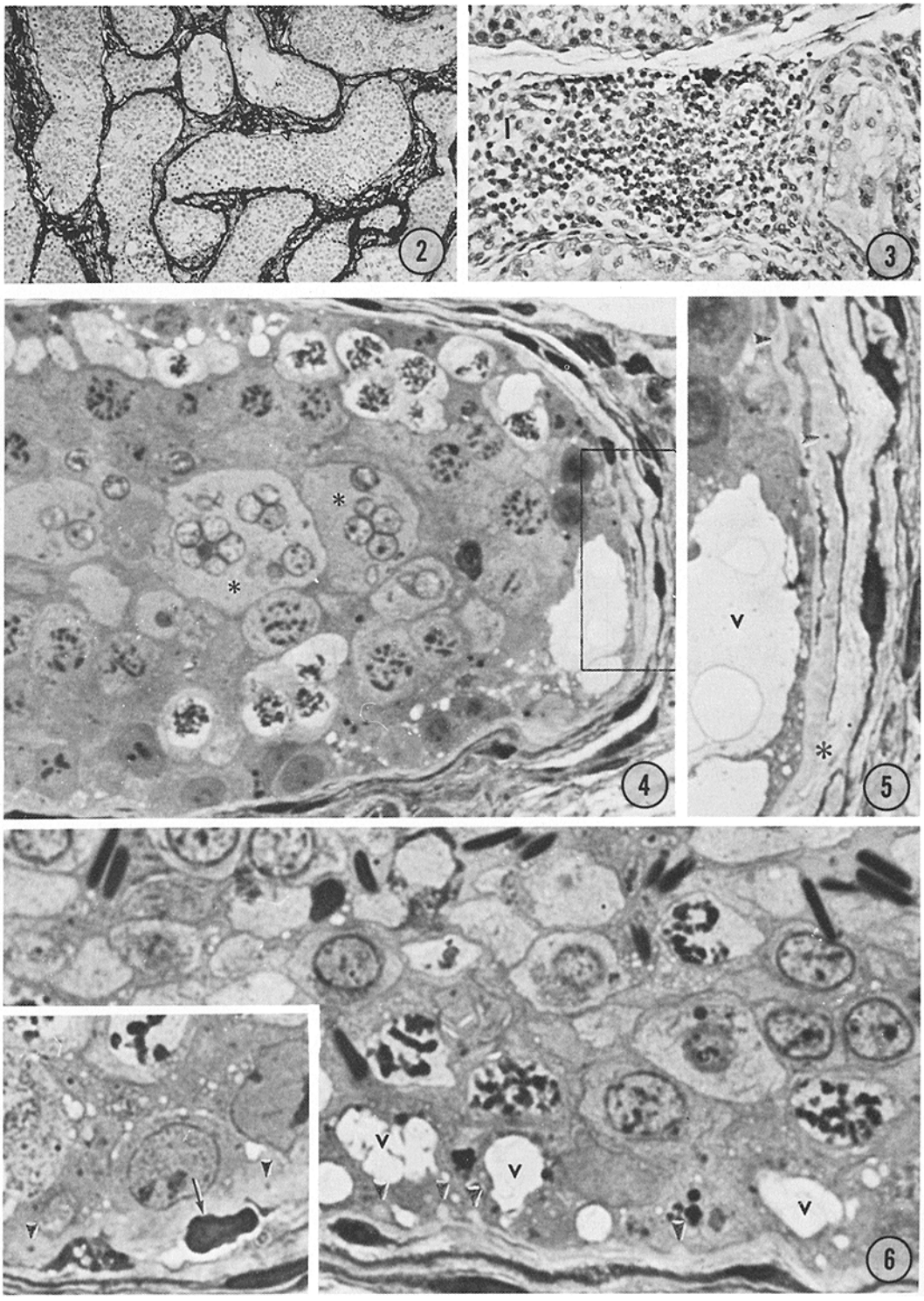
FIG. 2. Rabbit 37-10. Slight thickening of tubular basement membranes. Silver-methenamine staining. × 150.

FIG. 3. Infiltration of mononuclear cells in the interstitium (I) of rabbit 37-07. H & E × 250.

FIG. 4. Rabbit 38-12. The light micrograph shows absence of spermiogenesis and presence in the lumen of multinucleated spermatids (asterisks). The rectangular area in Fig. 4 is seen, at higher magnification, in Fig. 5. Toluidine-blue. × 800.

FIG. 5. The basement membrane (asterisk) is thickened and contains dense deposits (arrowheads). v, vacuole. × 1,600.

FIG. 6. Rabbit 37-10. Spikes (arrowheads) are seen on the epithelial side of the basement membrane. Several vacuoles (v) are present in the basal portion of the tubule. The inset shows part of a seminiferous tubule of rabbit 38-12. Arrowheads indicate dense deposits within the basement membrane. A macrophage (arrow) is near the basement membrane. Toluidine-blue. × 1,000.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*Morphological and Immunohistochemical Findings.* Rabbits with high and persistent levels of circulating antibody (group I) showed thickening of basement membranes of seminiferous tubules (Figs. 2, 4, and 5) and rare focal infiltrates of mononuclear cell in the interstitium (Fig. 3) in others. Occasionally, multinucleated spermatids (Fig. 4), vacuolization of the basal portion of the tubules (Fig. 5) as well as partial or total disappearance of Sertoli and spermatogenic cells were observed. In thin sections the main abnormalities of the basal lamina were: small areas of increased contrast (Figs. 5, and 6 inset), irregularities directed toward the epithelium (Fig. 6), and accumulation of mononuclear cells (Fig. 6 inset) and rare polymorphonuclear leukocytes.

By electron microscopy, the most characteristic finding was the deposit of electron-opaque material in the basement membranes of seminiferous tubules (Figs. 7–12 inset). In rabbits with less severe or initial lesions (group II), the deposits were found within the basement membrane proper (or lamina densa). In rabbits with more pronounced changes (group I) there was formation of new layers of epithelial basement membrane (Figs. 9, 10, and 12 inset) and development of "spikes" (Figs. 12, 13, and 16) directed toward the epithelium; the electron-opaque deposits, frequently surrounded by newly formed layers of epithelial basement membrane (Fig. 12 inset), were either localized within the spikes (Figs. 11, 13, and 16 inset) or between the collagenous layers of the basal lamina or in the basement membranes of interstitial vessels, in minimal amounts.

There was an increased number of lipid droplets in Sertoli cells (Fig. 7) and degenerative changes of the spermatids, with discontinuity of nuclear membranes, widening of the subacrosomal space, appearance of small vacuoles in the postnuclear region, and abnormal formation of the acrosome. Fusion of sperm nuclei with swelling, fragmentation, or loss of plasma membranes and acrosomal material (Figs. 14 inset, and 15) were also observed. Lymphocytes and monocyte-macrophages infiltrated the basal portion of the tubules (Figs. 7 and 12); the macrophages were frequently close to areas of the basement membrane which contained electron-opaque deposits, spikes, or both (Figs. 14, 16, and 16 inset).

Sertoli and spermatogenic cells had disappeared from tubules with most severe lesions (Fig. 17). Macrophages, monocytes, and lymphocytes, together with a few polymorphonuclear leukocytes, infiltrated the space between the collagenous basal lamina and the lamina densa. The manner in which these cells migrated through the basal lamina was not determined in this study. Parts of the lamina densa and the electron-opaque deposits were partially or completely surrounded by cytoplasmic projections of macrophages. In some areas, the lamina densa was disrupted or missing. Fragments of basement membrane or opaque material morphologically similar to dense deposits were seen within the cytoplasm of macrophages. The lumina of the tubules contained agglutinated and disrupted spermatozoa and macrophages engulfing and digesting sperm fragments. The collagenous basal lamina was thickened and showed increased amounts of collagen fibrils.

Granular deposits of rabbit IgG (Figs. 18–20) and C3 (Fig. 21) were demonstrated along the seminiferous tubules by IF. In rabbits with mild or initial

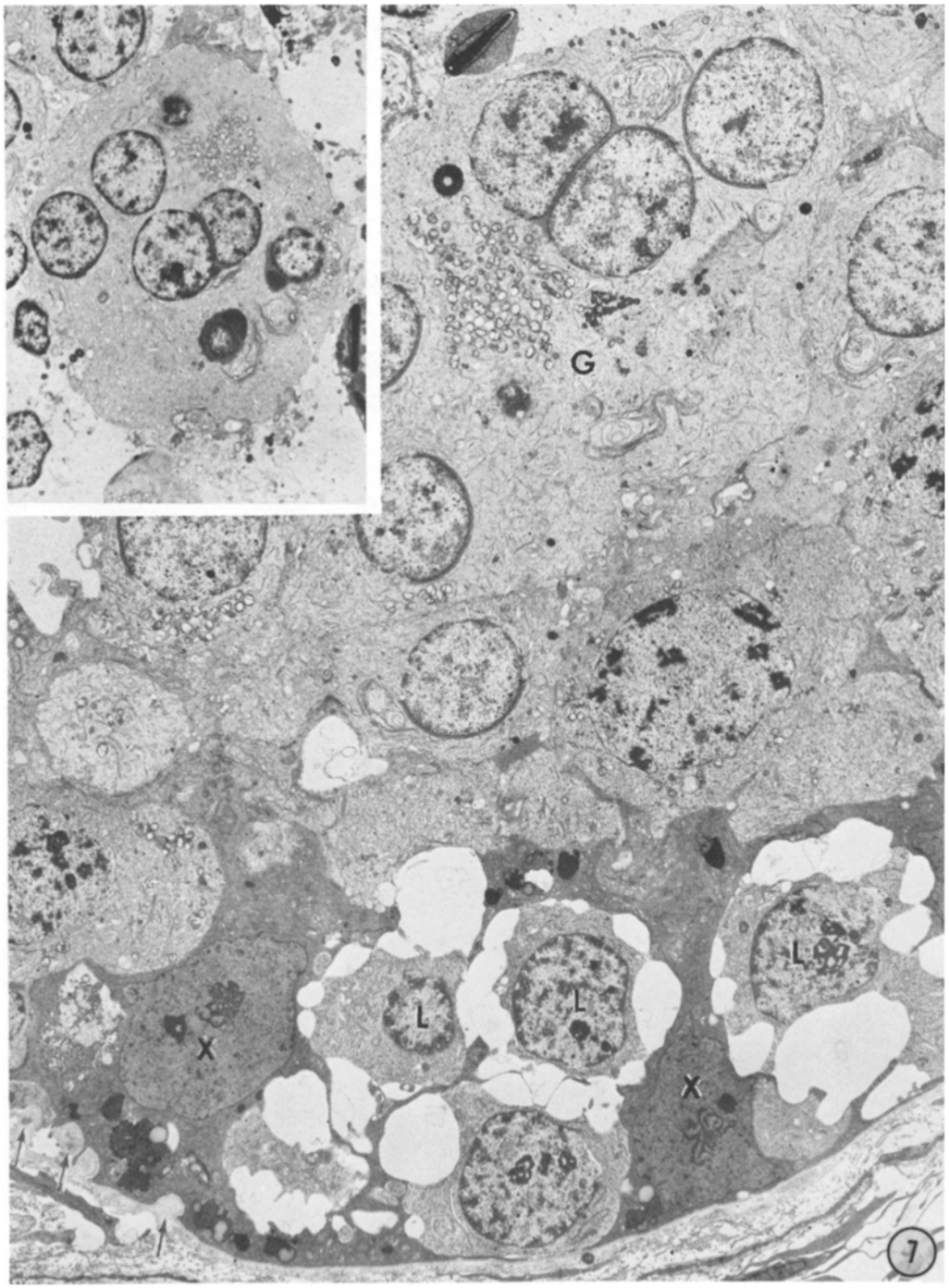
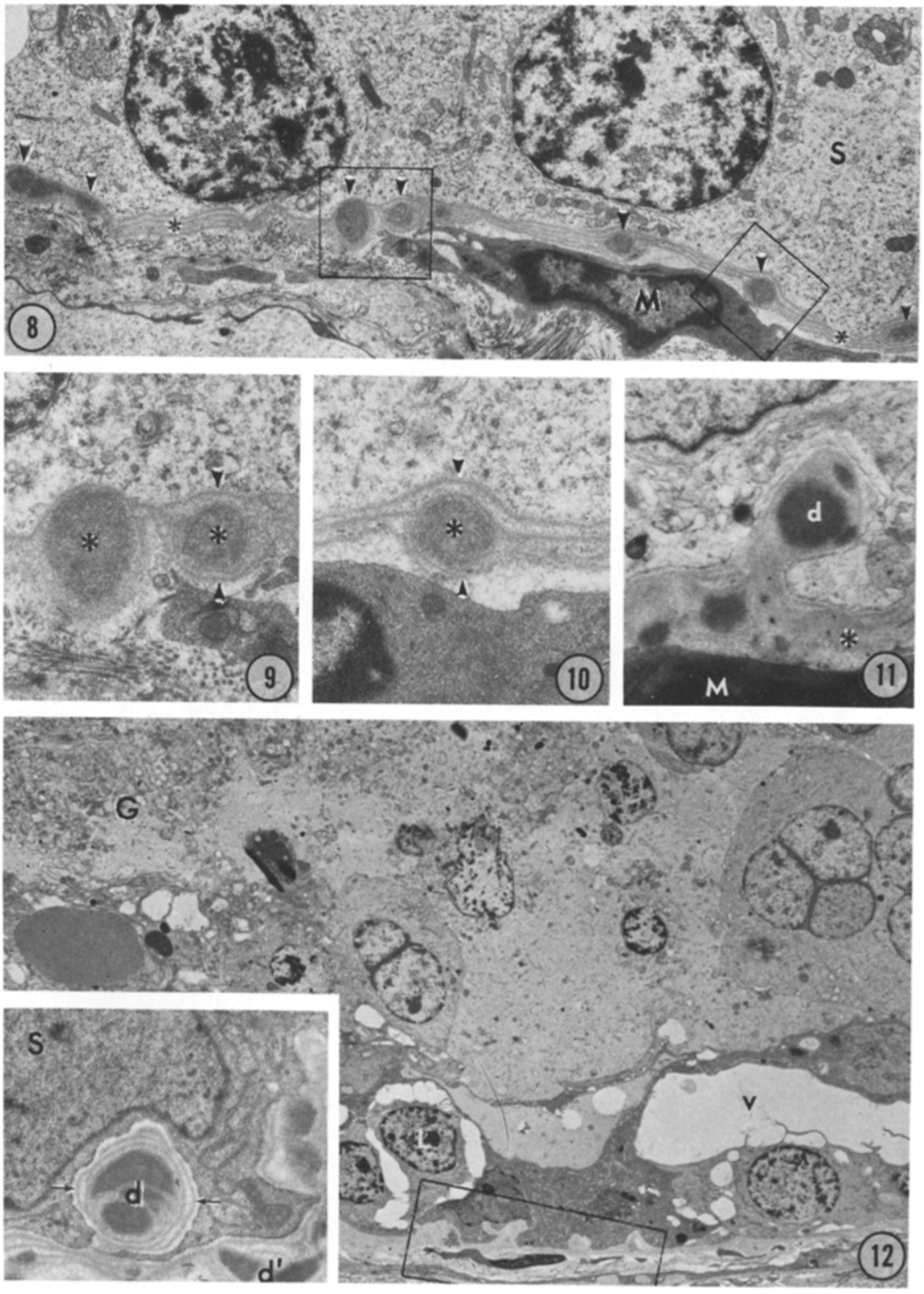



FIG. 7. Rabbit 40-19. Infiltrated lymphoid cells (L) are present between Sertoli cells (×) which contain lipid droplets. The arrows indicate spikes and electron-opaque deposits in the tubular basement membrane. G, multinucleated spermatid. × 4,000. The inset shows a multinucleated spermatid in the tubular lumen of rabbit 40-19. × 4,000.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changes, the deposits were seen along the lamina densa (Fig. 19) that appeared in proximity to the collagenous basal lamina; in contrast, in severely damaged tubules the immune deposits were separated from the collagenous basal lamina by a space containing macrophages (Fig. 19).

The results of the elution experiments are summarized in Table II. Immunoglobulin deposits in testicular sections were not removed by incubation in PBS for 1 h or longer at room temperature. In contrast, there was a partial elution of deposits, associated with marked alteration of tubular structures, after incubation in citrate buffer, NaSCN, and KI under the same conditions of time and temperature. Incubation in PBS for 1 h at 56°C greatly decreased the intensity of fluorescence of granular deposits without apparent damage of tubular structures. The fluorescence, however, was practically eradicated by the other buffers. These results led to the design of experiments investigating the possibility that immune deposits indeed contained antigen-antibody complexes. Thus, sections of testis from rabbit 37-07 and 37-10, partially eluted with PBS at 56°C for 1 h, were stained with fluorescein-conjugated rabbit antisperm antibody. Small granular deposits were detected along the basement membranes of the tubules (Fig. 2). The staining pattern corresponded to that of the IgG and C3 deposits; moreover, it corresponded to the position of the electron-opaque deposits seen by electron microscopy. Fluorescent deposits were not observed in sections which were similarly eluted but stained with fluorescein-conjugated gamma globulin from rabbits before vasectomy or from rabbits with sham-operation. Likewise, no fluorescent deposits were detectable in uneluted sections.

The rabbit antisperm antibody employed in the foregoing experiments for the detection of the antigens in granular deposits of rabbits with orchitis was used in indirect IF for identification of antigens in sections of normal rabbit testis as well as in isolated spermatozoa.

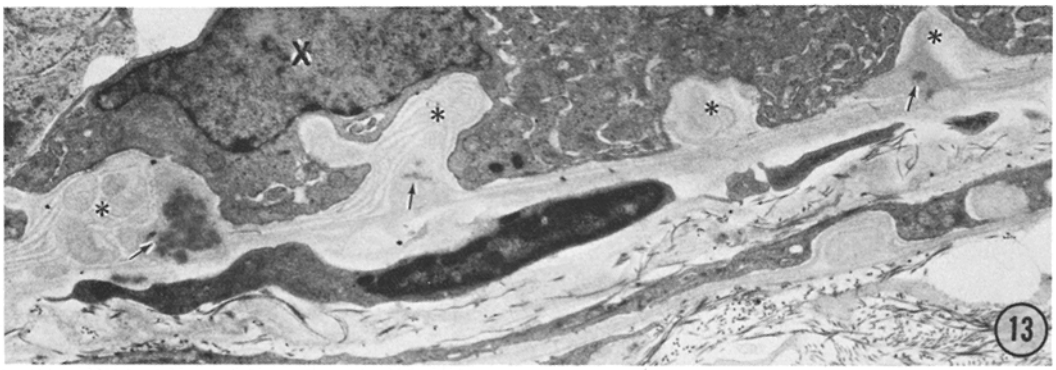
In frozen sections of normal rabbit testis the rabbit antisperm antibody reacted with the apical germinal cells; in isolated rabbit sperms it stained the acrosomal region (Figs. 24 and 26 inset). Parallel experiments of indirect immunoelectron microscopy were performed on isolated sperms. Ferritin-conjugated antibody was bound to the plasma membranes of the head of sperms (Figs. 25 and 26). In contrast, absence, or a minimal amount of ferritin only, was found in

FIG. 8. Rabbit 38-12. Electron-opaque deposits (arrowheads) are present within the basement membrane (asterisks) of a seminiferous tubule. S, spermatogonium, and M, macrophage. The boxed areas are seen, at higher magnification, in Figs. 9 and 10. × 7,000.

FIGS. 9 and 10. These show three electron-opaque deposits (asterisks) between the split layers (arrowheads) of the lamina densa. × 14,000.

FIG. 11. Rabbit 38-64. Deposits of marked electron opacity are present in the basement membrane (asterisk) and within a spike (d). M indicates part of a macrophage. × 14,000.

FIG. 12. Rabbit 37-10. The picture shows degenerative changes of germinal cells (G), vacuoles (v), and an infiltrated lymphoid cell (L). The boxed area, seen at higher magnification in Fig. 13, shows spikes on the epithelial side of the basement membrane. × 2,000. In the inset electron-opaque deposits (d') are seen in the basement membrane. Another deposit (d) is surrounded by newly formed concentric layers of epithelial basement membrane (arrows). S, spermatogonium. × 12,000.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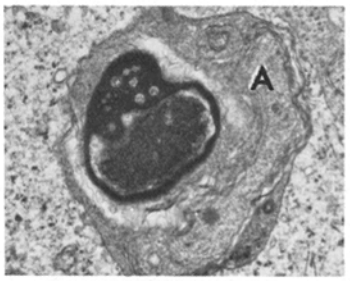
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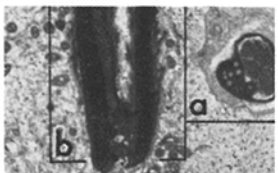
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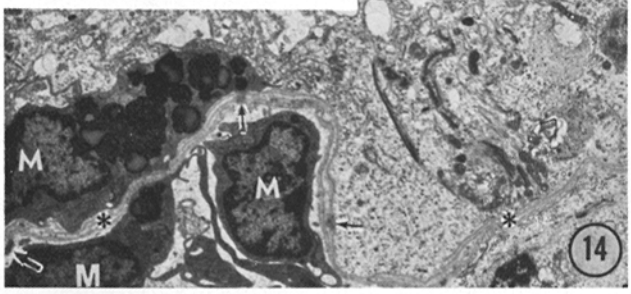
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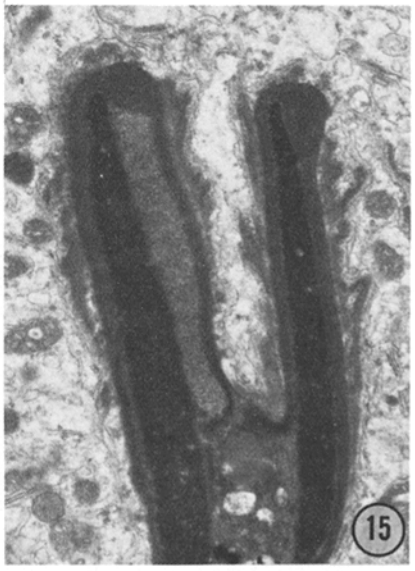
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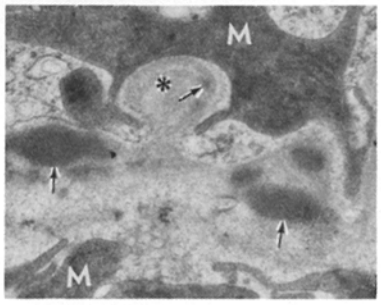
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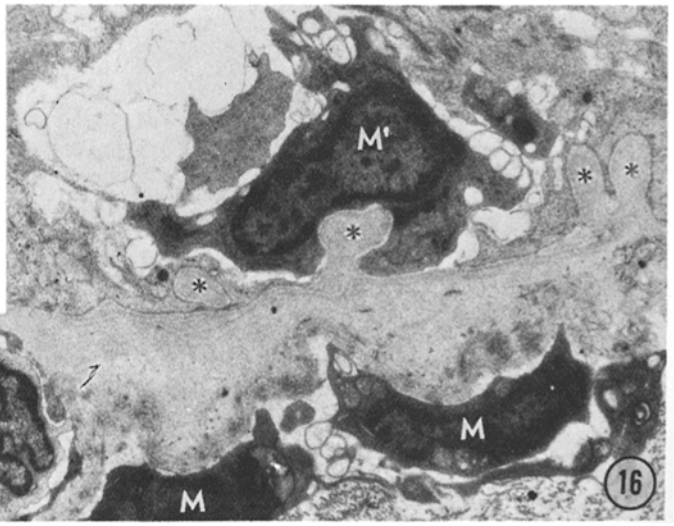
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the neck and tail regions (Figs. 26). When the plasma membranes of the head were fragmented or removed by virtue of poor fixation and cell handling, thus leaving the acrosome partially or fully exposed, ferritin granules then localized in the acrosomal material (Figs. 27 and 28). Controls for the foregoing experiments were carried out by prior incubation of normal testicular tissue, or isolated normal rabbit sperms, with antiserum to measles virus or with the serum from rabbit 37-07 before vasectomy, followed by treatment with fluorescein or ferritin-conjugated antibody to rabbit IgG. No specific localization was seen (Figs. 29 and 29 inset).

The renal glomeruli of rabbit 37-07 and 37-10 had increased amount of mesangial matrix and mild mesangial cell proliferation. Granular deposits of rabbit IgG (Fig. 30) and C3 (Fig. 31) were present in the mesangium and in peripheral capillary walls, in minor amounts. Corresponding opaque deposits were seen in the mesangium by electron microscopy. Staining of uneluted or partially eluted renal sections with the same fluorescein-conjugated rabbit antisperm antibody used for demonstration of sperm antigens in the testes of rabbits with orchitis, failed to detect such antigens in glomerular deposits. There was no histologic or immunofluorescent evidence of glomerular lesions in the other rabbits of group I.

The eluates obtained from the testes or from the kidneys of rabbits 37-07 and 37-10 were tested using indirect IF technique with normal rabbit testis or isolated sperms as substrates. In frozen sections of testis the testicular (Fig. 23) and renal eluates reacted with the apical germinal cells of the seminiferous tubules. When tested with smears or suspensions of isolated normal rabbit spermatozoa the testicular (Fig. 32) and the renal (Fig. 33) eluates reacted with the acrosome. Eluates from the testes or from the kidneys of rabbits with no circulating antibodies to sperm antigens or of rabbits with sham-vasectomy or normal rabbits did not stain sections of normal rabbit testis nor the acrosome of isolated sperms.

The testes of rabbits with transient and/or low levels of circulating antibody (group II) appeared normal by light microscopy. Only minimal and focal granular deposits of IgG and C3 were found along the basement membranes of seminiferous tubules. The testes of vasectomized rabbits with no circulating antibody (group III) were consistently normal by light, electron, and IF micros-

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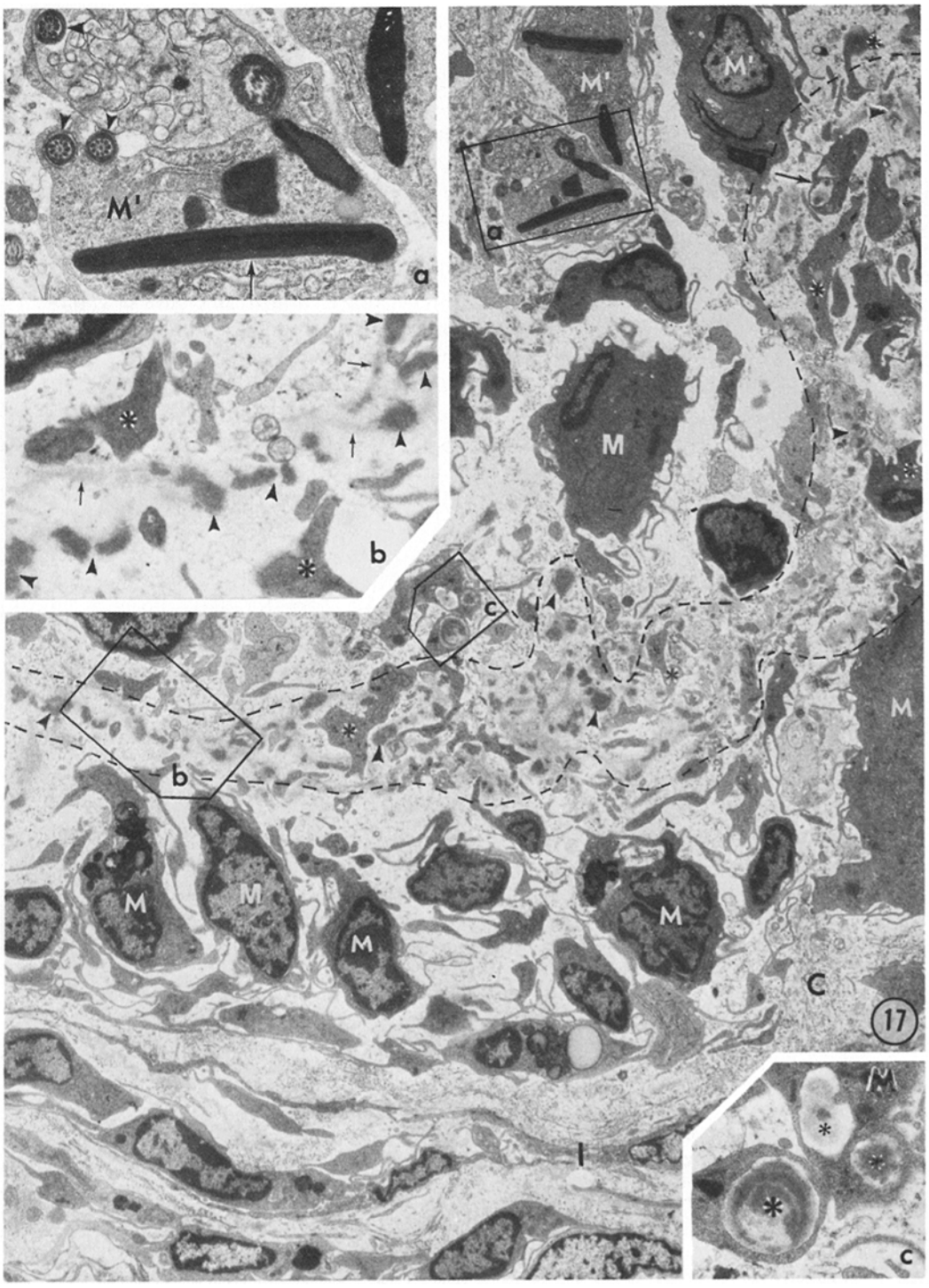
FIG. 13. Rabbit 37-10. Spikes (asterisks) on epithelial side of basement membrane. The arrows indicate electron-opaque deposits. X, Sertoli cell. × 8,000.

FIG. 14. Rabbit 37-07. Macrophages (M) are seen on both sides of the basement membrane (asterisks). The arrows indicate electron-opaque deposits. × 4,000. The inset (higher magnification of boxed area *a* shows a cross-section of a spermatozoon. The cytoplasm (A) is swollen and distorted. × 12,000.

FIG. 15. An enlargement of boxed area *b* of Fig. 14. There is fusion of two sperm heads and fragmentation of cell membranes and acrosomal material. × 16,000.

FIG. 16. Rabbit 37-10. Macrophages (M) are present on both sides of the basement membrane which is thickened and shows epithelial spikes (asterisks). One spike is partially phagocytized by a macrophage (M'). × 12,000. The inset shows a macrophage (M) seemingly engaged in phagocytosis of another spike (asterisk). Electron-opaque deposits (arrows) are present within the spike and in the basement membrane. × 15,000.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copy. Likewise, rabbits with sham-vasectomy (group IV) did not develop antibody and their testes were normal. There was no histologic evidence of lesions in the lung, liver, spleen, pancreas, intestine, and muscle in the rabbits of all the four groups.

## Discussion

The results of the present study show that the bilaterally vasectomized rabbits which have elaborated antibodies to sperm antigens, develop an orchitis associated with granular deposits of rabbit IgG, C3, and sperm antigens in the basement membrane of seminiferous tubules. The granular pattern of IF corresponds to opaque deposits seen in the same area by electron microscopy and is comparable to that observed in renal glomeruli of humans or animals with immune complex diseases (16).

The pathogenetic role of the immune deposits and the possibility that they contain antigen-antibody complexes is suggested by: (*a*) selective accumulation of IgG along the basement membrane of seminiferous tubules of vasectomized rabbits with high and persistent levels of circulating antibodies to sperm antigens; (*b*) absence of other serum components, such as albumin and fibrinogen, in the tubular deposits of the same animals; (*c*) concomitant presence of C3 and IgG deposits; (*d*) possibility to elute or dissociate the immunoglobulins from the tissue with chaotropic ion-containing buffers, low pH buffers, or heat; and (*e*) detection in partially eluted testicular sections of granular deposits of sperm antigens, in a location similar to that of IgG and C3, using fluorescein-conjugated specific antibody.

The studies designed to establish the specificity of antibodies contained in the sera or testicular eluates from rabbits of group I, showed that the immunoglobulin fractions bound in an identical manner to the germinal cells localized in the apical part of the tubules or to the acrosomes of isolated spermatozoa. The experiments of immunoelectron microscopy demonstrated that the same antibody was bound to the cell membrane of the sperm head and, more intensely, to the acrosomal and subacrosomal material. Thus, these results suggest that acrosomal antigens are the most important, if not the sole factors, involved in the formation of immune complexes responsible for orchitis.

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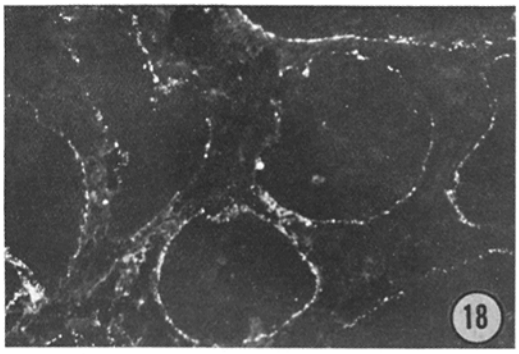
FIG. 17. Rabbit 37-10. Severe, terminal changes in a seminiferous tubule. The Sertoli and germinal cells have disappeared. Macrophages (M) are seen between the collagenous basement membrane (I) (containing an increased amount of collagen fibrils [C]) and remnants of the basement membrane proper (or lamina densa). The remnants of this latter structure, electron-opaque deposits (arrowheads), and pseudopodia of macrophages (asterisks) are seen in the space between the two dotted lines. Two arrows indicate parts of macrophages that phagocytize electron-opaque deposits. × 4,000. The inset *a* shows, at higher magnification, macrophages (M') which have ingested parts of spermatozoa; the arrow indicates the nucleus of a sperm and the arrowheads point to parts of sperm tails. × 12,000. The inset *b* illustrates fragments of lamina densa (arrows), electron-opaque deposits (arrow heads), and pseudopodia of macrophages (asterisks). × 12,000. Inset *c* shows three electron-opaque deposits (asterisks) surrounded by pseudopodia of a macrophage (M). × 12,000.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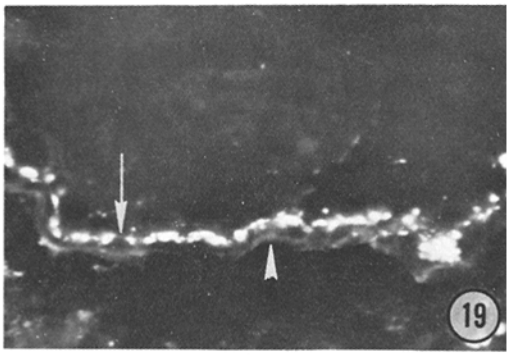
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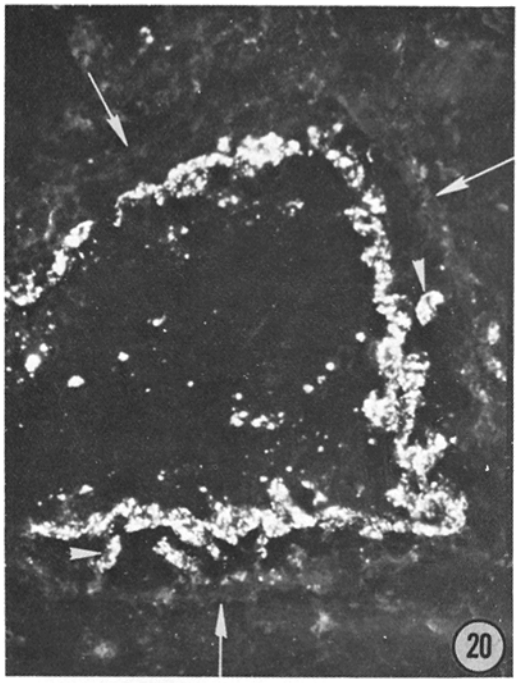
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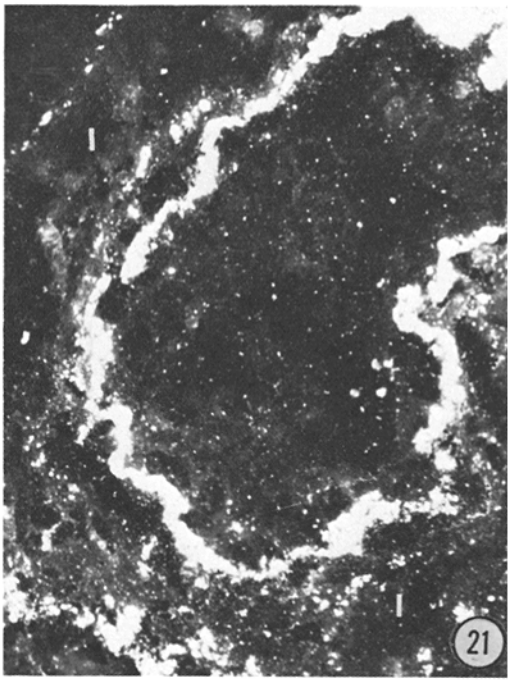
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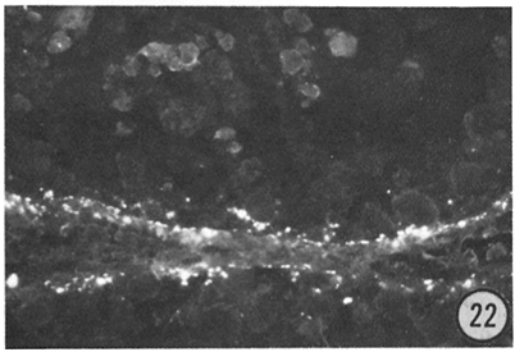
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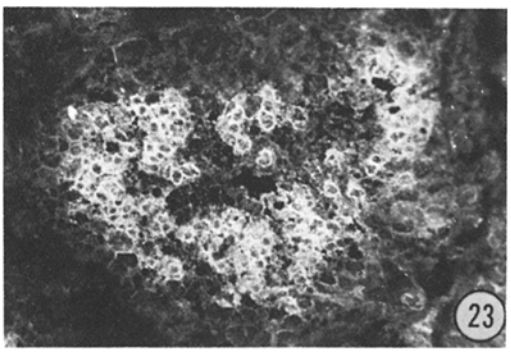
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TABLE II
*Effect of Elution Procedures on Immunoglobulin Deposits in Testis*

| Elution buffers | Incubation for 1 h | |
|---|---|---|
| | Room temperature | 56°C |
| PBS | +++* | + |
| Citrate buffer, 0.02 M, pH 3.2 | ++ | ± |
| NaSCN 2 M | + | − |
| KI | + | − |

* (+), Amounts of IgG, detectable by IF, remaining after elution.

A direct correlation was observed between levels and persistence of circulating antibody to sperm antigens, amount of immune deposits in the testis, and severity of histologic lesions. The tubular changes were characterized by electron-opaque deposits in the basement membranes, formation of new layers of epithelial basement membrane, development of "spikes," which probably represented excessive production of epithelial basement membrane in response to focal injury (17, 18), and overall thickening of the basal lamina. These alterations are similar to "membranous" lesions produced by antigen-antibody complexes in glomerular capillary walls of patients with membranous glomerulonephritis (19), of rabbits with chronic serum sickness glomerulonephritis (20), and of rats with Heymann nephritis (21). The membranous changes were accompanied by tubular infiltration of lymphocytes, monocytes, and a few polymorphonuclear leukocytes. Since only rabbits with a long-standing vasectomy were examined in this study, it was impossible to elucidate the precise mechanism of damage of seminiferous tubules and especially whether the initial destruction of

FIG. 18. Rabbit 38-12. Section of testis stained with fluorescein-conjugated goat antirabbit IgG. Granular deposits are present around the seminiferous tubules. × 150.

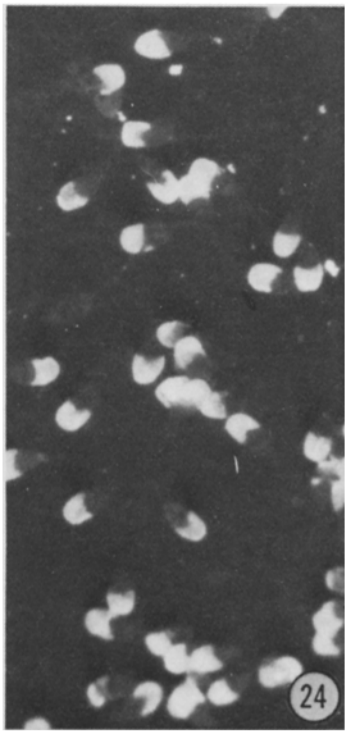
FIG. 19. Rabbit 38-12. Granular deposits of IgG are seen along the basement membrane proper (arrow) which is in close proximity to the collagenous basal lamina (arrowhead). × 600.

FIG. 20. Rabbit 37-07, shows a tubule with more severe damage. The granular deposits of IgG are separated from the collagenous basal lamina (arrows) by a space containing macrophages (arrowheads) that have phagocytized immune deposits. × 600. Comparable features are shown by electron microscopy in Fig. 16.

FIG. 21. Rabbit 37-07. Granular deposits of rabbit C3 around a seminiferous tubule and in the interstitium (I). Small deposits of C3 are also present between tubular cells. × 600.

FIG. 22. Rabbit 37-07. Section of testis eluted with PBS at 56°C for partial removal of immunoglobulin deposits. The section was then stained with fluorescein-conjugated antibody to acrosomal antigens which showed small granular deposits along the basement membrane. × 600.

FIG. 23. Section of a normal rabbit testis stained with the testicular eluate from rabbit 37-07. Fluorescence is localized in the cells of the apical part of a seminiferous tubule. × 250.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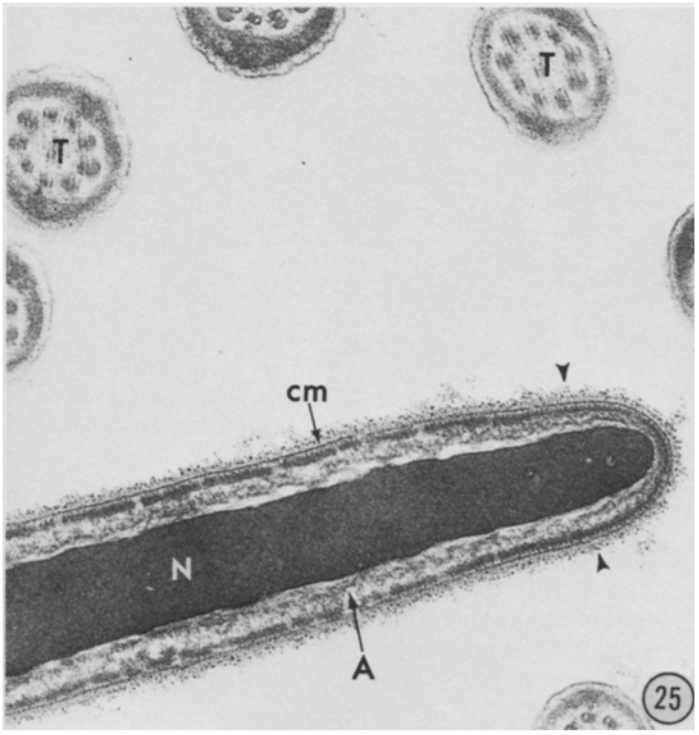
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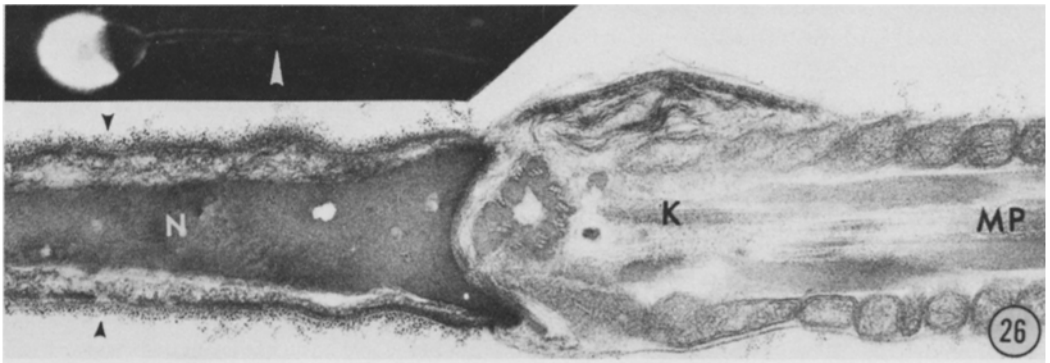
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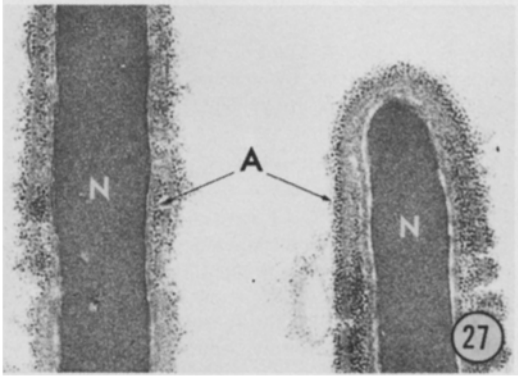
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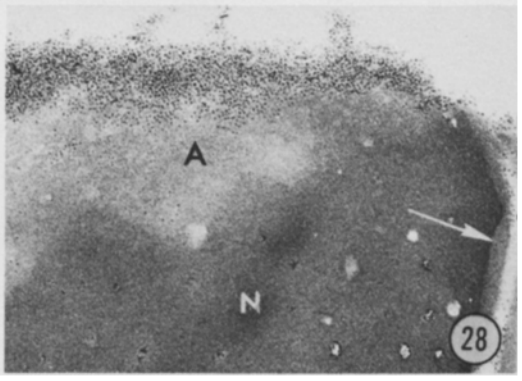
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28

the lamina densa and of the germinal epithelium was due to injury resulting from material released from polymorphonuclear leukocytes (22) or to phagocytic activity of monocyte-macrophages (23).

The rare infiltrates of mononuclear cells seen in the interstitium of rabbits of group I might result in part from a delayed hypersensitivity reaction triggered by antigens of the basement membrane or epithelial cells. An alternative explanation is that mononuclear cell infiltration is mediated by humoral antibody (24) or by antigen-antibody complexes, attracting mononuclear cells with complement receptors (25).

The pathogenesis of membranous immune complex orchitis, postulated in vasectomized rabbits of group I, would probably be similar to that of an Arthus reaction in which antigen-antibody complexes result from the union of circulating antibody with sperm antigens as they diffuse out of the seminiferous tubules. Soluble immune complexes preferentially precipitate within the basement membrane as a consequence of "sterical exclusion" which decreases their solubility during filtration through polysaccharide media (26, 27). A comparable *in situ* formation of immune complexes has been described in rats injected with homologous kidney suspension (28) and in experimental thyroiditis of guinea pigs (29) or mice (30).

The initial immune response which follows long-term vasectomy is conceivably due to the disintegration of spermatozoa that lack an exit passage (31) and release their products into circulation and thereby, provide the stimulus for antibody formation (32). The specific antibodies and complement may contribute to further damage of spermatozoa which are still being produced (33, 34) since serum proteins can easily cross a blood-testis barrier made more permeable by vasectomy (35) or by inflammatory reaction (36).

In addition to primary membranous orchitis, a secondary tissue damage might result from the immune complexes formed in circulation when sperm antigens are released in large amounts from damaged seminiferous tubules. Under this condition, the antigens that have reacted with circulating antibod-

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FIG. 24. Sperm from a normal rabbit incubated with rabbit serum containing antiacrosomal antibody (rabbit 37-07, Table I) and then stained with fluorescein-conjugated goat antirabbit IgG. Fluorescence is present in the acrosomal caps. × 400.

FIGS. 25–28. These show the results of experiments in which sperms from normal rabbits were first treated with the serum containing antiacrosomal antibody (rabbit 37-07, Table I) before incubation with ferritin-conjugated goat anti-rabbit IgG.

FIG. 25. Ferritin (arrowheads) is localized on a cell membrane (cm) of a sperm head. Ferritin is not bound to the cell membrane of the tail (T). A, subacrosomal space; and N, nucleus. × 35,000.

FIG. 26. Shows a longitudinal section of the central part of a sperm. Ferritin is localized on the surface of the sperm head (arrowheads). Only a few ferritin granules are bound to the neck (K) and to the midpiece (MP). × 35,000. The inset illustrates one sperm treated in the same manner as in Fig. 24. Fluorescein-conjugated antibody is localized in the acrosomal part of the spermatozoon. The tail (arrowhead) is negative. × 1,000.

FIGS. 27 and 28. Cross or oblique sections of sperm heads, respectively. As a consequence of poor fixation and handling, the cell membrane and parts of the acrosomal membranes have disappeared. Ferritin is localized in the acrosomal or subacrosomal material (A). N, nucleus. The arrow of Fig. 28 indicates the basal plate of the sperm. × 35,000.

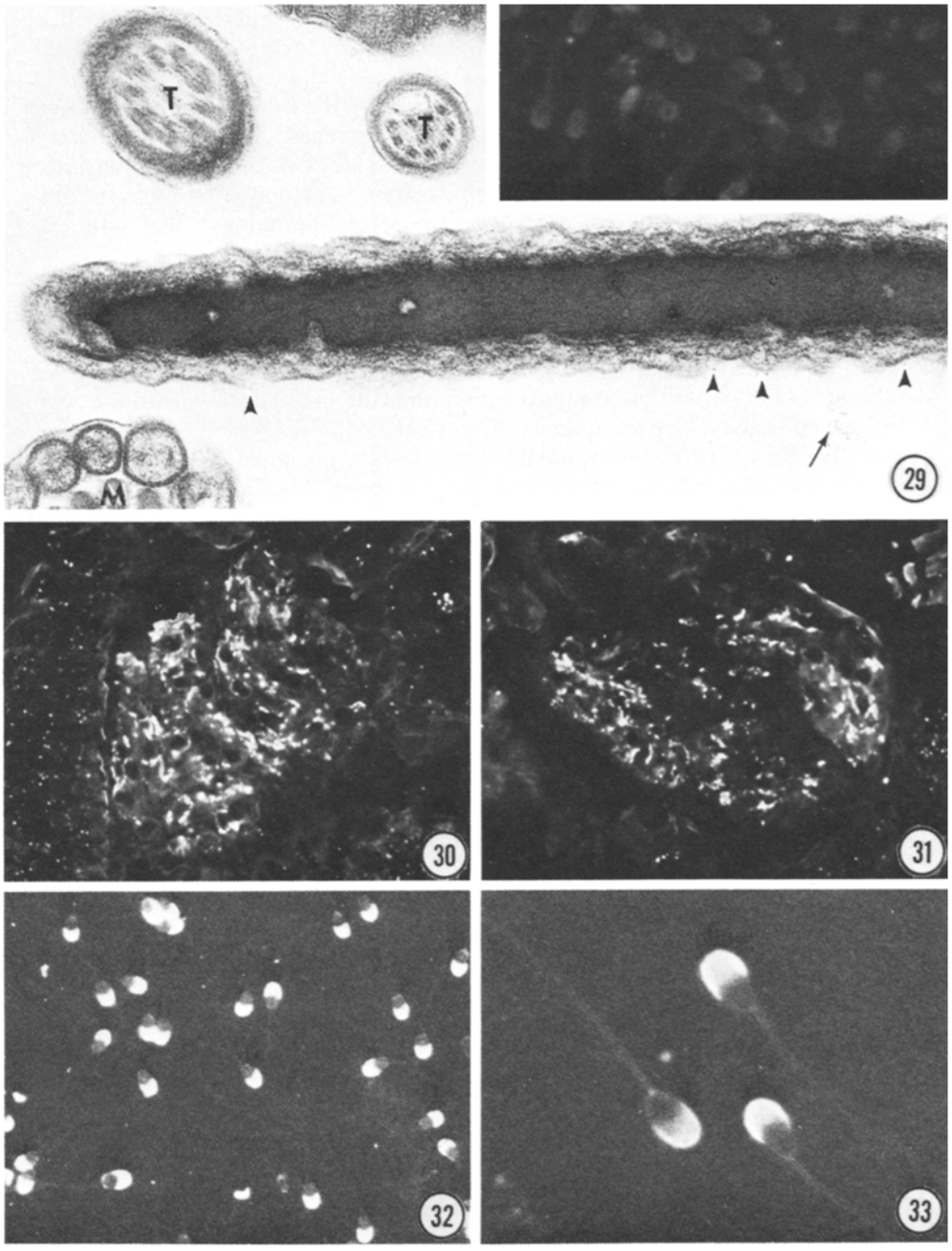


FIG. 29. The photograph shows the results of an experiment in which sperms from a normal rabbit were incubated with the serum obtained from rabbit 37-07 before vasectomy and then treated with ferritin-conjugated antibody to rabbit IgG. Only a few ferritin granules are bound to the cell membrane of the sperm head (arrowheads). The arrow indicates unbound ferritin granules; T, cross-sectioned tails; and M, a midpiece. × 35,000. The inset shows the results of a similar experiment performed with fluorescein-conjugated goat antirabbit IgG. The sperms are not stained. × 400.

ies, initiate a sequence of events similar to that of serum sickness (37). Two rabbits of group I, with higher levels of antibodies and severe membranous orchitis, developed a mild glomerulonephritis accompanied by granular deposition of immunoglobulins and complement in mesangium and, in minor amounts, in peripheral capillary walls. Opaque deposits were seen in the mesangium by electron microscopy. Acrosomal antigens could not be demonstrated in glomerular structures together with IgG and C3 by direct immunofluorescence; this could signify that IgG and C3 only, were present in renal glomeruli. It is also possible, however, that the antigens involved in the formation of immune complexes localized in the kidneys were not easily detectable by IF due either to their low concentration, to denaturation, or to blanketing by antibody excess (38). The elution from renal glomeruli of antisperm antibodies is consistent with the hypothesis that the same antigen-antibody complexes found in the testes are also localized in the kidneys. If confirmed, this phenomenon would be comparable to that observed in experimental thyroiditis of the mouse when the amount of thyroglobulin available for formation of complexes is artificially increased (39).

Vasectomy, the most common method of permanent male sterilization, involves surgical intervention in a healthy individual. An obvious concern is whether this procedure causes long-term local or systemic disease. Scattered reports have increased this concern suggesting a correlation between vasectomy and damage of the testis or diseases in organs far from the surgical site, such as thrombophlebitis, arthritis, and even glomerulonephritis (40). The present results suggest that further studies of testis and kidney are necessary in order to clarify the possible side effects of antigen-antibody complexes in vasectomized men who frequently develop high levels of antibodies to sperm antigens (41).

## Summary

The results of the present study show that bilaterally vasectomized rabbits with high levels of antibodies to sperm antigens frequently develop an orchitis associated with granular deposits of rabbit IgG and C3 in the basement membranes of seminiferous tubules. The immune deposits correspond in location to electron-opaque deposits seen by electron microscopy. The "membranous orchitis" is characterized by thickening of tubular basement membranes, accumulation of macrophages and a few polymorphonuclear leukocytes, and destruction of the basal lamina, of the Sertoli and spermatogenetic cells.

The pathogenetic role of the immune deposits and the possibility that they

FIG. 30. Section of kidney from rabbit 37-07 stained with fluorescein-conjugated antibody to rabbit IgG. Granular deposits are seen in glomerular capillary walls and in the mesangium. × 400.

FIG. 31. Kidney section from 37-07 stained with fluorescein-conjugated antibody to rabbit C3. Granular deposits are present in glomerular structures. × 400.

FIG. 32. Sperms from a normal rabbit incubated with gamma globulin eluted from the testes of rabbits 37-07 and 37-10 and then stained with fluorescein-conjugated goat antirabbit IgG. Fluorescence is shown in the acrosome of the sperms. × 400.

FIG. 33. Experiment similar to that illustrated in Fig. 32, but performed with gamma globulin eluted from glomeruli of rabbits 37-07 and 37-10. Fluorescent staining is localized in the acrosomal caps. × 800.

contain antigen-antibody complexes is indicated by: (*a*) selective accumulation of IgG and C3 granular deposits along the basement membranes of seminiferous tubules in rabbits producing high and persistent levels of antibodies to sperm antigens; (*b*) the elution of immunoglobulins from tissues with chaotropic ion-containing buffers, acid buffers, or heat; (*c*) the observation that the immunoglobulins accumulated in the testis contain antibody to sperm antigens; and (*d*) the demonstration of sperm antigens in a location similar to that of IgG and C3. It is postulated that sperm antigen-antibody complexes are formed in the basement membranes of seminiferous tubules when antigens leaking out of the tubules react with specific antibody coming from the circulation.

In two rabbits with higher levels of circulating antisperm antibodies and severe orchitis, granular deposits of IgG and C3 were also present in renal glomeruli. Immunoglobulins eluted from the kidneys contained antibody with antisperm activity. These findings are consistent with the hypothesis that in some vasectomized rabbits extratesticular lesions may develop by a mechanism comparable to that of chronic serum sickness.

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