IMMUNOLOGICAL EFFECTS OF THERMAL INJURY

I. Inhibition of Spermatogenesis in Guinea Pigs*

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It is generally agreed that thermal injury in patients and experimental animals may be associated with a variety of immunological abnormalities (1). Burned subjects have been shown to accord prolonged survival times to skin allografts (2), and this change has been associated with a diminution of their ability to exhibit delayed hypersensitivity reactions (3). In addition, the studies of Rosenthal (4) and other workers (5–11) have provided evidence for the formation of anti-skin antibodies in burned men, horses, dogs, rabbits, rats, and mice. Recently, McCarthy and associates (12, 13) have described antibodies to autologous erythrocytes in burned rats, detectable by direct antiglobulin tests. A hemagglutinin directed against syngeneic erythrocytes was then demonstrated in the thoracic duct lymph of burned rats (14). In addition, "heterophile" antibodies against rat erythrocytes and human gamma globulin were detected in guinea pigs and rabbits exposed to repeated thermal injury, and in some patients with severe skin burns (15). It has been suggested that the appearance of auto- and heteroantibodies after thermal injury may be a consequence of the release of intracellular antigens and/or alteration of autologous antigens (16).

These considerations have raised the possibility that thermal injury might be of value as an experimental model for further investigations of mechanisms of induction of autoimmune responses. In the present study, controlled burns of one testis have induced the formation of testis-specific antibodies in guinea pigs. In addition, pathological changes have been noted in the germinal components of the contralateral testis. This response bears a similarity to the results of immunization with suspensions of testis incorporated in complete Freund's adjuvant (17). The pertinence of the results obtained by thermal

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injury to those noted after active immunization with testicular antigens will be described and discussed.

Materials and Methods

Experimental Animals and Method of Thermal Injury.—Male outbred guinea pigs of the Hartley Strain, weighing 450–500 g and maintained on a standard Purina pellet diet, were used. Testicular damage was produced under general ether anesthesia. Hair was removed from the lower abdomen and scrotum with electrical clippers. Under sterile precautions, an incision measuring approximately 20 mm was made over the surface of the left testis, penetrating the scrotal sac and tunica vaginalis. The testis, surrounded by the tunica albuginea, was delivered into the wound, and its entire thickness was punctured four times with a Bi-active Electrode (Model No. 789CC, Birtcher Corp., Los Angeles, Calif.). The electrode was connected to a Birtcher Hyfrecator (Model No. 709), delivering an electrical current at a rheostat setting of 75, given with each puncture for 4 sec, and yielding an average temperature of 220°C at the burn site. The testis was returned to the scrotal sac, and the wound was closed in layers with interrupted 4.0 silk sutures. Particular care was taken not to disturb the right testis or tunica vaginalis during exposure and burning of the left testis. 39 guinea pigs were subjected to thermal injury by this technique; 34 other animals received similar treatment, but only with application of the unheated electrode. Serum samples were obtained from each animal by cardiac puncture before the experiment, and at weekly intervals thereafter for the following 8 wk. In addition, a number of animals was exsanguinated at various intervals after injury. All serum samples were stored at −20° until used.

Preparation of Tissue Extracts.—Frozen testes from mature exsanguinated guinea pigs of the Hartley Strain and from mice, rats, rabbits, and dogs were purchased from the Pel-Freez Co., Rogers, Ark. The organs were freed of all surrounding connective tissue, including the tunica albuginea; they were minced with fine scissors, and processed in a Virtis Homogenizer for 15 min at 6°–8°C. A 40% (w/v) homogenate was then prepared in 0.14 M saline solution. This suspension was disrupted at 20,000 cps for 1 min at 4°–6°C, using an ultrasonic disintegrator (Biosonik, BronWill Scientific, Rochester, N.Y.). After sonication, the suspension was centrifuged at 57,348 *g* for 30 min at 5°C in a Spinco Model L ultracentrifuge. The supernatant solution was removed and adjusted to a protein concentration of 10 mg/ml. This preparation, termed "testicular extract," was stored at −20°C. Similar extracts were prepared from guinea pig brain, heart, lung, liver, and spleen. These organs were obtained from normal animals exsanguinated in the laboratory.

For some experiments, testicular extract was prepared from the right testis of each guinea pig, which was removed before burning the left testis. This was the "autologous" testicular extract.

Serological Reactions.—

(*a*) *Doube diffusion gel precipitation:* This test was performed in Petri dishes containing 1% Difco Noble agar with 0.01% thimerosal. The wells containing the sera were 3 mm deep, with a diameter of 3 mm; those containing the tissue extracts were 3 mm deep, with a diameter of 5.5 mm. The diffusion distance between the antigen- and antibody-containing wells was 3 mm.

(*b*) *Absorption of antisera:* 1 ml samples of serum were mixed with 10–100 mg of dry powder obtained by lyophilization of tissue extracts of guinea pig, mouse, rat, rabbit, and dog testes, and of guinea pig brain, heart, lung, liver, and spleen. The mixture was shaken on an electric shaker for 30 min and left for 1 hr at room temperature. The preparation was then centrifuged at 29,000 *g* at 4°C for 30 min, and the absorbed serum was removed and stored at −20°C.

(*c*) *Immunoelectrophoresis:* The modification described by Scheidegger (18) was used, with 1% Difco Noble agar in barbital buffer at pH 8.6 and ionic strength of 0.075. Rabbit antiserum to guinea pig gamma globulin was obtained from Hyland Laboratories, Los Angeles, Calif.

(*d*) *Passive cutaneous anaphylaxis (PCA) test:* The method described by Ovary and associates (19, 20) was used; each serum sample was tested in three guinea pigs. For this purpose, undiluted serum samples were injected intradermally in 0.1 ml volumes at six sites on the shaved backs of albino guinea pigs weighing approximately 250 g. 4 hr later, the animal received an intravenous injection of 1.0 ml of a mixture consisting of equal volumes of tissue extract and Evans Blue Dye. The occurrence of an antigen-antibody reaction was evidenced by the appearance of bluish areas at the corresponding skin test sites. These usually developed within 10 min after intravenous injection of the antigen-dye mixture. However, all animals were observed for at least 6 hr; at that time, they were sacrificed, and the subcutaneous surfaces were inspected.

TABLE I

Detection of Testis Antibodies by Double Diffusion Gel Precipitation

Serum samples	Number of animals studied	Number of animals which formed antibodies to guinea pig testis	% of positive reactions
Before injury	39	0	0
Days after injury			
7	25	13	52
14	33	22	66
21	29	20	68
28	18	9	50
35	16	4	25
42	16	2	12
56	16	0	0

(*e*) *Histological study:* Samples of burned and contralateral testes were excised and fixed in 10% formalin solution. The sections were stained with hematoxylin and eosin for histological study.

RESULTS

Serological Reactions.—The results of double diffusion gel precipitation tests utilizing guinea pig testicular extract and serum samples obtained at various times after testicular injury are summarized in Table I. None of 39 serum samples obtained before burning gave a positive reaction with testicular extract. In contrast, many sera obtained from burned animals gave precipitation tests with this extract. The proportion of such positive sera increased from 52% (13 of 25 animals) at 7 days after injury to 68% (20 of 29) animals at 21 days. The incidence of positive reactors fell to 12% (2 of 16 guinea pigs) in animals tested 42 days after injury. No animals had antibodies at 56 days after burning. Fig. 1 illustrates a study of sequential serum samples obtained from a burned guinea pig; sera obtained before injury and 56 days after burning gave no

reactions with testicular extract. In contrast, postburn samples obtained at 7, 14, 21, and 35 days after injury formed sharp precipitation lines with testicular extract; these lines merged in reactions of complete identity. All attempts to obtain complement fixation tests with testicular extracts and postburn sera were unsuccessful.

The results of serum antibody studies in animals exposed to injury with the unheated electrode are presented in Table II. Some of these animals formed

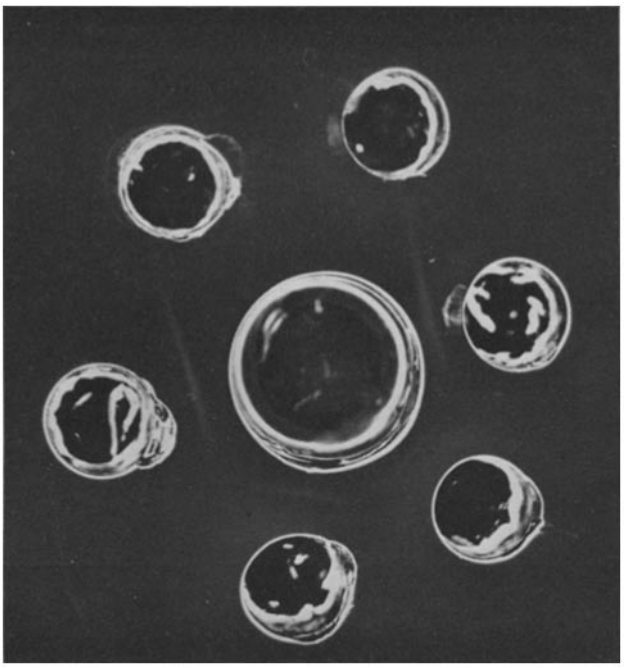

FIG. 1. Double diffusion gel precipitation test. Central well: guinea pig testicular extract. Peripheral wells, clockwise, beginning with the uppermost well: Sequential serum samples obtained from a guinea pig before injury and at 7, 14, 21, 35, and 56 days after thermal injury to the testis.

anti-testis antibodies detectable by double diffusion gel precipitation. The frequency of positive results was considerably lower, however, than that observed in the group of burned animals. The highest incidence of positive reactions occurred at 14 days. At that time, 15% of the animals (4 of 26) had formed testis antibodies. The precipitation lines formed with testicular extract by sera obtained from such animals regularly merged in reactions of identity with lines formed by postburn sera.

Specificity of the Serological Response to Testicular Injury.—The autoimmune nature of the serological response to thermal injury of the testis is illustrated in

Fig. 2. A postburn serum formed lines of precipitation with both autologous and homologous testicular antigens. These lines merged in reactions of complete identity. Similar results were observed with sera obtained from five other burned guinea pigs. Absorption of positive sera with autologous or homologous testicular antigen completely removed the activity of such sera in the double diffusion gel precipitation test.

Positive sera were also tested against extracts of guinea pig brain, heart, liver, lung, and spleen. None of these extracts gave reactions with the postburn sera, pointing to the organ specificity of the antibody under study. The sera were also tested against testicular extracts obtained from mice, rats, rabbits, and dogs. None of these antigens formed lines of precipitation with potent sera which were active against guinea pig testicular extract. Absorption of such serum

TABLE II

Testis Antibody Responses After Cold Electrode (27°C) Injury: Double Diffusion Gel Precipitation Test

Serum sample	No. of animals studied	No. of animals whose sera reacted with guinea pig testicular extract by double diffusion gel precipitation	Positive reactions
			%
Before Injury	34	0	0
Days after Injury			
7	15	2	13
14	26	4	15
21	12	1	8
28	10	1	10

samples with heterologous testicular antigens also consistently failed to affect their reaction with guinea pig testicular extract. These data indicated that the antigen detected by burn sera is species restricted.

Immunoglobulin Nature of Postburn Antibodies.—Selected serum samples giving strong reactions with testicular extract were studied by immunoelectrophoresis against testicular extract and against a rabbit anti-serum to guinea pig gamma globulin.

The testicular extract formed a single line of precipitation with burn sera. This line was located in the gamma globulin region, and its position corresponded to the location of the line formed by the faster antigen of the two antigenic components recognized by rabbit anti-guinea pig gamma globulin serum.

Postburn sera were also studied by the passive cutaneous anaphylaxis (PCA) test. Fig. 3 illustrates the results obtained with sequential serum samples taken from one injured guinea pig. Positive reactions were noted at skin test sites prepared with sera collected at 7, 14, 28, and 42 days after injury, but not

at test sites prepared with sera obtained before burning or 49 days after injury. Similar results occurred in this test in two other guinea pigs prepared with the same serum samples. PCA tests performed utilizing sera obtained from four other burned animals also gave similar results.

The PCA test was used for an assessment of the organ and species-specificity of the postburn antibodies. Absorption of sera with extracts of guinea pig brain, heart, liver lung, and spleen, or with testicular antigens prepared from pooled testes of mice, rats, rabbits, or dogs did not alter the reactivity of such sera with guinea pig testicular extract in the PCA test. In addition, attempts to

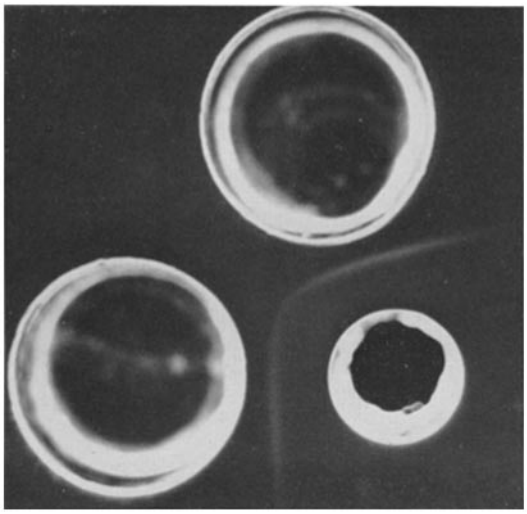

FIG. 2. Double diffusion gel precipitation test. Lower right well: Serum sample obtained 14 days after injury. Upper well: Autologous testicular extract. Lower left well: Pooled guinea pig testicular extract.

induce PCA reactions by intravenous challenge with extracts of guinea pig organs other than the testis, or with testicular extracts obtained from other species were also unsuccessful.

Pathological Sequelae of Thermal Injury to the Testis.—The histological appearance of a normal guinea pig testis is illustrated in Fig. 4. Note the active spermatogenesis, numerous spermatozoa in the lumen and normal interstitial cells. The microscopic pattern observed at 14 days in burned testes included multiple zones of necrosis, surrounded by massive infiltration with polymorphonuclear neutrophiles and mononuclear cells. Complete aspermatogenesis occurred throughout the testis, even in areas which were not in direct contact with the burning electrode. A very few small interstitial accumulations of lymphocytes and plasma cells were also found in those areas.

The acute inflammatory changes subsided during the 3rd and 4th wk, with progression of fibrosis, continued degeneration of all testicular elements, appearance of multiple giant cells, and deposition of hemosiderin. By the 8th wk, burned testes were reduced to small fibrotic masses containing occasional multinucleated giant cells. There was no evidence of extension of this inflammatory reaction beyond the confines of the tunica vaginalis.

The contralateral testis appeared grossly normal. The histological changes noted in 32 right testes removed at various periods after thermal injury to the left testes are summarized in Table III. Four of seven organs removed 7 days

TABLE III

Testicular Damage Associated with Thermal Injury to the Contralateral Testis

Time of study	Grade of testicular damage observed*				Total no. of animals
	0	1+	2+	3+–4+	
Normal testis	17	–	–	–	17
7 days after injury	3	4	–	–	7
14 days after injury	–	2	1	3	6
21–42 days after injury	7	2	2	–	11
56–63 days after injury	4	2	2	–	8

* The grade of testicular damage was assessed by the criteria of Freund, Lipton, and Thompson (16), and includes:

0, no evidence of damage.

1+, very few mature spermia present; immature spermia in the lumen; swelling and vacuolization of a few of the spermatogenic cells, some of these being exfoliated.

2+, may or may not show a few mature spermia; swelling vacuolization, and exfoliation of fairly large number of spermatogenic cells.

3+, no mature spermia present; swelling, vacuolization, and exfoliation of a large number of spermatogenic cells.

4+, damage ranging from partial to complete devastation of the tubules, with only Sertoli cells and the basement membrane remaining.

after injury showed slight diminution of spermatogenesis, increases in the number of immature spermia in the lumen of the seminiferous tubules, and moderate swelling of the spermatocytes. 14 days after burning, the right testes showed varying degrees of damage in all six animals studied. These ranged from the mild changes described above, which were noted in two instances, to more severe, although irregular changes, including marked diminution of spermatogenesis, degeneration and exfoliation of spermatogenic cells, swelling, vacuolization and exfoliation of spermatocytes and spermatogonia, appearance of large multinucleated giant cells, and areas of focal necrosis. There were also mucoid eosinophilic amorphous masses in some of the tubules. In some areas, only the Sertoli cells and the basement membranes of the seminiferous tubules remained intact. Such alterations were noted in three of six animals at this time (Fig. 5). Only minimal focal accumulations of lymphocytes and plasma

cells were found (Fig. 6). 7 of 11 testes studied at 21–42 days after injury were intact. The other four testes showed mild degrees of inhibition of spermatogenesis, increases in immature forms, exfoliation of spermatocytes, patchy necrosis of seminiferous tubules, and occasional multinucleated giant cells. These changes were particularly apparent in two testes studied 3 wk after injury. Four of eight testes studied at 56–63 days after injury had no evidence of damage, with the possible exception of occasional areas of fibrosis; the other four organs showed changes similar to those described in animals studied at 21 days after injury. There was no evidence of a direct correlation between the presence of testis autoantibodies and histological damage to the testis.

17 testes removed from normal animals matched for age and weight with the experimental animals had a normal histological appearance. Serial study of testes removed at similar periods after cold-probe injury to the contralateral organ gave no evidence of histological alterations smilar to those described in the experimental group.

DISCUSSION

Experimental models for the sensitization of the mammalian host to sperm have been known since 1900 (21). The immunological nature of this type of response (22, 23) was not well understood, however, until the careful studies of Freund, Lipton, and Thompson (17, 24, 25), who produced aspermatogenesis and damage to the germinal cell components of guinea pig and rat testes after immunization of recipients with testicular extract in complete Freund's adjuvant. This work formed the basis for further study of a type of auto-immune organ damage, which has been termed "experimental allergic orchitis" by Brown, Glynn, and Holborow (26, 27). The original reports of Voisin et al. (22, 23) and of Freund et al. (17, 24, 30) have recently been confirmed and extended by Katsh and Bishop (28), Waksman (29), Bishop and associates (30, 31), Chutna and Pokorna (32), and Boughton and associates (33, 34). More recently, Mancini (35) has documented similar lesions in human volunteers immunized with suspensions of autologous or homologous human testicular extract and complete Freund's adjuvant.

It is generally agreed that experimental allergic orchitis and aspermatogenesis may be induced by immunization with autologous or homologous adult testicular homogenate in Freund's complete adjuvant. This reaction is highly organ- and species-specific, and is accompanied by the development of cutaneous hypersensitivity of the immediate and of the delayed type to testicular extracts (36). It is also associated with the appearance of a variety of humoral antibodies (32).

Results of this study indicate that thermal injury to guinea pig testes can induce an auto-immune response similar to that observed after immunization with autologous or homologous testicular tissue. A high proportion of guinea

pigs exposed to thermal injury of the testis formed antibodies against a testicular antigen. These antibodies were organ- and species-specific, i.e., they reacted only with testicular antigens of guinea pig origin. The autoantibody nature of this response was assessed by demonstrating reactions with extracts of the animal's own contralateral testis. The antibodies could be demonstrated by precipitation and by PCA tests, but not by complement fixation. Their electrophoretic properties were comparable to those of fast-moving gamma globulins. The serological activity and electrophoretic mobility of such antibodies therefore justify their classification as γ-1-globulins (20).

Testicular injury by the unheated electrode results in the production of testis antibody in a small number of animals. The incidence and duration of such responses were not comparable to those obtained with thermal injury. The identity of the antibodies observed after both types of injury would, however, lend support to the concept that, in both instances, the appearance of antibodies was a consequence of the release of intracellular tissue antigens which are not normally available to the immunological apparatus of the host.

A significant number of burned animals showed pathological changes in the germinal components of the contralateral testes. Such changes were similar to those described after immunization with testicular tissue. There was, however, no correlation between the presence of testis antibodies and damage to the contralateral testis. This observation is consistent with the studies of Bishop and Carlson (31), who have provided evidence that testis antibodies from actively sensitized guinea pigs do not induce damage to testes after passive transfer to a normal recipient. Although it has been suggested by Waksman (36) that the mechanisms implicated in this type of testicular injury may be cellular rather than humoral in nature, the paucity of mononuclear infiltrates observed in most studies, including the present one, would appear to militate against this possibility.

The appearance of humoral antibodies in response to burns of the testis is in harmony with the responses described after active sensitization of guinea pigs with testicular extract in complete Freund's adjuvant. The results are also in keeping with the reports by Shulman et al. (37) of the development of tissue-specific autoantibodies after extreme cold injury to the prostate gland in rabbits.

These results raise the possibility that thermal injury may be comparable in its effects to those observed after the use of adjuvants for the induction of immunological organ damage. Controlled thermal injury of an organ may therefore provide a useful experimental model for further studies of autoimmunity in the mammalian host. The results also suggest that, under certain conditions, thermal injury may set in motion an autoimmune process leading to organ damage. This consideration may be of relevance to further studies of the clinical status of the severely burned human subject.

SUMMARY

Unilateral thermal injury to guinea pig testes has elicited the formation of organ- and species-specific antibodies against a testicular antigen. The antibodies were of an IgG_1 nature, and reacted with autologous as well as homologous testicular extracts.

A significant number of burned animals showed pathological changes in the contralateral testis, which were similar to those observed after the induction of experimental allergic orchitis by active immunization with testicular tissue.

These results indicate that thermal injury may be associated with autoimmunization of the host by the injured organ.

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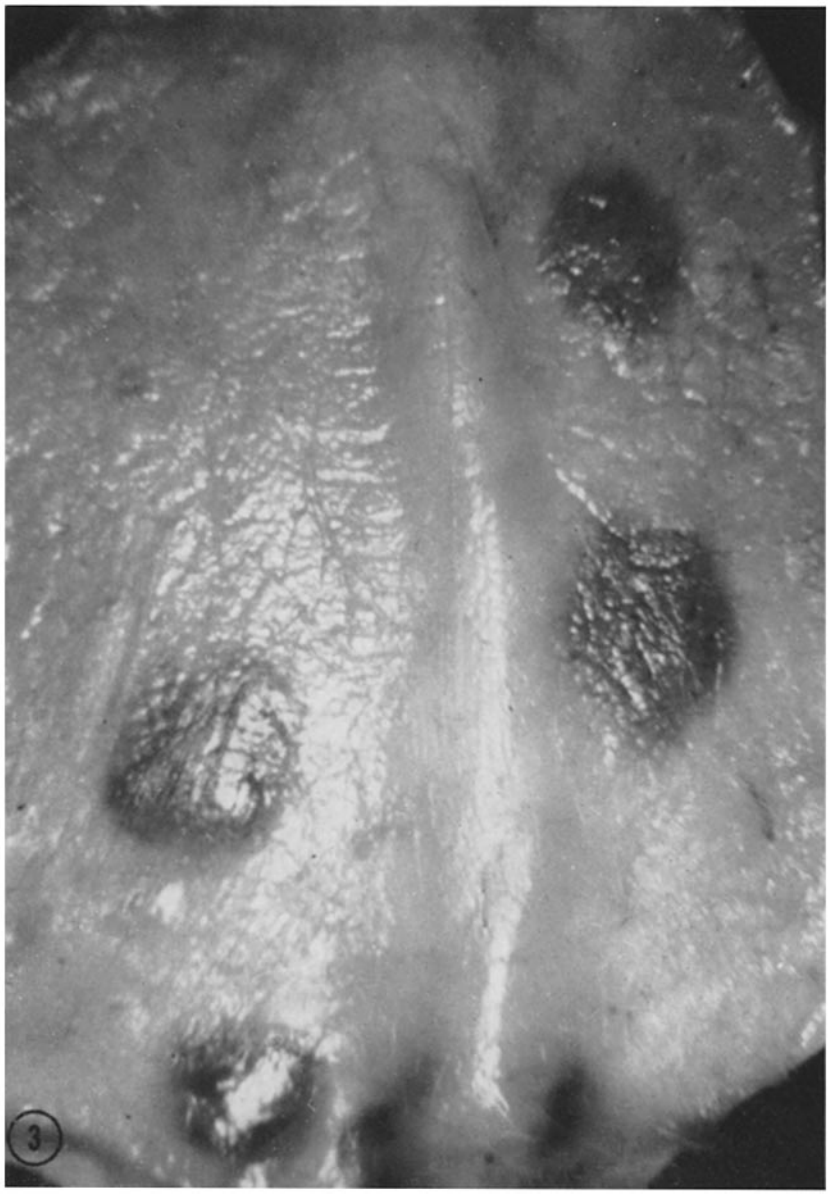

FIG. 3. Passive cutaneous anaphylaxis test. Six skin test sites were prepared simultaneously with serum samples obtained from a guinea pig before injury, and at 7, 14, 28, 42, and 49 days after burning of the testis. Beginning with the upper left, the skin sites were prepared respectively with serum samples obtained from a guinea pig before injury, and at 7 and 14 days after burning. Beginning with the upper right, the sites were prepared with serum samples obtained at 28, 42, and 49 days after injury. Note the positive reactions at the skin test sites prepared with sera obtained at 7, 14, 28, and 42 days after injury, and no responses at the sites prepared with serum samples obtained before burning or 49 days later.

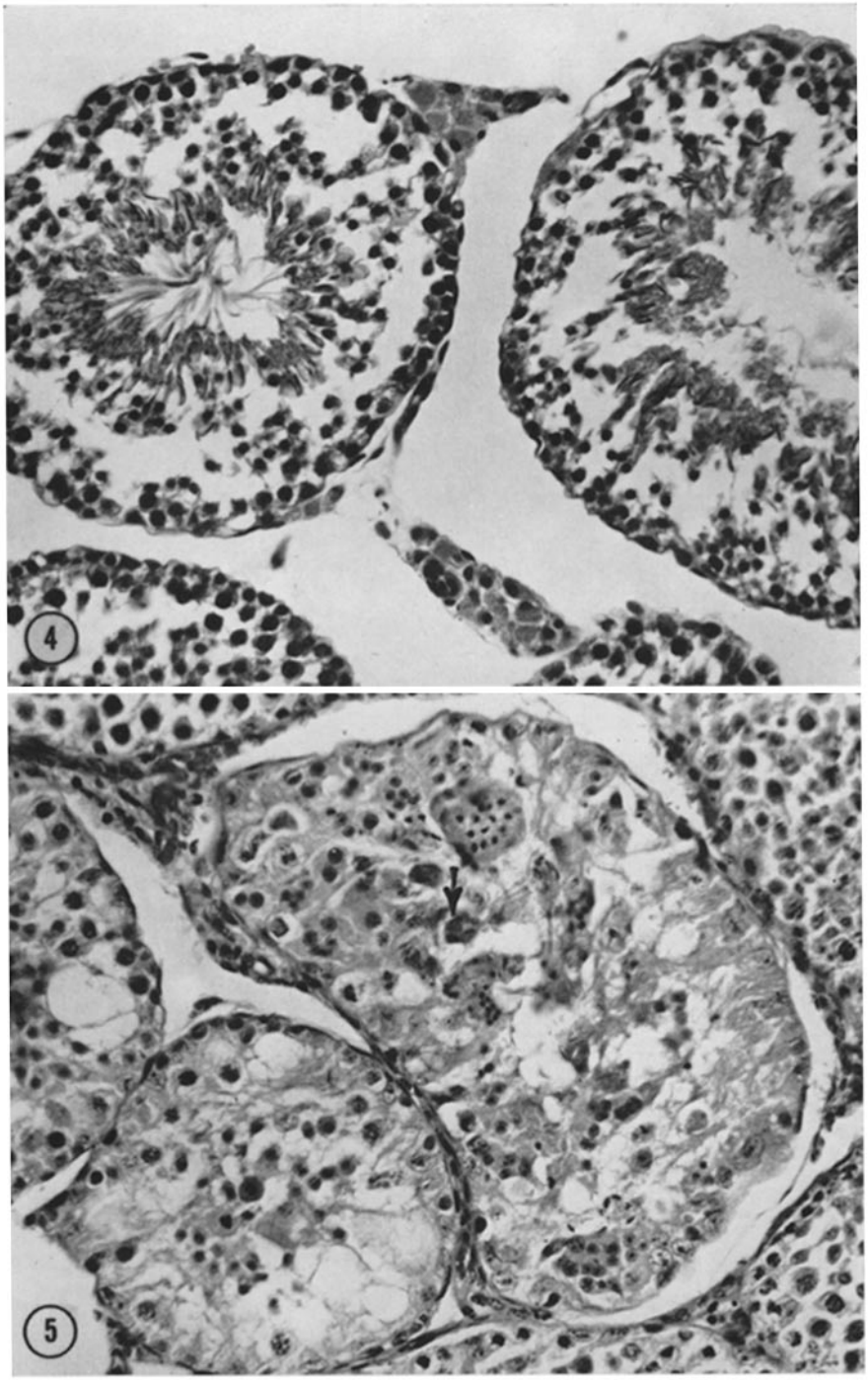

FIG. 4. Cross-section of normal testis, showing seminiferous tubules containing all stages of maturation of the germinal epithelium, from spermatogonia to spermatozoa. Hematoxylin and eosin stain, × 250.

FIG. 5. Cross-section of right testis obtained from a guinea pig 14 days after thermal injury to the left testis. Note that the seminiferous tubules contain numerous degenerated cells, some multinuclear giant cells, and absence of spermatogenesis. The tubules showed no tendency to separate during preparation of the histologic sections. Hematoxylin and eosin stain, × 250.

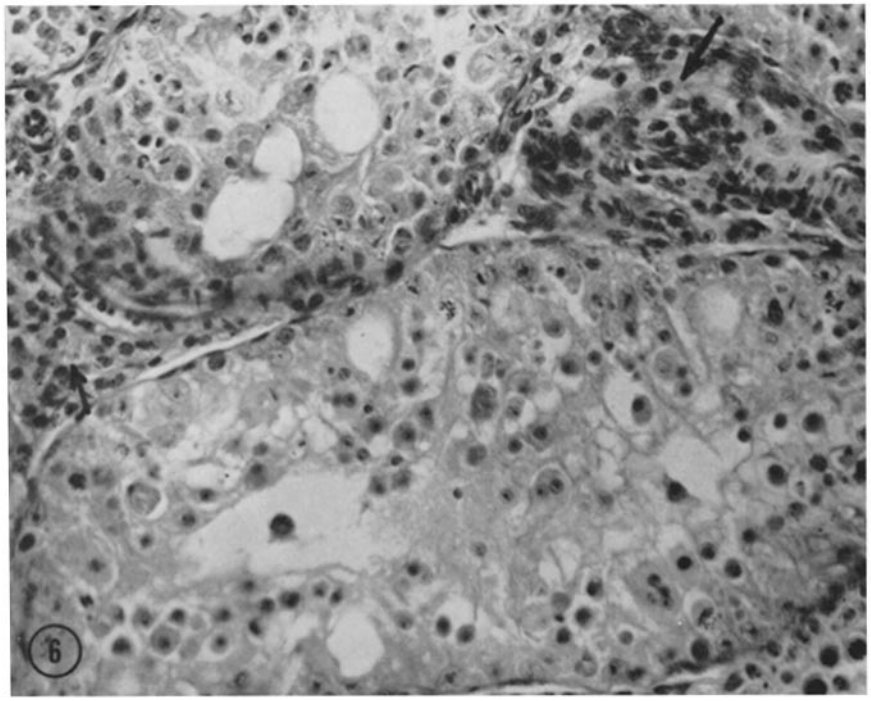

FIG. 6. Cross-section of right testis obtained from a guinea pig 14 days after thermal injury to the left testis. Note that, in addition to the degenerative changes in the seminiferous tubules, two small foci of interstitial mononuclear cell infiltration are seen; the one to the right contained a few plasma cells. Hematoxylin and eosin stain, × 250.