GLOMERULONEPHRITIS INDUCED IN SHEEP BY INJECTIONS OF HETEROLOGOUS GLOMERULAR BASEMENT MEMBRANE AND FREUND'S COMPLETE ADJUVANT*· ‡

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Plates 26 to 30

(Received for publication, March 29, 1962)

Although an immunologic basis for the pathogenesis of human acute and chronic glomerulonephritis has enjoyed wide popularity because of certain clinical observations (1–9) and laboratory experiments (10–14), there has been, until recently, no convincing laboratory model to produce glomerulonephritis in animals by means of an *autoimmune* mechanism.

Most attempts to produce experimental animal nephritis by immunological models have centered chiefly on the production of nephritis by single or repeated intravenous injections of foreign protein (15–17), antigen-antibody complexes (18), or anti-kidney antibodies (13). Heteronephrotoxic sera have been considered to be highly species specific (19, 20), but Steblay and Lepper (21) have shown that rabbit antihuman glomerular basement membrane serum will produce nephritis in dogs. We have confirmed this original observation and extended it to include numerous other examples of cross-reactions. All the above models are valuable in contributing to our understanding of how alternate experimental methods may damage glomeruli. However, they all fail to provide evidence for the crucial step postulated in the autoimmune theory, namely, the *self*-production of antibodies and/or antibody-bearing (sensitized) cells by the host specific for an antigen which is a normal constituent of its own glomerular tissue. The *self*-produced antibody and/or sensitized cell is capable of initiating injury to the antigen-containing tissue.

Many efforts have been made to produce a plausible autoimmune model. Diverse autologous, homologous, or heterologous kidney preparations with various adjuvants have been injected into several species. Such efforts have been largely fruitless; and one early report of success (22) has not been confirmed (23). Recently, Heymann (24) has reported the production of the nephrotic syndrome in rats by repeated intraperitoneal injection of homologous or autologous rat kidney tissue in Freund's complete adjuvant; and Hess (25) has reported the transfer of this rat renal disease by means

* This work has been supported (in part) by research grant H-4785 from the National Institute of Health, United States Public Health Service, the Chicago Heart Association, and the Schering Corporation.

‡ Presented in part at the Hahnemann Symposium on Inflammation and Diseases of Connective Tissue in Philadelphia, December, 1960 (35).

of lymph node cells from donor rats with induced disease into healthy tolerant recipients.

An experimental model which appears to satisfy the requirements for an autoimmune mechanism has been described for a growing list of readily reproducible laboratory diseases. These experimental diseases have been produced by injections of suitably prepared mammalian tissues incorporated in Freund's complete adjuvant into certain susceptible species. The tissues used for producing specific organ damage include central and peripheral nervous tissue, uvea, adrenal, testis, and thyroid. In some cases (central and peripheral nervous tissue, thyroid, lens, and uvea) heterologous tissue can be used (26). Regardless of the nature of the antigenic stimulus, whether auto-, iso-, homo-, or heteroimmunization has been used, the host's own tissue constituents, antigenically related to the injected antigen, serve as the target organ.

In an effort to produce an experimental autoimmune glomerulonephritis based on the preceding model, glomerular basement membrane, prepared from kidneys of human, monkey, rabbit, rat, or dog, was incorporated with Freund's adjuvant and injected by various routes into several different species. This paper reports evidence for the successful production of a fatal, fulminating extracapillary glomerulonephritis in adult female sheep which is remarkably similar to various stages of glomerulonephritis in man.

Materials and Methods

Human glomeruli were isolated from kidneys obtained from patients who underwent sudden violent death. Monkey, rabbit, rat, and dog glomeruli were obtained from healthy animals sacrificed for their kidneys. Glomerular basement membrane (GBM) was prepared from isolated glomeruli by the previously described (27) slightly modified method of Krakower and Greenspon (28). Human glomeruli preparations contained the least amount of tubules, capsules and interstitial tissue, while the rabbit preparations contained the most tubules and capsules. Preparations containing large amounts of tubules and capsules were discarded.

The GBM was homogenized in isotonic saline with the sonic oscillator[1] for 3 to 5 minutes and made up to a concentration of 20 to 50 mg (wet weight) per ml. Merthiolate was added to give a final concentration of 1:10,000. The homogenized suspension was slowly added to Freund's complete adjuvant[2] to form a 1:1 water-in-oil emulsion. The final concentration of GBM in the emulsion varied from 10.3 mg per ml to 26.7 mg per ml, but most preparations contained about 25 mg per ml emulsion.

A human placenta was obtained shortly after birth, freed of membranes and washed free of blood in the gross. It was homogenized in a Waring blendor with equal weights of saline and then incorporated with an equal volume of Freund's adjuvant. Additional saline was added until the final concentration of the placenta in the water-in-oil emulsion was 5 gm per 30 ml. Because of the high concentration of large tissue particles, this preparation did not form a very stable or smooth emulsion.

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[1] Raytheon sonic oscillator, model S102A, 50 watt, 9 kc; Raytheon Co., Waltham, Massachusetts.

[2] Freund's complete adjuvant: Arlacel A., 15.0 ml; bayol F, 85.0 ml, *Mycobacterium butyricum* (Difco), 100 mg. Arlacel A: Atlas Powder Co., Wilmington. Bayol F: Penola Division, Detroit.

Young Hampshire ewes, weighing about 120 to 140 pounds, were obtained from different herds through a local veterinarian. The sheep were housed in three different environments and given food from three different commercial sources. There were five different experimental groups of animals. The following results are the composite of these different experiments. A total of thirty-two sheep were employed.

After having been found free of overt disease and with normal urine and blood urea nitrogen, the sheep were injected every 2 weeks with 6 to 15 ml of emulsion containing approximately 100 to 240 mg of GBM. Each sheep received GBM from a single species. The average amount of GBM per injection was approximately 150 mg. It was given by a combination of intramuscular, subcutaneous, and intradermal routes at each administration. For example, 10 ml of emulsion might be divided as follows: 5 ml intramuscularly in one hind leg, 2 ml subcutaneously in the axillary and inguinal regions on the same side, and 1 ml intradermally in the skin of the dorsum of the neck.

A total of 30 ml of placental emulsion was similarly given by multiple routes every 2 weeks until sacrifice.

Periodic urinalyses and blood urea nitrogen determinations were performed on some of the sheep during the course of immunization. The urine from some of the sheep was collected in metabolism cages. An occasional casual specimen was obtained by chance from a sheep that urinated spontaneously. The urine protein was determined by the heat and acetic acid method and by the sulfosalicylic acid method. Blood urea nitrogen, serum albumin, globulin, and cholesterol were determined prior to injection and at death in most of the sheep.

At death, complete autopsies were performed, bladder urine analysed, and the kidneys carefully examined in the gross and microscopically. Kidney and other tissues were fixed in ice cold 10 per cent formalin and stained with hematoxylin and eosin, periodic acid–Schiff reagent with alcian blue and Masson's trichrome stain.

RESULTS

Experiment I. Regular Production of a Fatal, Fulminating Glomerulonephritis.—

In this experiment, ten sheep were injected with human or animal GBM and Freund's adjuvant until they died. Each sheep received 412 to 1419 mg of GBM from a single species in 3 to 6 injections. All sheep died 38 to 90 days after injections began. There was a high-degree of uniformity in the results despite the variations in species origin of antigen, concentration of antigen in emulsion, or the amount of emulsion injected

The sheep died in uremia from renal failure characterized by blood urea nitrogen elevation greater than 350 mg per cent; 3 to 4+ proteinuria in urine obtained from the bladder or metabolism cage; and microscopic evidence of severe glomerulonephritis. The results are summarized in Table I.

The clinical course of the disease in these sheep appeared to be very short. It was not unusual for a sheep to appear healthy within hours before death. One sheep, No. 2-7, gave birth to a live lamb 3 days before dying from severe renal damage in uremia. Some of the sheep became weak, lethargic, or lost weight a few days before death. Two sheep developed tarry stools and at autopsy had tarry stool in the gut. One sheep developed dyspnea and had marked pulmonary edema at death. None of the sheep appeared edematous during life or at autopsy.

The pathologic changes in the kidneys of all the sheep were similar. In the gross the kidneys were large, pale, swollen, gray-brown, with petechiae scattered over the cortical and cut surfaces. The capsule stripped easily and the cortical surface was smooth.

TABLE I

Comparison of Renal Histology, Blood Urea Nitrogen, Proteinuria, and Day of Death with Type of Antigen, Amount, Concentration and Number of Injections

Sheep No.	Antigen	Total amount of antigen (wet wt.)	Amount of emulsion*	Concentration of antigen in emulsion	Total No. of injections	Day of death	Bladder urinary protein	Blood urea nitrogen	Renal histology‡
		mg	*ml/injection*	*mg/ml*				*mg/100 ml*	
1	Human GBM	480	6	26.7	3	42	4+	ND	Chronic glomerulonephritis
2	Human GBM	1419	6	26.7	6	90	3+	372	Chronic glomerulonephritis
3	Human GBM	480	6	26.7	3	44	ND§	ND	Chronic glomerulonephritis
4	Human GBM	480	6	26.7	3	38	4+	428	Chronic glomerulonephritis
1-7	Human GBM	815	10	19.5	4	52	4+	378	Chronic glomerulonephritis
5	Monkey GBM	412	10	10.3	4	67	ND	240‖	Chronic glomerulonephritis
7	Rabbit GBM	483	10	16.1	3	58	3+	390	Chronic glomerulonephritis
2-A	Rat GBM	1032	6	43.0	4	82	ND	ND	Chronic glomerulonephritis
3-7	" "	638	7	25.0	4	45	4+	355	Chronic glomerulonephritis
1-8	Dog GBM	840	15	13.8	4	50	ND	448	Chronic glomerulonephritis
9	Human placenta	25 gm	30	1 gm/6 ml	5	100¶	trace	21	Normal limits
1-2	Human placenta	40 gm	30	1 gm/6 ml	8	112¶	0	14	" "
8	Freund's adjuvant only	0	5–6	0	4	100¶	0	21	" "
1-4	Freund's adjuvant only	0	10	0	8	112	1+	50	Focal pyelonephritis
1-5	Freund's adjuvant only	0	10	0	8	112	ND	17	Normal limits
2-2	Freund's adjuvant only	0	10	0	9	118	1+	22	" "
2-3	Freund's adjuvant only	0	10	0	9	118	3+	9	Questionable changes**
2-4	Freund's adjuvant only	0	10	0	9	118	trace	22	Normal limits
2-9	Freund's adjuvant only	0	7	0	10	126	0	19	" "

* All injections were given by multiple portals: intramuscular, subcutaneous, and intradermal.

‡ All kidneys grossly were swollen, gray-brown, and contained petechiae on surface in group given GBM and adjuvant.

§ ND = Not done.

‖ This determination was done 6 days before death.

¶ All the control sheep in the group given Freund's adjuvant alone were sacrificed, and all sheep given human placenta and Freund's adjuvant were sacrificed.

** Changes were present in the glomeruli which were difficult to interpret. No petechiae were present.

Microscopic findings were characterized by various stages of glomerular obsolescence (Fig. 3). Fibrocellular proliferation of the glomerulus obliterated Bowman's space and made it difficult to delimit the various parts of the glomerulus from itself and the surrounding interstitial connective tissue. There was a conspicuous increase of interstitial connective tissue and some areas of interstitial cellular infiltration. Casts of various types and blood were present in tubules. Tubular atrophy and dilatation were present. Various degenerative changes were seen in the tubules. Postmortem changes sometimes obscured these histologic changes.

As controls, seven sheep were given Freund's adjuvant alone. One of these sheep had a blood urea nitrogen of 50 mg per cent at sacrifice and renal histology compatible with pyelonephritis, presumably unrelated to the experimental procedure. Of the remaining six sheep, three had 1+ bladder-urinary protein for which no renal lesion could be found. One sheep had 3+ proteinuria and mild glomerular changes which were difficult to interpret. All the kidneys of the control sheep were of a normal borwn-purple color and size and did not show any petechiae on their surfaces (Figs. 1 and 2).

Two sheep were given injections of human placenta and Freund's complete adjuvant. They were sacrificed 100 to 112 days after the first of 5 and 8 injections, respectively. The kidneys were of normal size and color and had no petechiae. The bladder urine contained a trace and no protein, respectively, and the blood urea nitrogen was normal.

It may be noted that the first four sheep in Table I (Nos. 1, 2, 3, 4) all received the same antigen in an identical immunization schedule. Although three of these sheep died within 38 to 44 days after the first of three injections, the fourth sheep died only after 90 days. The long-delayed death of this sheep illustrates that a variable host factor may be present.

In summary, the chief result of this experiment was the regular production of a fatal, fulminating diffuse subacute to chronic glomerulonephritis within 38 to 90 days after the first of 3 to 6 injections of human, monkey, rabbit, rat, or dog GBM in Freund's complete adjuvant. The quantity of GBM used from each species was of the same order of magnitude. The total weight of GBM used varied from 412 to 1419 mg of GBM wet weight. A combination of intramuscular, subcutaneous, and intradermal injections that totaled about 6 to 15 ml of emulsion was given at each administration. Freund's complete adjuvant alone or in combination with human placental tissue (under the conditions of the present experiment) were unable to produce these results.

Experiment II. To Study the Effect of Varying the Route of Injection, Number of Injections, and Presence of Freund's Adjuvant.—The preceding experiment established that adult female sheep injected with heterologous GBM and Freund's adjuvant regularly develop a fulminating glomerulonephritis. The second experiment was designed to examine the effect of varying the route of injection, number of injections, and presence of Freund's adjuvant in producing glomerulonephritis.

The same preparation of pooled HGBM was used throughout this experiment. One sheep was given *one* injection of 50 ml of emulsion (25 mg HGBM/ml emulsion) by intramuscular, subcutaneous, and intradermal routes. A total of 1.25 gm of HGBM was given. Two sheep were given intradermal injections of HGBM in saline *without adjuvant.* 1 ml of HGBM in saline (concentration of 33.2 mg/ml) was injected into each of seven different intradermal

locations every 2 weeks. One sheep was given a total of 7 ml of emulsion (containing about 175 mg of HGBM wet weight) intramuscularly in two locations; another sheep was given a total of 7 ml of emulsion subcutaneously in two locations, and three sheep were given 1 ml intradermally in each of seven different sites every 2 weeks (concentration was 25 mg/ml in one sheep and 10 mg./ml. in two sheep.) The sheep were followed with serial urinalyses and blood urea nitrogen and sacrificed when judged to have detectable nephritis.

The results of this experiment are summarized in Table II. A combination of HGBM and Freund's adjuvant by *any single* route was effective in producing a severe nephritis. The times of sacrifice were estimated to be a few days before spontaneous death would have occurred. All sheep had proteinuria, elevated BUN, petechiae on kidneys and marked glomerular lesions. The disease progressed rapidly once proteinuria occurred and the blood urea nitrogen increased in an accelerated fashion once azotemia began (*vide infra*). Although there were not enough sheep to compare statistically the efficiency of each route, it should be noted that all three sheep given intradermal injections had developed a more fulminating disease than either of the sheep injected by the other routes. It should be particularly noted that sheep 4-4 died within 27 days after only 2 injections and a total of 120 mg of GBM. This finding is in keeping with a previously described greater efficiency of the intradermal route in producing reactions of delayed hypersensitivity and autoallergic diseases (29).

Two sheep were given a total of 3.5 gm of HGBM in saline by the intradermal route in 20 different injections (a total of 7 ml divided in 7 different locations at each injection) and have been followed for 10 months (at the time of this writing) and have not developed proteinuria or an elevated blood urea nitrogen. The injection of HGBM *without adjuvant* is apparently incapable of producing nephritis, at least in a comparable period of time.

The single sheep given one injection of HGBM in Freund's adjuvant developed 2+ proteinuria on the 70th day in a casual urine specimen. The sheep was sacrificed with a normal BUN because we were interested in obtaining an early lesion. Histopathologic evidence for glomerulonephritis was slight. In the gross there were only a few petechiae on the kidney. There was evidence of abnormal glomerular permeabiliy which consisted of blood and casts in tubules. However, the glomerular lesions were either very early or mild or both and were not diffuse, but consisted of focal acute proliferative glomerulonephritis.

In summary, the chief results of this experiment were to demonstrate that subcutaneous, intramuscular, or intradermal routes of injection were all effective in producing an extracapillary glomerulonephritis. There is suggestive evidence that the intradermal route may be the most efficient in producing an earlier onset of death. Human GBM alone, without adjuvant, was ineffective in producing the disease, at least after 20 injections over a period of 10 months. Finally, one injection of HGBM in Freund's adjuvant by all portals appears to have produced an early and/or mild focal proliferative glomerulonephritis at the time of sacrifice. Nothing can be said about the minimum amount of HGBM needed to produce disease, since the smallest amount used, a total of 120 mg wet weight, given in two intradermal injections (10 mg/ml emulsion) was sufficient to produce a fatal nephritis in 27 days.

Experiment III. The Onset of Proteinuria, Onset and Rate of Progression of Azotemia; Serum Proteins and Serum Cholesterol; and Serial Histopathology.—In this experiment an attempt was made to ascertain certain features of the course of the disease.

Results of serum biochemical determinations:

TABLE II

Comparison of Production of Nephritis with Route of Injection, Number of Injections, and Absence of Freund's Adjuvant

Sheep No.	Total amount of antigen* (wet wt.)	Amount of emulsion	Concentration of antigen in emulsion	Total No. of injections	Day of sacrifice	Bladder urinary protein	Blood urea nitrogen at sacrifice	Renal histology	Route of injection
	mg	*ml/injection*	*mg/ml*						
2-5	700	7	25	4	54	3+	112	Subacute glomerulonephritis	Intramuscular
2-6	875	7	25	5	66	3+	58	Acute to subacute glomerulonephritis	Subcutaneously
2-7	525	7	25	3	46	4+	420	Subacute to chronic glomerulonephritis	Intradermally
3-2	1250	5	25	1	70	3+	24	Early‡ glomerulitis	Multiple portals§
4-3	180	6	10	3	41‖	4+	340	Subacute to chronic glomerulonephritis	Intradermally
4-4	120	6	10	2	27‖	4+	375	Subacute glomerulonephritis	Intradermally
2-8	3500	0	0	20	10 months	0	19	Normal	Intradermally¶
2-8A	3500	0	0	20	10 months	0	24	"	Intradermally¶

* All sheep were injected from the same pool of human glomerular basement membrane preparation.

‡ No definite glomerular lesion was seen; evidence of abnormal glomerular permeability was demonstrated by red cells and protein in Bowman's space and lumen of tubules.

§ 40 ml was given intramuscularly, 8 ml subcutaneously, and 2 ml intradermally.

‖ Died.

¶ These injections were given without Freund's adjuvant; the antigen was suspended in saline at a concentration of 33.2 mg per ml saline. A total of 7 ml of suspension was given at each injection: 1 ml was given in each of 7 different intradermal sites.

The most striking change in the serum biochemical determinations is the increase in blood urea nitrogen in the animals given heterologous GBM and adjuvant (Table III). One control sheep which recieved Freund's adjuvant alone had a BUN of 50 mg per cent but this was probably explained by a pyelonephritis presumably unrelated to the experimental procedure.

The sheep were arbitrarily sacrificed at different values of blood urea nitrogen to provide renal lesions of increasing severity or duration and are listed according to their increase in blood urea nitrogen determinations (Table III). There was no correlation between the BUN

TABLE III

Correlation of Renal Histology with Blood Urea Nitrogen. Comparison of Blood Urea Nitrogen, Serum Cholesterol, Bladder Urine Proteinuria, and Renal Histology with Kind of Antigen and Day of Sacrifice

Sheep No.	Antigen	Day of sacrifice	Bladder urinary protein	Renal histology	Preinjection blood chemistries		Blood chemistries at death or sacrifice	
					Blood urea nitrogen	Cholesterol	Blood urea nitrogen	Cholesterol
					mg per cent	*mg per cent*	*mg per cent*	*mg per cent*
2-6	Human GBM	66	3+	Acute to subacute glomerulonephritis	27	66	58	83
1-6	" "	84	4+	Subacute glomerulonephritis	22	55	91	91
2-5	" "	54	3+	Subacute glomerulonephritis	31	54	112	62
3-3	Rat GBM	74	ND	Subacute glomerulonephritis			147	
1-1	Rabbit GBM	63	ND	Subacute glomerulonephritis	14	116	186	82
1-0	Human GBM	50	3+	Subacute glomerulonephritis	9	75	300	98
1-3	Rat GBM	50	ND	Subacute glomerulonephritis	14	95	345	85
1-7	Human GBM	52*	4+	Subacute to chronic glomerulonephritis	19	56	378	68
2-7	" "	46	4+	Subacute to chronic glomerulonephritis	23	64	420	119
1-8	Dog GBM	50*	ND	Chronic glomerulonephritis	25	38	448	63
1-A	Human GBM	77	2+	Early‡ glomerulonephritis	10	70	22	65
3-2	" "	70	2+	Early§ glomerulitis	12		24	
9	Freund's adjuvant only	100	0	Normal limits	26	50	21	30
1-4	Freund's adjuvant only	112	1+	Focal pyelonephritis	18	71	50	46
1-5	Freund's adjuvant only	112	ND	Normal limits	15	58	17	81
2-2	Freund's adjuvant only	118	1+	Normal limits	20	68	22	52
2-3	Freund's adjuvant only	118	3+	Questionable changes	13	37	9	44
2-4	Freund's adjuvant only	118	trace	Normal limits	23	55	22	33
2-9	Freund's adjuvant only	126	0	Normal limits	27		19	

‡ Evidence of early glomerular inflammation with cellular proliferation and presence of polymorphonuclears in glomerulus; abnormal glomerular permeability was present.

§ No objective changes in glomeruli were seen; evidence of abnormal glomerular permeability consisted of red cells and protein in Bowman's space and lumen of tubules.

* Died spontaneously.

and day of sacrifice or death or any other serum biochemical determination. Serum albumin decreased and serum globulin increased in similar fashion in both the treated and control groups of sheep.

Although the serum cholesterol appears to be slightly increased in the group of sheep

treated with heterologous GBM, this difference is not statistically significantly different from the control group. The sheep did not appear edematous during life or at death. They did not, therefore, have the nephrotic syndrome.

Onset of proteinuria (incubation or latent period) and azotemia:

Serial urinary protein determinations were made on urine samples from some of the sheep. In column 3, Table IV, are tabulated the day after injection that the last negative urine for

TABLE IV

The Experimentally Determined Range, in Days, of the Onset of Proteinuria and Azotemia and the Estimated Day of Onset of Proteinuria and Azotemia

Sheep No.	Antigen	Range in days onset of proteinuria	Estimated day of onset of proteinuria	Estimated or actual day of death	Time of latent period	Range in days of onset of azotemia	Estimated day of azotemia	Duration of time to onset of azotemia
					per cent			*per cent*
1-6	Human GBM	64 to 77	71	92*	77	76 to 80	78	85
1-7	" "	10 to 30	20	52‡	39	29 to 42	37	72
1-8	Dog GBM	36 to 44	40	50‡	80	29 to 42	41	82
2-5	Human GBM	40 to 44	42	65*	65	1 to 45	43	66
2-6	" "	40 to 46	43	79*	55	59 to 66	60	76
2-7	" "	14 to 34	24	47*	51	1 to 45	35	75
1-0	" "	—	—	53*		42 to 49	44	83
1-1	Rabbit GBM	—	—	68*		55 to 62	57	84
1-3	Rat GBM	—	—	51*		1 to 42	41	81
5	Monkey GBM	31 to 60	45	65‡		1 to 60	50	77
3-2	Human GBM	1 to 70	<70	70§		>70	>70	
1-A	" "	1 to 77	<70	77§		>77	>77	
4-3	" "	16 to 22	19	41‖	45	30 to 34	31	74
4-4	" "	12 to 20	16	27‡	59	14 to 21	17	63

Per cent latent period calculated as follows: (days to onset of proteinuria/days to onset of death) × 100.

Per cent time to onset of azotemia as follows: (days to onset of azotemia/days to onset of death) × 100.

* Estimated.

‡ Died.

§ Sacrificed.

‖ Sacrificed when moribund.

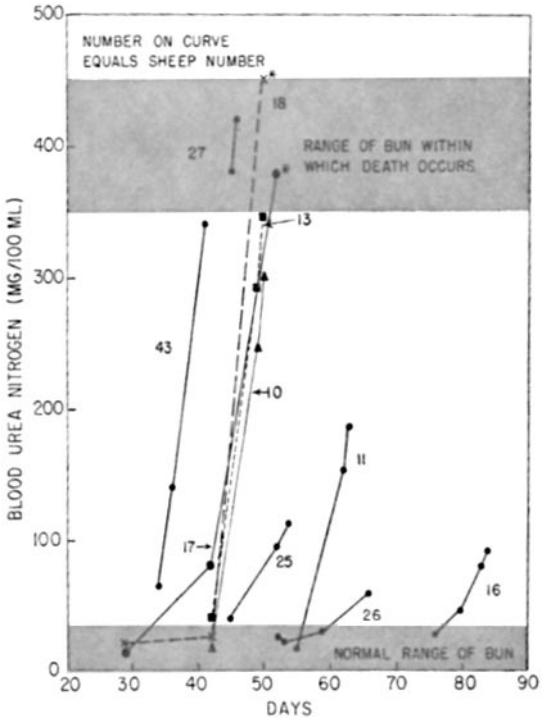
protein was obtained and the day the first positive urine for protein was obtained. This is given as the range of days within which proteinuria must have developed. The midpoint of this range was considered the estimated day of onset of proteinuria.

The range of these midpoints which approximate the onset of proteinuria (latent period) was 16 to 71 days. The latent period thus estimated varied from 39 to 80 per cent of the total time from first injection to death with an average value of 59 per cent.

The onset of proteinuria, as estimated, was found to occur on the average about 23 days before death with a range of 10 to 32 days among the animals which died. The time from onset of proteinuria to death may then be relatively short.

The day after injection on which the last normal BUN was determined and the first eleva-

sion was found similarly gives the experimentally determined range of days within which azotemia had its onset. These days are given in column 7, Table IV. The actual experimental curves of increase of nitrogen retention in nine sheep are given in Text-fig. 1 where sufficient serial BUN determinations have been performed to give a picture of the time of onset and rate of progression of nitrogen retention. These curves were used to estimate the day of onset of azotemia and day of death in sheep with infrequent data as follows: the experimentally



TEXT-FIG. 1. Curves illustrating the rate of increase of nitrogen retention for an individual sheep are shown. Although the onset of azotemia (BUN 35) varies widely, from 32 to 75 days for the sheep on this graph, the slopes of the progressive increase in BUN are quite similar in form, and it can be easily seen that with a few experimental points, a curve can be extrapolated parallel to these curves extending to a hypothetical onset (BUN = 35) and estimated day of death (BUN = 400).

determined values of BUN and day of occurrence were plotted on a graph as in Text-fig. 1. A curve was "fitted" through the experimental points similar in shape to experimentally determined curves. The fitted curve was then extrapolated to BUN values of 35 (onset) and 400 (elevation at which death arbitrarily was assumed to occur). This gives an estimated range of onset of azotemia of about 17 to 78 days after the first injection. The period of time to onset of azotemia is estimated to vary from 63 to 84 per cent of the total time from first injection to death.

The onset of azotemia is estimated to occur about 9 to 22 days before death with an average value of about 13 days. A rising blood urea nitrogen was thus indicative of terminal renal failure with imminent death.

Once nitrogen retention occurred, the daily rate of increase in blood urea nitrogen appeared to accelerate in a progressive fashion so that the higher the blood urea nitrogen, the more rapidly it rose each day. The sudden progressive acceleration of nitrogen retention and rapid culmination in death defines the fulminating character of this induced sheep renal disease.

Serial histopathology of the renal lesions:

Although a detailed description of the evolution of the renal lesion will be given elsewhere, a brief description of the pertinent histologic changes will be given here.

As would be expected, the severity of the renal lesions of the sacrificed animals is proportional to the height of the blood urea nitrogen elevation (Table III). The earliest lesions from animals with proteinuria and normal blood urea nitrogen appeared to be an acute extracapillary proliferative glomerulonephritis with casts and blood in tubules, colloid droplet degeneration of tubules, and increased numbers of polymorphonuclear leukocytes in glomerular loops. The earliest changes were primarily in the glomeruli with increased glomerular permeability and secondary degenerative changes in tubules. No significant change in tubular architecture or interstitial tissue was seen at this stage.

As the disease further progresses, some of the glomeruli become scarred and obsolescent and begin to resemble those seen in chronic glomerulonephritis (Figs. 7 and 8).

In the subacute stage there is marked fibro epithelial proliferation of the glomerulus (crescent formation) which gradually obliterates Bowman's space and makes it difficult to delimit the various parts of the glomerulus from itself and the surrounding interstitial connective tissue. There is a conspicuous increase in interstitial connective tissue and interstitial cellular infiltrates, chiefly mononuclear cells but occasionally polymorphonuclear cells. Casts of various types and blood are present in tubules. Atrophy and dilatation of tubules were present. The sheep which died had the most advanced renal lesions and usually had varying numbers and degrees of glomerular obsolescence (Figs. 4, 5, and 6).

In summary, proteinuria and azotemia began, on the average, about 23 days and 13 days, respectively, before death. The rate of increase of azotemia was progressive and accelerated. The course of the disease was fulminating. Serum albumin decreased and serum globulin increased in both treated and control sheep. Serum cholesterol values were not satistically significantly different before and after injection either in treated or control (Freund's adjuvant alone) sheep. The characteristic histologic lesion was an extracapillary glomerulonephritis with severe and progressive formation of crescents. The crescents eventually involved all glomeruli and the glomeruli rapidly developed glomerular obsolescence. Marked tubular and interstitial changes occurred secondary to the glomerular lesions.

Experiment IV. Some Immunologic Studies on Sera from Sheep Which Developed Nephritis.

A. Passive transfer studies in rats and dogs:

Sera were obtained from sheep after injections with either rat or dog GBM. The antisera were heated to 56°C. for ½ hour and then absorbed for 2 hours at room temperature with an equal volume of rat or dog washed red cells. About 0.5 ml of sheep anti-rat GBM serum was injected intravenously into 24 rats weighing about 170 gm. An immediate proteinuria and

detectable glomerular lesions were present within a few hours in all the rats (30). The sheep anti-dog GBM serum was injected intravenously into six dogs at a dosage of 0.5 ml/pound dog weight and produced an immediate nephritis within 6 hours in all the dogs. It was evident that the serum of a sheep with fulminating nephritis contained potent nephrotoxic antibodies for the species producing the antigen used in immunizing the sheep.

B. Fluorescent-labeled antibody studies:

Antisera were obtained from sheep that had been injected with either rat or human GBM and Freund's adjuvant until a fulminating nephritis developed. Gamma globulin fractions of the antisera were conjugated to fluorescein isothiocyanate according to the method of Riggs *et al.* (31). 4 μ sections of human or rat kidney were cut from frozen, unfixed pieces of human or rat kidney on a cryostat. The fluorescein conjugates were passed through a Dowex resin column and absorbed with mouse liver powder and human or rat blood. Staining of sections with fluorescein conjugates and inhibition controls were performed as described by Coons (32).

Sheep anti-rat or anti-human GBM fluorescein-labeled gamma globulin stained the basement membranes of the glomeruli, tubules, Bowman's capsules and intertubular capillaries and certain extracellular structures in the media and adventitia of arteries of rat or human kidney tissue sections, respectively, *in vitro* (33) (Fig. 9).

Antibodies which are capable of localizing on the basement membrane structures of the heterologous kidney antigen are evidently circulating in the serum of a sheep with fulminating nephritis.

Passive transfer studies of sheep antisera in lambs and fluorescein-labeled antibody studies with autologous and homologous sheep kidney tissue sections are in progress and will be reported later.

DISCUSSION

It is well known that Freund's adjuvant alone may disseminate within the body and form lesions in various organs (34). The possibility that adjuvant alone might produce some glomerular injury and increased glomerular permeability accounting for the presence of mild proteinuria (1+) in three sheep and 3+ proteinuria and focal glomerular lesions in another control sheep was considered. Two sheep were given injections of placenta and Freund's adjuvant without producing proteinuria or renal lesions. The injection of a combination of heterologous GBM and Freund's adjuvant, therefore, has a unique effect in inducing a new, previously unknown, uniformly fatal glomerulonephritis in sheep.

The experimental model used to produce this new experimental glomerulonephritis is responsible for inducing a number of so called "autoimmune diseases" and consists of injecting certain susceptible species with suitably prepared tissue in Freund's adjuvant. Either autologous, homologous, or heterologous tissue has been used, but regardless of the antigenic stimulus, the host's *own* immune response is directed at some antigenic constituent of its own tissue in the target organ, closely related to the antigen injected.

Waksman has aptly coined the term "autoallergic" for this group of labora-

tory autoimmune diseases and has characterized their properties in a comprehensive review (26). We wish to classify the sheep nephritis described in this paper as having an autoimmune basis as previously discussed (35).

Attempts to passively transfer the nephritis in sheep and other species by sheep anti-rat GBM, anti-dog GBM, and anti-human GBM sera are in progress. Preliminary attempts to transfer nephritis by these sera in sheep have been unsuccessful so far. On the other hand, these same antisera will produce immediate severe nephritis when injected intravenously into rats (30) or dogs (*vide supra*). Although it is well known that such heterologous anti-kidney sera are nephrotoxic for the species providing the kidney, this is the first example that we are aware of where the animal which produced the nephrotoxic serum developed nephritis *itself*. Moreover, the observation that the highly potent nephrotoxic sera is not able, at least so far, to produce nephritis when injected intravenously into other sheep, suggests that the circulating nephrotoxic antibodies for rat or dog kidney may not have a role in the sheep disease.

Gamma globulin obtained from sera of sheep injected with human GBM, conjugated to fluorescein isothiocyanate, can stain the BM structures of human kidney (*vide supra*). Such antisera are also, in preliminary studies, incapable of transferring nephritis to other sheep.

It may be concluded that the circulating antibodies which localize in human kidney or which are nephrotoxic for rats and dogs are present in high concentration in the sera of sheep with nephritis which was induced by the corresponding GBM antigens. It is, however, purely speculative as to whether such demonstrated localizing or nephrotoxic antibodies have any role in the sheep nephritis. Waksman (26) has pointed out that passive transfer of serum from animals with autoallergic diseases has consistently failed to produce disease in the same or *different* species. It is thus important to remember that, whereas successful transfer of nephritis by serum from sheep with nephritis to other species appears to be the first exception to this generalization, we have not demonstrated that such serum antibodies are important in the pathogenesis of the sheep disease. Conclusive proof that the sheep nephritis is produced by an autoimmune mechanism rests upon successful passive transfer of the nephritis in the *same* species by serum antibody and/or sensitized cells.

Heymann (24) has described a renal lesion in rats resulting from intraperitoneal injections of rats with autologous or isologous rat whole kidney emulsion and Freund's adjuvant. Several observations in his results and those presented in this paper are either different or in marked contrast: the renal lesions in the sheep were quite different from the rat lesions which were described or published by Heymann; the rats developed a nephrotic syndrome with characteristic serum biochemical changes and a histological lesion of membranous glomerulonephritis; the diseased rats lived much longer than 3 months after the first injection, whereas the maximum duration of life in the sheep was 90 days after

the first injection; the rat disease could be produced only by intraperitoneal injection. Parenteral routes were said to be ineffective. We did not try intraperitoneal injections in the sheep but any of the portals described in this paper were effective. Heymann has further reported that tubercle bacilli were effective but *Mycobacterium butyricum* as adjuvant was not. On the other hand, we have found *Mycobacterium butyricum* to be quite effective in sheep. Heymann used autologous or isologous whole rat kidney emulsion as antigen in contrast to the heterologous GBM antigen preparations used in this report. The renal disease in the rats has been reported by Hess (25) to be transferred by lymph node cells to suitably tolerant recipient rats. This would appear to establish the rat disease as having an autoimmune basis and being mediated by sensitized cells.

The fatal nephritis described in this paper, however different it may be from the above induced rat nephrotic syndrome, is most probably based on an autoimmune mechanism. In the absence of successful passive transfer of an experimental disease in the same species by serum antibody and/or cells, Waksman (26) has listed the following criteria as characterizing the immunologic basis for the mechanism of the autoimmune diseases: the production of lesions in organs corresponding to the tissues used in immunization, presence of a latent period, specificity of lesion in that it occurs only in regions where antigen is accessible in suitable concentrations, histologic appearance resembling the tuberculin reaction, diminution of the autoallergic state with time, anamnestic reaction and susceptibility of the disease to steroid therapy. Not all of these criteria apply to the present sheep disease, but based upon consideration of the above kind of criteria used to characterize the autoallergic diseases, the most reasonable explanation for the observed sheep kidney damage is an autoimmune mechanism.

It has been frequently postulated that the important tissue antigens in the autoallergic diseases are characterized as being separated from the blood or lymph stream by more or less anatomic or physiological barriers. The body is presumably not really tolerant to the "autoantigen" but merely harboring a hidden antigen, sheltered from exposure to the antibody-forming centers.

However, in the case of the immunizing antigen in autoimmune sheep nephritis, the antigen used was a greatly concentrated or purified preparation of human or animal GBM. This is a tissue constituent normally exposed to the blood stream through fenestrations in the glomerular endothelium. The human GBM has been reported to be present (Dr. Robert Vernier, personal communications) in the fetal kidney at 2 months. The presence of glomerular basement membrane structures in the fetus suggests that the fetus has ample opportunity to develop tolerance to glomerular basement membrane constituents. Further, the adult animal with its blood stream normally intimately exposed to GBM, would be expected to be highly tolerant indeed of its own GBM. However, heterologous GBM might vary sufficiently to appear "foreign" enough to the host animal to provide an immune response which could cross-react with

"closely related" antigens in the host's own kidney GBM. Further, the act of preparing GBM might sufficiently alter the important antigen in the basement membrane preparation so as to render it somewhat more "foreign" and yet sufficiently related to produce the cross-reacting response.

If this be so, we might predict that the autologous and homologous GBM preparations, unless sufficiently altered by the process of preparation, might not be as potent an antigen in producing autoimmune nephritis as heterologous GBM. We are in the process of testing this hypothesis by injecting sheep with autologous and homologous sheep GBM and Freund's adjuvant.

Recently, Weigle (36) reported that immunological tolerance induced in rabbits by neonatal injections of bovine serum albumin (BSA) can be terminated by a series of injections of certain heterologous serum albumins which cross-react with BSA. Injections of heterologous albumins, distantly related to BSA, were more effective in producing a loss of tolerance for BSA than albumins closely related were. If it is assumed that tolerance induced in neonatal rabbits to BSA is similar to tolerance induced during fetal life to their own body constituents, *e.g.* glomerular basement membrane, then injections of heterologous albumins which cross-react with the albumins used to induce tolerance are analogous with injections of heterologous GBM in sheep which are tolerant to their own GBM. It is possible that animals may lose tolerance to their own body constituents by some manner as yet unknown by exposure to antigens which cross-react with their own body constituents; *i.e.*, which have simultaneously some degree of similarity and yet foreign quality. Whether the heterologous GBM induces an immune response which cross-reacts with autologous GBM, or whether the immune response is somewhow induced by the autologous constituents is unknown.

The reason for the parallel changes in serum albumin, globulin, and cholesterol of the GBM-treated group of sheep and the control (Freund's adjuvant alone) group is unknown. The increase in globulin in the treated group may be explained by the increase in gamma globulin from immunization. The parallel rise in gamma globulin in the control group may be due to the non-specific inflammatory effect of Freund's adjuvant or its ability to produce an immune increase in gamma globulin. Prolonged immunization has been reported to produce a marked increase in serum gamma globulin and a fall in serum albumin concentration. This has been demonstrated by Bjorneboe (37) following hyperimmunization of rabbits with pneumococcal vaccine.

Thus the parallel hypoalbuminemia in the control sheep may be due to an immunizing effect of Freund's adjuvant alone. Perhaps the *Mycobacterium butyricum* in the adjuvant or altered tissue at site of injection acts as an antigen. The changes in serum cholesterol are not statistically significant although there appears to be a trend towards elevated serum cholesterol in the GBM-treated group.

The explanation for the length and variation in the latent period is specula-

tive. It appears to be due to variation in time for the autoimmune mechanism to get started. Once started, however, the renal lesion progresses rapidly. The curves of increase of nitrogen retention indicate a rapidly accelerating process; and the rate of increase of BUN plus the rapid evolution of the renal lesion from acute through subacute to chronic lesions resulting in death from uremia characterize the disease as a fatal fulminating one.

The early lesion in the typical autoallergic diseases is characterized by a perivenous collection of mononuclear cells (38). These cells are thought to be the mediators of tissue damage in areas of sequestered antigen by some unknown mechanism. On the contrary, in the sheep nephritis, the antigen is part of the glomerular basement membrane and directly exposed to the immunologic mediator of damage. The lesion starts then in the glomerular capillaries. The extraordinary feature of the lesion is the marked fibroepithelial proliferation (crescent) which appears to originate largely from Bowman's capsule.

It is unlikely that the technique described in this paper is related to an antigen-antibody complex mechanism such as Dixon has employed in producing glomerulonephritis in rabbits (17). The bulk of evidence in the autoallergic disease suggests each disease is mediated by cells (sensitized or antibody-bearing), but the evidence in the sheep nephritis is incomplete for either vector. Decisive experiments to determine whether antibody and/or sensitized cells are the vector mediating the glomerular damage are in progress.

SUMMARY

Sheep injected every 2 weeks with heterologous GBM and Freund's adjuvant by any one or combination of the following routes: intramuscular, subcutaneous, or intradermal, develop uniformly a fulminating, extracapillary glomerulonephritis, invariably fatal within 27 to 90 days after the first injection.

The chief histologic feature is marked fibroepithelial proliferation of Bowman's capsule with crescent formation. The appearance of the lesions resembles the acute, subacute, and chronic stages of human glomerulonephritis, and depends on when the animal was sacrificed.

Freund's adjuvant or heterologous GBM alone does not produce such a nephritis. The combination of placental tissue and Freund's adjuvant under the present experimental conditions was also unable to produce a nephritis.

The clinical course, increase in nitrogen retention, evolution of renal lesions, and death, all describe a fulminating disease. The disease most characteristically resembles fatal, fulminating human subacute glomerulonephritis.

The changes in serum proteins, decrease in serum albumin, and increase in serum globulin, occurred approximately the same in both the GBM-treated and the control adjuvant group. Similar changes have been reported from hyperimmunization alone, and so it is not clear how much these changes are due to immunization and how much is due to the nephritic process. The changes in serum cholesterol were not considered statistically significant.

Circulating serum antibodies which localized (by fluorescent antibody technique *in vitro*) on basement membrane structures of the heterologous donor kidney antigen or which produced nephritis in the heterologous donor species (rat and dog) were found in serum of sheep sick or dying of nephritis. The passive transfer of nephritis by serum antibodies marks the first successful instance of transfer of nephritis by serum antibody to a heterologous species from an animal which had developed nephritis *itself*. The serum antibodies involved in the transfer of disease to the donor species appear to be unrelated to the mediators of nephritis in the sheep and may represent only the previously known heteronephrotoxic antibodies. By various biologic criteria the sheep nephritis presumably occurs by an autoimmune mechanism. However, it is not known whether the sheep nephritis is mediated by sensitized cells and/or antibodies.

The latent period was estimated to end about 16 to 71 days after the first injection. Azotemia was estimated to begin about 17 to 78 days after the first injection. Proteinuria and azotemia began approximately 23 and 13 days before death. The rapid progression to a fatal termination defined the fulminating character of this disease.

The author is indebted to Dr. Mark H. Lepper, Department of Preventive Medicine, University of Illinois College of Medicine, Chicago, and to Dr. M. Edward Davis, for invaluable assistance; to Dr. Robert Jennings and the Armed Forces Institute of Pathology for aid in interpreting the histopathology; and to Mr. Ulrich Rudofsky for technical assistance.

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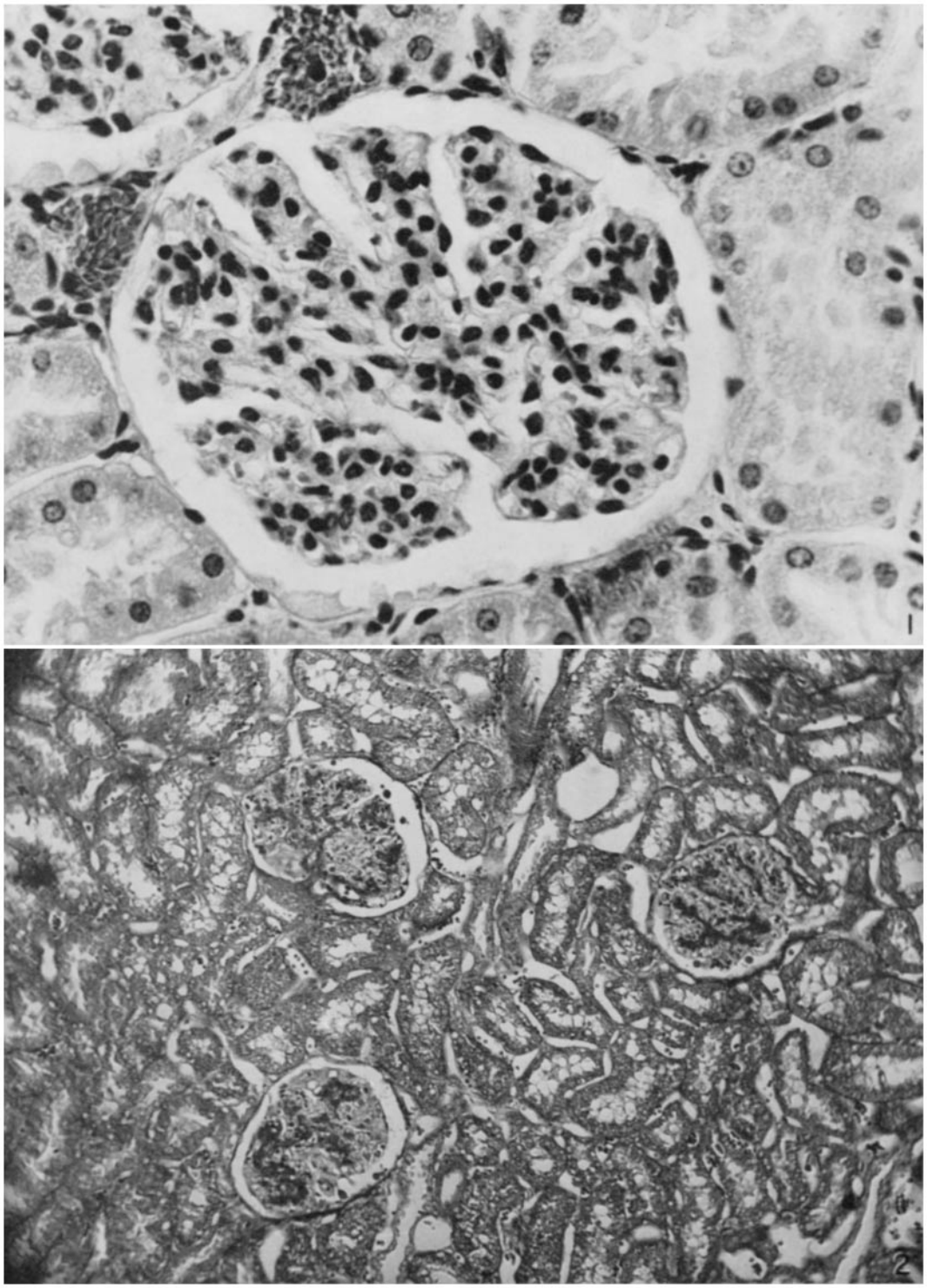
EXPLANATION OF PLATES

PLATE 26

FIG. 1. Kidney of sheep picked at random from stockyards. Sheep estimated to be in cold room 5 to 6 hours after death. Normal tubular, capsular, and glomerular architecture can be seen. Postmortem changes obscure some details. Hematoxylin and eosin. × 750.

FIG. 2. Kidney of a control sheep, No. 9, given 6 injections of Freund's adjuvant alone. It was sacrificed 100 days after injections began. The section shows a normal pattern of kidney architecture and demonstrates the normal basis of reference for size and structure of glomeruli, capsules, and tubules. Note lack of interstitial connective tissue and interstitial cellular infiltrates. Masson's trichrome stain. × 200.

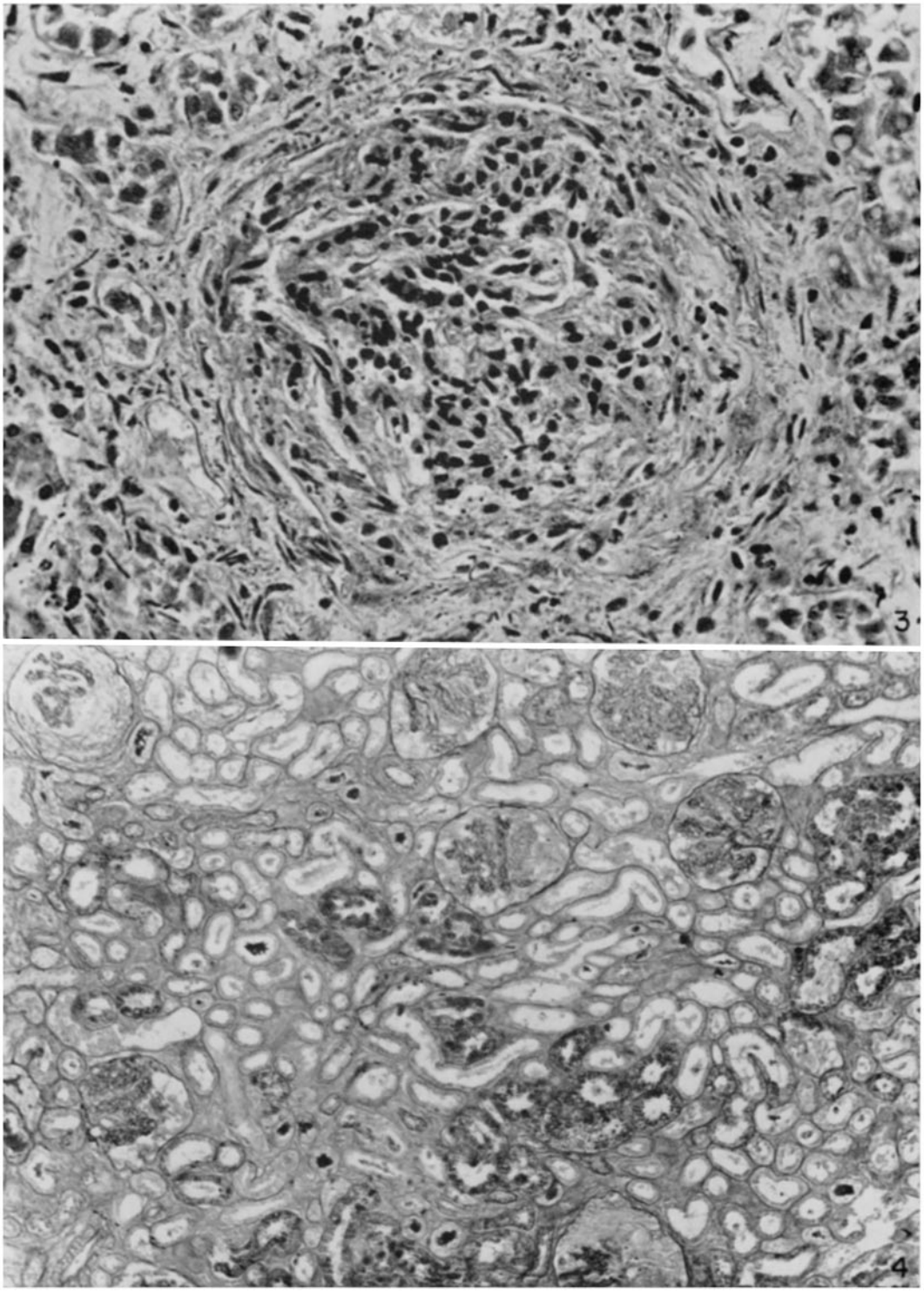


(Steblay: Glomerulonephritis induced in sheep)

PLATE 27

FIG. 3. Kidney taken from sheep 2A which was injected 4 times with a total of 1032 mg of rat GBM. The sheep was found dead on day 82. It had melena shortly before death and tarry stool was present in the gastrointestinal tract at autopsy. Postmortem changes obscure the picture but the marked increase in connective tissue inside the glomerulus, in the periglomerular area, and intertubular interstitium is evident. Hematoxylin and eosin. × 550.

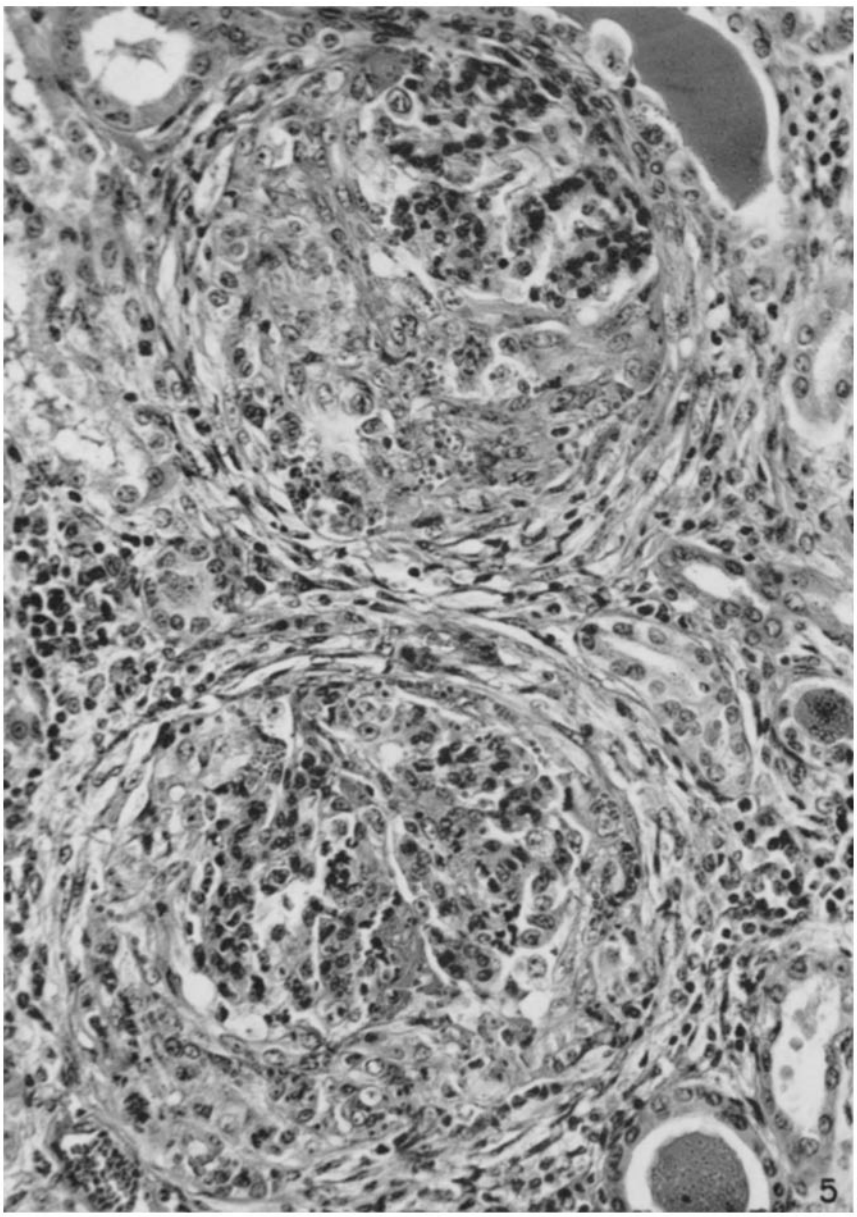
FIG. 4. Kidney section from sheep 1-6 which was sacrificed on day 84 after 6 injections of a total of 1192 mg of human GBM. Most glomeruli have varying degrees of fibroepithelial proliferation of Bowman's capsule (early crescent formation). There is hyaline droplet degeneration and casts in some of the tubules. There are focal areas of interstitial fibrosis but tubular architecture is relatively intact. The BUN was 91. This is an early proliferative extracapillary glomerulonephritis. PAS. × 100.



(Steblay: Glomerulonephritis induced in sheep)

PLATE 28

FIG. 5. Kidney section from sheep 1-0 which was injected 4 times with a total of 912 mg of human GBM. The sheep was sacrificed on day 50 with a BUN of 300. Marked fibroepithelial proliferation of Bowman's capsule (crescent formation) is present. The glomerular tuft is compressed, hypercellular, and areas of necrosis and infiltration of polymorphonuclear cells can be seen in it. Blood and casts are present in the tubules. There is an increase in connective tissue and mononuclear cell infiltration in the interstitial spaces. The similarity to human subacute glomerulonephritis is evident. Hematoxylin and eosin. × 320.

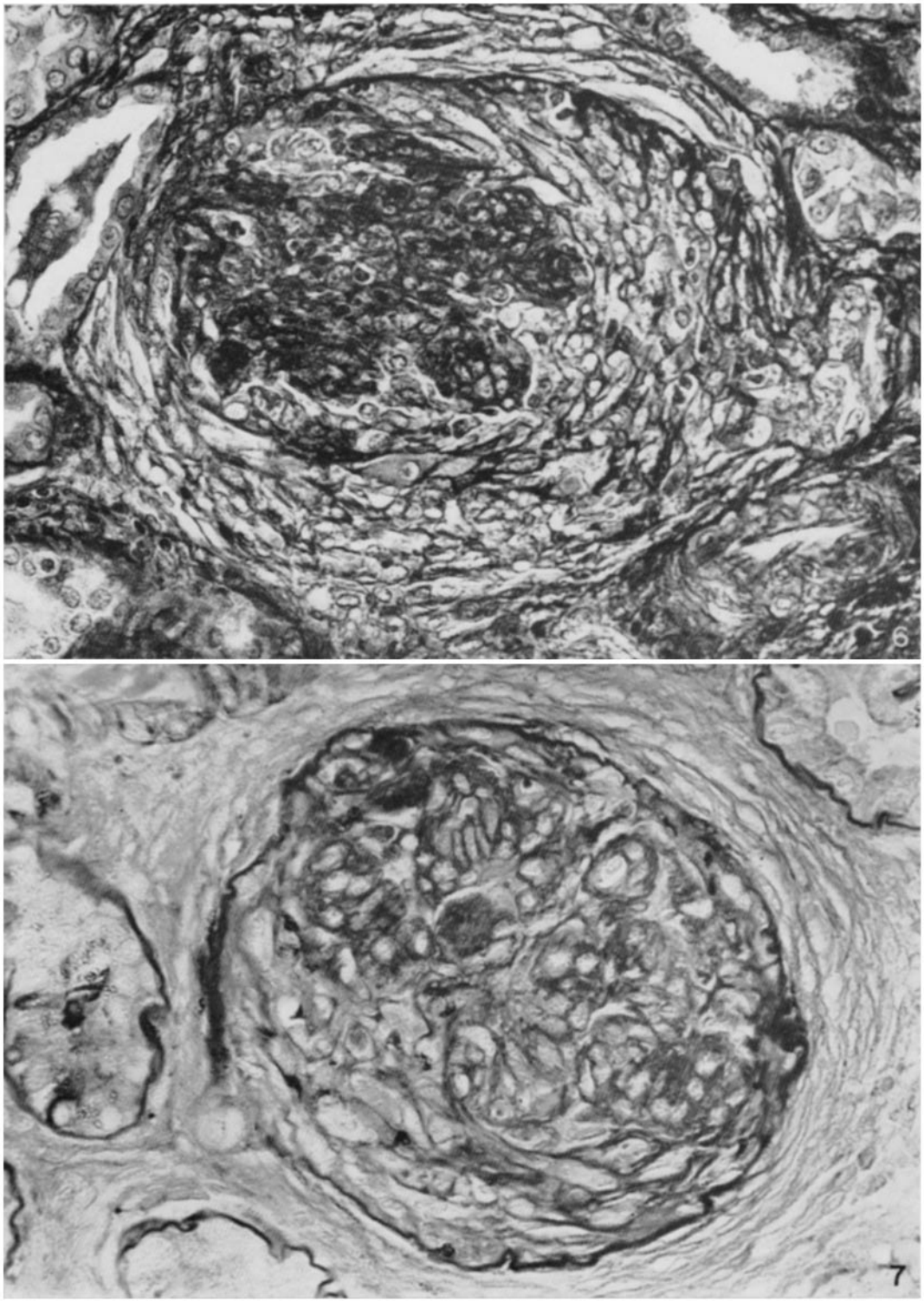


(Steblay: Glomerulonephritis induced in sheep)

PLATE 29

FIG. 6. Kidney section from sheep 1-3 which was injected 4 times with a total of 952 mg of rat GBM. The sheep was sacrificed on day 50 with a BUN of 345. There is marked fibroepithelial proliferation inside the original Bowman's capsule. The fibrillary part of the newly proliferating connective tissue can be seen in the extensive crescent which is compressing the original glomerular tuft. Masson's trichrome. × 500.

FIG. 7. Kidney from sheep 2-7 which was sacrificed 46 days after the first of 3 injections of a total of 525 mg of HGBM. The most prominently staining membrane is probably the original Bowman's capsule. New fibers can be seen arising or proliferating from it. The extensive fibroepithelial proliferation obliterating Bowman's space and fusing with the extensive periglomerular connective tissue can be seen. Dilated tubules are present. This glomerulus is obsolescent. PAS. × 700.

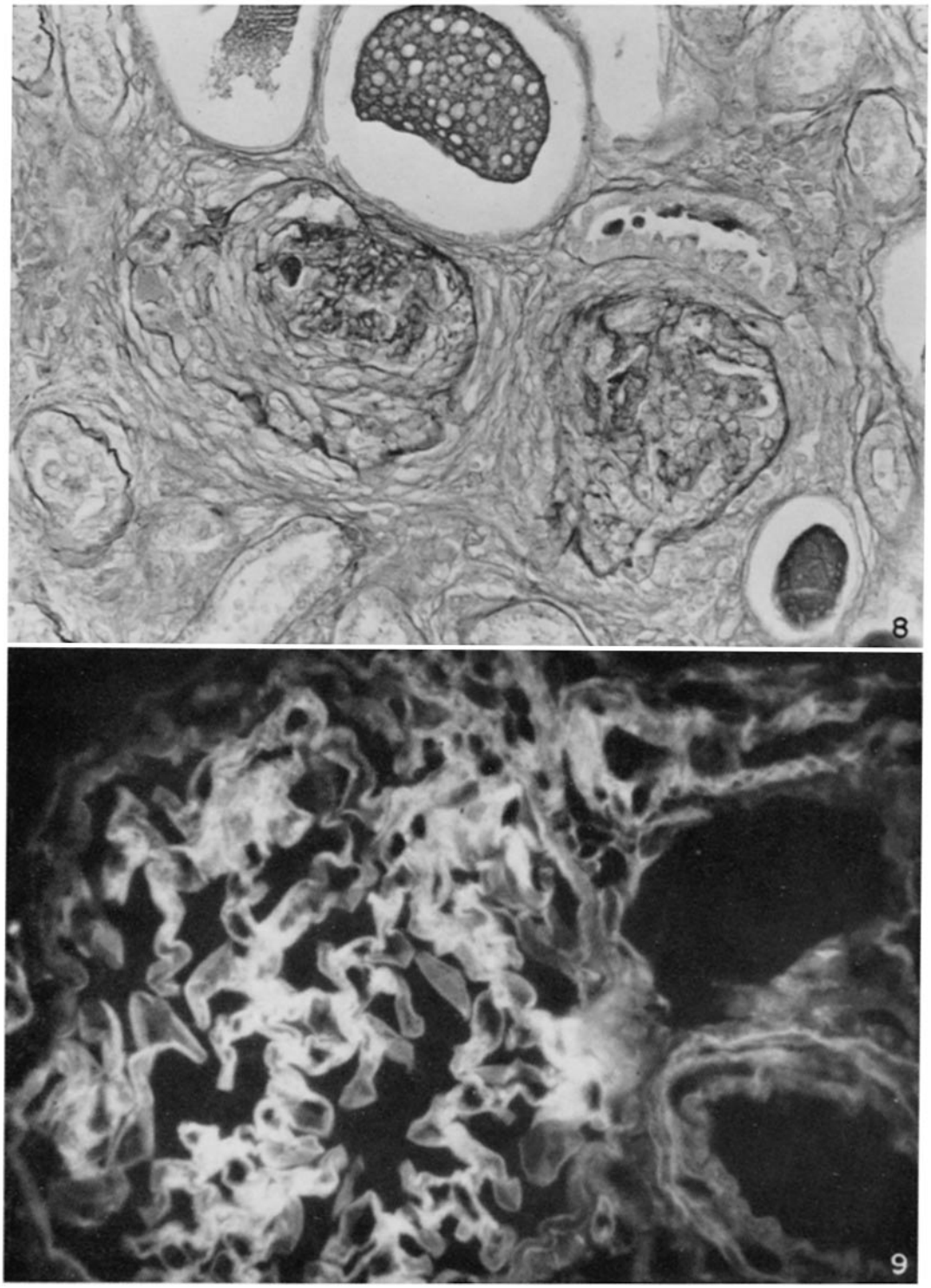


(Steblay: Glomerulonephritis induced in sheep)

PLATE 30

FIG. 8. Kidney section taken from sheep 1-0 which was sacrificed 50 days after the first of 4 injections of a total of 912 mg of HGBM. The BUN was 300. Two obsolescent glomeruli can be seen. Remnants of the original Bowman's capsule can be seen with irregular and disrupted outlines. The extensive fibrous proliferation inside and outside the glomerulus can be seen. Casts are present in some of the dilated tubules. PAS. × 350.

FIG. 9. Human kidney section treated with fluorescein-labeled sheep anti-human GBM gamma globulin. The basement membrane of Bowman's capsule, glomeruli, tubules, and intertubular capillaries and certain extracellular structures in the media and adventitia of arteries are specifically stained with the fluorescent conjugates.



(Steblay: Glomerulonephritis induced in sheep)