A ROLE OF POLYMORPHONUCLEAR LEUKOCYTES AND COMPLEMENT IN NEPHROTOXIC NEPHRITIS*, ‡

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In acute immunologic injury of tissues one of the common features noted is the accumulation of polymorphonuclear leukocytes (polymorphs) along with antigen, antibody, and host complement (C′) at the site of damage. This has been noted in venules of the Arthus phenomenon, in arteries in serum sickness, and in glomeruli of acute nephritis both in experimental animals and in human beings. In early tuberculin reactions polymorphs are also a common feature. Immunologic tissue injury in this respect is quite similar to other forms of acute inflammation.

Studies of the pathogenetic mediators of the immunologic inflammatory injury have been concerned primarily with C′ and polymorphs. Removal of circulating polymorphs from several species of animals has prevented the development of Arthus reactions (1–3). Further, it has recently been found that the arteritic lesions in serum sickness are dependent upon polymorphs (4). In addition to polymorphs, C′ has been observed by fluorescent antibody techniques in many immunologic reactions associated with antigen and antibody. At least one role of C′ in the Arthus phenomenon appears to be the attraction of polymorphs to the immunologic deposits (5). Strong evidence favors a role of C′ in nephrotoxic nephritis (reviewed and extended in reference 6), but the nature of its action in this disease is yet unclear.

The purpose of the present study was to evaluate to what extent the glomerular injury of acute nephrotoxic nephritis (NTN) may be dependent upon the local accumulation of polymorphs and the relationship of C′ to this accumulation.

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Materials and Methods

Animals.—For induction of nephrotoxic nephritis, Sprague-Dawley rats weighing 150 to 180 gms and New Zealand rabbits weighing 1.2 to 1.5 kg were employed. Urine protein was determined by the sulfosalycilic acid method (6). Normal urine protein levels in rats were less than 20 mg/24 hours (6). Normal rabbits of this weight gave less than 5 mg protein in urine/24 hours.

Antigens.—Kidney antigens, used to obtain nephrotoxic antiserum, were prepared from fresh or frozen rat and rabbit kidneys as previously described (7, 8). Human gamma globulin (HGG) fraction II was obtained from the American Red Cross through the courtesy of Dr. J. L. Pert. Rabbit gamma globulin (RGG) was obtained from Pentex, Inc., Kankakee, Illinois.

Antibodies–Nephrotoxic Globulin (NTG).—Nephrotoxic antibodies to rat kidney antigens were obtained from rabbits and ducks immunized to the insoluble portion of rat kidney (6). After absorption with rat erythrocytes and plasma, the gamma globulin fraction of the rabbit sera was obtained by column chromatography using diethylaminoethyl cellulose with a 0.0175 M phosphate buffer at a 7.2 pH. In most of the experiments the amount of kidney-fixing antibody was known. Kidney-fixing antibody (KFAb) was determined as before (9) and expressed in μg of antibody. The amounts of antibody varied from 150 to 351 μg (10 to 22 mg of total protein). A crude globulin preparation of duck NTG obtained by ammonium sulfate fractionation and containing 224 μg/ml was used. Antibodies to rabbit kidney antigens were obtained from sheep after repeated monthly injections of 5 ml of 10 per cent suspensions of the kidney antigen in incomplete Freund's adjuvant. The antisera obtained were absorbed twice with rabbit plasma, packed red blood cells, and buffy coat using 0.2 ml plasma and 1 ml cells per 10 ml serum. The absorbed sera were then fractionated with ammonium sulfate (50 per cent) to obtain the globulin fraction. Antibodies to rabbit fibrin were obtained by preparing fibrin from plasma with drop-wise addition of bovine thrombin, washing the resulting fibrin clump, and immunizing guinea pigs with the fibrin incorporated in Freund's adjuvant. Antisera obtained were absorbed with thrombin-treated rabbit serum. This yielded antisera that gave a single band in the β1-region in immunoelectrophoresis (IEP) when tested against rabbit plasma. Rabbit serum failed to yield precipitin bands in IEP with the antisera to fibrin. Antibodies to *rabbit gamma globulin* (RGG) were prepared in sheep as previously described (3). The RGG used for injection was first subjected to DEAE column chromatography using 0.01 M Na phosphate buffer at pH 8.0. The first protein fraction eluted was employed. Antisera from sheep yielded a single band in IEP in the γ-globulin region when developed with whole rabbit serum and occasionally a second faint band lying in the β2-region. *Anti-sheep gamma globulin* was obtained from Antibodies, Inc., Davis, California. It yielded a single band in the γ-region in IEP with whole sheep serum used as antigen Antibodies to *HGG* were prepared in rabbits as previously described (10). Antibiodies to *guinea pig*, *rat*, and *rabbit* complement or β 1C-globulin (anti-C′) were prepared and characterized as before (11).

Depletion of Polymorphs.—(*a*) Rats were depleted of polymorphs by one, or more injections of antipolymorph serum. Antibodies to rat polymorphs (antipolymorphs) were prepared by injecting rabbits with rat polymorphs obtained from the peritoneal cavity of rats following instillation of 0.1 per cent glycogen by a method adapted from that of Cohn and Hirsch (12). 90 to 100 per cent polymorphs were obtained, and following washing twice in 0.15 M saline in the cold, they were incorporated into incomplete Freund's adjuvant for immunization. Approximately 2×10^8 polymorphs were injected per rabbit in a single injection. The rabbits were exsanguinated 3 weeks later. This early response antiserum was found more efficacious in depleting rat polymorphs than that antiserum obtained after subsequent immunizations. The antisera so obtained were absorbed with plasma, packed rat red blood cells, and platelets obtained from rats depleted of polymorphs to avoid absorption of specific antipolymorph

antibody. The ratio of absorbing cells and plasma to antiserum was the same as that noted above in the absorption of nephrotoxic serum. The antisera were further absorbed with rat mesenteric and popliteal lymph node cells, using the nodes of 1 rat of 300 to 350 gm for approximately 20 ml antiserum. The antiserum was then tested by injecting graded amounts from 0.5 to 2.0 ml intraperitoneally in 150 gm rats. Only those antisera were employed that rendered rats deficient of polymorphs (less than 250/mm^3 of blood) at doses below 1.5 ml in a 16 hour period. The antisera were maintained in the frozen state (−20°C) at all times when not in use to avoid the development of toxicity.

(*b*) Rabbits (1 to 1.5 kg) were depleted of polymorphs by the injection of mechlorethamine HCl (nitrogen mustard, HN_2), using 1.75 mg/kg body weight. By the morning of the 3rd day postinjection, all rabbits exhibited polymorph counts below 200/mm^3 of blood. In both rats and rabbits, reversed passive Arthus sites were placed on the skin to signal the return of polymorphs. To produce the Arthus reactions, 50 μg anti-HGG N were used in rats intradermally and 100 μg antibody N in the rabbit, along with 3 mg HGG N intravenously/kg. If polymorphs reentered the circulation within 24 hours of the administration of Arthus reactants, edematous, and hemorrhagic reactions developed at the skin site. Polymorphs in rats were noted to return to approximately half normal values on the average 6 to 8 hours after injection of NTG and frequently to normal vaules by 24 hours. By contrast, in the 1 to 1.5 kg rabbits, polymorph counts never rose above 150/mm^3 24 hours after injection of NTG (*i.e.*, between days 3 and 4 after injection of HN_2). The effect of anti-rat polymorph serum on the formed blood elements other than polymorphs was not marked, however HN_2 treated rabbits had levels of mononuclear cells only 40 per cent of normal. Platelets were abundant in smears, and in rabbits, basophils ranged between 0 and 6/mm^3 in both polymorph depleted and control animals. The effect of polymorph depletion on C′ levels is given in the Results section.

Complement Depletion.—C′ depletion in rats was carried out using heat aggregated human gamma globulin (agg HGG) (13). An intravenous injection of 3 mg agg HGG N was given 30 minutes prior to the injection of nephrotoxic globulin and 0.8 mg N 30 minutes after. Blood was collected just before the injection of nephrotoxic globulin for C′ studies from both experimental and control animals. It was placed immediately in EDTA (0.01 M final volume) in order to prevent further C′ fixation *in vitro* by persisting agg HGG (14). The cells were then centrifuged away. When carrying out the analysis of C′ in such plasmas, $CaCl_2$ and $MgCl_2$ were added to equal the molar concentration of EDTA in the sample of plasma being analyzed.

Analysis of Complement Levels ($C'H_{50}$).—The quantitative C′ analysis described by Osler (15) was employed in the case of rats. In rabbits a modification of this technique was used to increase the sensitivity (4). Normal $C'H_{50}$ values for rabbits ranged from 75 to 125 U/ml.

Preparation of Polymorph Granules (Lysosomes) from Rabbits.—The method of Cohn and Hirsch was employed (12).

Immunoelectrophoresis (IEP).—The Scheidigger (16) adaptation of the method of Grabar and Williams (17) was used.

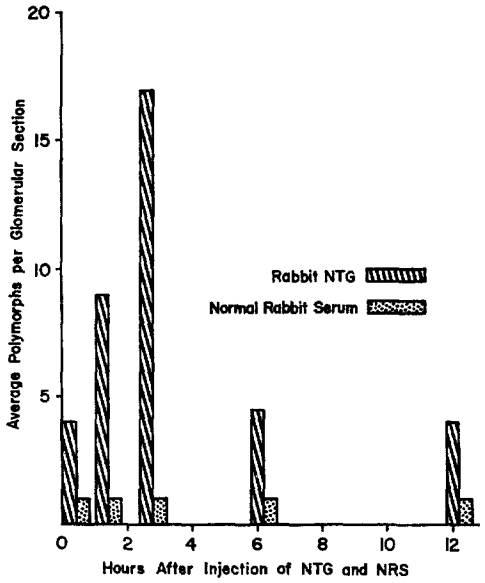
Fluorescent Antibody Technique.—The fluorescent antibody technique of Coons and Kaplan with minor modifications was employed (18). Controls used routinely were previously described (3).

Counts of Polymorphs in Glomeruli.—Sections of kidneys of rats and rabbits were embedded in paraffin, cut at 5 microns, and stained with hematoxylin and eosin. Counts of polymorphs were made in at least 15 glomeruli sectioned centrally ranging in position from the medullary-cortical junction out to the capsule. The average counts per glomerulus are reported.

RESULTS

The Presence of Polymorphs in Renal Glomeruli at Various Times after Injection of Nephrotoxic Globulin (NTG).—Rats were injected with an amount of

NTG known to yield an average of 130 mg proteinuria in the first 24 hours or, for purposes of control, with an equivalent amount of normal rabbit globulin and were sacrificed after 20 minutes, 1, 2.5, 6, and 12 hours. Microscopic sections of the kidneys revealed an elevation in the numbers of polymorphs in glomeruli between 20 minutes and 12 hours after injection of NTG. A peak accumulation was observed at 2.5 hours as noted in Text-fig. 1 and Figs. 1 and 2. Counts ranged as high as 60 polymorphs per section of a glomerulus at 2.5 hours with an average of 17. Endothelial cells at 6 and 12 hours after injection were swollen in many glomeruli and at times appeared separated from the



TEXT-FIG. 1. Numbers of polymorphs in 5 micron sections of glomeruli at different times after the injection of nephrotoxic globulin and normal rabbit serum in rats.

basement membrane. Rats injected with normal globulin failed to demonstrate an increase in polymorphs in glomeruli, even though the total white counts, and notably the numbers of polymorphs circulating were elevated to the same degree as those in the NTG injected rats (Text-fig. 1). In addition, observations on the lungs of the experimental and control rats microscopically revealed a marked and equal concentration of polymorphs in the pulmonary alveolar capillaries in the two groups between 1 and 6 hours. Alveolar edema or other alterations were not observed in the lungs of either group. Sections of adrenal gland and testis failed to reveal an increase in numbers of polymorphs.

By fluorescent antibody techniques in rats sacrificed at 2.5 hours, the NTG was readily detected in glomeruli of injected rats, concentrated apparently

along the basement membrane. Rat C′ was also found concentrated along the basement membrane. Neither was found when rats were injected with identical amounts of normal rabbit globulin. In rats injected with NTG, the NTG and rat C′ were not found in the pulmonary vessels with the exception of occasional peribronchial venules.

In further experiments, rats injected with NTG were sacrificed at 1 to 6 days following injection. Little or no polymorph concentration was observed during these days, including the period in which the rats underwent the second stage of the nephrotoxic disease, *i.e.*, following production of antibody against the NTG.

Electron microscopic studies: Electron microscopic studies of rats injected with NTG 2.5, 6, and 12 hours previously were carried out to find if alterations in the polymorphs or the glomerular structure could be observed. Examination of well over 100 polymorphs localized in glomeruli 2.5 and 6 hours after injection of NTG revealed that the polymorphs infiltrated the fenestrae of the endothelial cells, gaining intimate contact with the basement membrane (Figs. 3 and 4). This is similar to the changes noted by Winemiller, Steblay, and Spargo (19). Frequently, complete displacement of the endothelial cell by the polymorph from the basement membrane was found (Fig. 5).

At six hours, many polymorphs had disappeared leaving behind denuded glomerular basement membrane. Other than physical displacement, however, marked damage of the endothelial cells was not apparent, and by 12 hours, the endothelial cells had regained their former position along the basement membranes with the exception of occasional small subendothelial spaces separating basement membrane and endothelial cells.

Ultrastructural alterations of the polymorphs were not apparent. The peripheral cytoplasm of the polymorphs was occasionally clear, and devoid of organelles. However, granule lysis and phagocytic vacuoles were never seen. Nor was there found either a discharge of lysosomes or other cytoplasmic organelles to the surface or disruption of the cytoplasmic membrane itself.

Examination of the basement membranes with the electron microscope at 2.5 and 6 hours after injection of NTG also failed to reveal any dramatic alteration in its structure. The lamina densa was intact in a continuous electron dense band (Fig. 5), and differences could not be distinguished between zones of the basement membrane in intimate contact with polymorphs and basement membrane in capillary loops unaffected by polymorphs. Fibrin deposits were not observed along the basement membrane, in cells, or in the capillary lumina. However, irregular clear spaces separating the endothelial cells and the basement membrane were occasionally seen as described previously (20).

Inhibition of Proteinuria in Rats by Polymorph Depletion.—Attempts were carried out to determine if the polymorphs in the glomeruli in early NTN contributed to the pathogenesis of the structural damage of the glomerulus with

resulting proteinuria. NTG was injected into rats depleted of polymorphs by prior injection of specific rabbit anti-rat polymorph serum. Normal rats, serving as controls, were given normal rabbit globulin instead of antipolymorph serum and were then injected with NTG. The levels of proteinuria were followed in both groups, along with counts of levels of circulating polymorphs as recorded in Table I.

Polymorph depletion in rats injected with 150 to 201 μg of KFAb was ac-

TABLE I

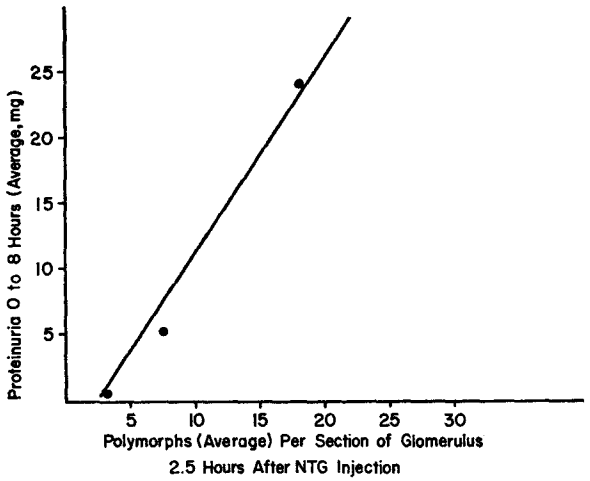
Effect of Polymorph Depletion on Early Proteinuria in Rats Injected with 150 μg of Rabbit Kidney-Fixing Antibody

Rat No.	Circulating polymorphs time 0 (per mm³)	Proteinuria			
		Day 1	Day 4	Day 8	Day 18
		mg P/24 hrs.	*mg P/24 hrs.*	*mg P/24 hrs.*	*mg P/24 hrs.*
1	69	1.0	1.6	8.0	135.0
2	0	3.1	2.7	24.0	288.0
3	0	1.9	6.6	81.5	180.0
4	0	2.0	5.2	84.0	40.0
5	0	5.0	1.0	5.0	105.0
6	0	3.7	6.0	27.0	180.0
7	0	30.7	24.0	49.5	180.0
Average 24 hr. proteinuria.....		6.8	6.7	40.0	158.3
8	2019	19.8	44.4	200.0	150.0
9	3040	31.4	21.0	100.0	264.0
10	3922	4.9	28.0	60.0	102.0
11	3839	146.4	150.0	84.0	105.0
12	1886	68.0	70.0	100.0	204.0
13	2130	27.4	15.0	72.0	135.0
Average 24 hr. proteinuria.....		49.6	54.7	102.7	160.0

companied by a profound inhibition of proteinuria during the first 24 hours. The latter dose was known to produce just maximal proteinuria. Urine volumes were comparable to the control groups. A representative experiment is shown in Table I. In this experiment an average of 6.8 mg of protein was excreted in the urine of the polymorph depleted group while an average of 49.6 mg was excreted in controls. In the polymorph depleted group, a single rat (No. 7) accounted for most of the combined values. Fluorescent antibody studies of both groups of rats revealed no difference in the intensity or pattern of distribution of rabbit GG or host C′ in glomeruli when rats were examined at 2.5 or 24 hours after the injection of NTG. The inhibition of proteinuria by poly-

morph depletion continued throughout the first week of the nephritis as noted in Table I. With the development of the second stage of NTN, however, proteinuria reached a degree comparable to that of the control group.

In an attempt to find if a correlation existed between the numbers of polymorphs that accumulated in glomeruli and the amount of proteinuria that developed, rats were injected with graded doses of NTG (212, 132, and 79.5 μg KFAb) and sacrificed at either 2.5 hours for histologic study of the glomerulus or at 8 hours for the degree of proteinuria. The results, presented in Text-fig. 2, indicated that when considerable numbers of polymorphs accumulated at 2.5 hours in the glomeruli a corresponding brisk proteinuria developed. On the



TEXT-FIG. 2. Comparison of numbers of polymorphs in 5 micron sections of rat glomerulus 2.5 hours after injection of nephrotoxic globulin and the amount of proteinuria appearing by 8 hours.

other hand, at the lower dosage levels, when few polymorphs were seen, little or no proteinuria developed, the theoretical line drawn in the figure passing nearly through 0.

Polymorph depletion in rats injected with 289 to 350 μg KFAb was accompanied by a partial inhibition of proteinuria during the first 24 hours. Inhibition of proteinuria varied from 24 to 53 per cent depending on the dose of antibody injected. When urine was collected during the first 8 hours it was constantly noted that the inhibition of proteinuria was marked. Between 8 and 24 hours however, injury of the glomeruli in polymorph depleted rats apparently had occurred as revealed by proteinuria equal to slightly more than half that of the controls. A representative experiment is shown in Table II. As noted in Table II an average of 245.7 mg of protein was excreted per animal

in the first 24 hours while an average of 59.4 μg per animal was excreted in the polymorph depleted rats. In another experiment it was found that there was little or no accumulation of polymorphs in the glomeruli when polymorph depleted rats were examined at 4, 8, 12, and 24 hours post-NTG injection. However, circulating polymorphs rose during this time and reversed passive Arthus reactions, placed on the skin to signal the return of polymorphs, had become positive by 8 hours. Histologic examinations of rats injected with these doses of antibody revealed severe glomerular basement membrane alterations with the presence of focal PAS-positive deposits in the glomeruli by 24 hours. As in

TABLE II

Effect of Polymorph Depletion on Early Proteinuria in Rats Injected with 351 μg of Rabbit Kidney-Fixing Antibody

Polymorph depleted		Control	
No. of rats	Average proteinuria 0 to 24 hrs.	No. of rats	Average proteinuria 0 to 24 hrs.
	μg		*mg*
5	59.4 (17–93)	8	245.7 (50–360)

TABLE III

Deposition of I^{131} NTG in Kidneys of Normal and Polymorph-Depleted Rats

	No. rats	Per cent of activity injected remaining in kidneys		Tissue bound I^{131} NTG*
		Average	Range	
				per cent
Polymorph depleted.....	4	2.87	2.80–2.98	1.90
Normal.....	3	2.57	2.36–2.85	1.70

* Per cent of I^{131} NTG in perfused kidneys minus per cent of I^{131} normal rabbit globulin left in perfused kidneys.

the previous group there was no difference in the intensity or pattern of distribution of rabbit GG or rat C′ in control and polymorph depleted groups when examined 24 hours after NTG injection; focal deposits of fibrinogen and rat gamma were also noted in the capillary walls of these rats.

Control studies on polymorph depleted rats: Studies were undertaken to find if measurable factors other than the polymorphs were affected by the anti-polymorph serum. As noted in Materials and Methods, no apparent decrease in platelets occurred. A decrease in mononuclear cells occurred from average values of 11,700 to 18,500 in normal rats to 6700 to 14,200 in polymorph depleted rats, depending upon the pool of anti-rat polymorphs employed. Hemolytic C′ levels of the blood at the beginning of the experiments showed 30.3

$C'H_{50}$ units (8 rats) in the polymorph depleted group and 44 $C'H_{50}$ units in the normal group (5 rats). Overlap of $C'H_{50}$ levels existed between the two groups. In addition, as noted above, abundant C′ was found with the NTG in the glomeruli in both groups along the basement membrane by fluorescent antibody techniques.

Tests were performed to find if in the polymorph depleted rats, a decreased amount of NTG localized in the kidneys in comparison with the normals. Accordingly, NTG and control normal rabbit globulin (NRG) were labeled with I^{131} and injected into polymorph depleted and control rats as noted in Materials and Methods. Counts of the perfused kidneys are shown in Table III. After subtracting the amount of I^{131} NRG not removed by perfusion the amount of I^{131} NTG bound in the kidneys was approximately the same in polymorph-depleted and normal rats.

Tests were also carried out to find if the peripheral vascular beds in the

TABLE IV

Effect of Polymorph Depletion on Proteinuria in Early Nephrotoxic Nephritis in Rabbits

No. rabbits	PMN/mm³	Proteinuria		
		0 to 24 hrs.	24 to 48 hrs.	6 to 7 days
		mg	*mg*	*mg*
5	<100	0	4	85
5	4340	1847	1151	491

polymorph-depleted rats were able to respond normally to certain exogenous stimuli. 5-hydroxytryptamine (5.0 μg/ml) and compound 48/80 (10 μg/ml) were injected intradermally (0.1 ml/site) into 3 polymorph depleted and 2 control rats within 10 minutes of an intravenous injection of 0.5 ml 1 per cent Evan's Blue. 30 minutes after the injection of Evan's Blue, the skin was reflected for examination and revealed blueing zones measuring 8 to 10 mm diameter at the 5-hydroxytryptamine sites and 10 to 11 mm at the 48/80 sites in both polymorph depleted and control rats.

Inhibition of Proteinuria in Rabbits by Polymorph Depletion.—Rabbits were depleted of polymorphs by a prior injection of HN_2 and were then injected with varying doses of nephrotoxic globulin. Urine protein levels were obtained at 9 to 10 hours after injection, at 23 to 24 hours and on subsequent days. Specimens were obtained by catheter at the end of the time period to insure complete sampling. The results are presented in Table IV. As noted, using a dose of NTG that brought about a total of 1847 mg protein in the urine in the first 24 hours in the normal rabbits, a complete inhibition of proteinuria was found in the polymorph depleted animals. This inhibition continued through the second

day and, as in rats, through the entire first stage of NTN even though polymorphs began returning to the circulation (Table IV). However, proteinuria did develop when the rabbits began producing antibody to the NTG.

When the dosage of NTG was increased it was found that 2 of 5 polymorph depleted rabbits developed a significant degree of protein in the urine by 10 hours after injection while 3 of 5 failed to exhibit more than slight proteinuria until after 10 hours had elapsed (Table V). Thus, as was the case in rats depleted of polymorphs and injected with large doses of NTG, a lag was noted

TABLE V

Delayed Development of Proteinuria in Polymorph-Depleted Rabbits with a Large Dose of Nephrotoxic Globulin

	Proteinuria*			
	0 to 10 hrs.	11 to 24 hrs.	Total 0 to 24 hrs.	24 to 48 hrs.
	mg	*mg*	*mg*	*mg*
Polymorph depleted	1365	500	1850	390
	30	1000	1030	60
	12	175	187	435
	1025	1650	2675	840
	18	240	258	135
Average	490	710	1200	373
Normal controls	420	600	1020	1650
	900	2300	3400	1900
	1023	1450	2473	3120
	3500	1200	4700	750
Average	1461	1388	2896	1855

* Proteinuria developing during the listed time periods following the injection of NTG. Obtaining specimens for the total period was assured by final catheterization.

between the initial interaction of antibody and glomerular basement membrane and the development of proteinuria. During the first 24 hours the circulating blood was free of polymorphs and the Arthus sites, placed on the rabbits at the beginning of the experiment did not flare during the testing period. Differential counts of formed blood elements are noted in Materials and Methods. 5 rabbits injected with normal sheep globulin instead of NTG failed to show any protein in the urine. When even larger amounts of NTG were employed, proteinuria did develop within the first 9 hours, in an amount 55 per cent of that found in normal rabbits (7 rabbits in each group). This dose of NTG was found to be lethal to normal animals within 3 days. At this dose level, the depletion of polymorphs reduced the amount of protein in the urine by close to 40 per cent in the first 24 hours.

As in the rats, rabbits sacrificed at 2 hours after injection of NTG demonstrated abundant heterologous (sheep) gamma globulin and host C′ along the basement membrane of the glomerulus. No difference in the amount of fluorescence could be distinguished between polymorph-depleted and normal rabbits. Levels of hemolytic C′ at the time of injection of NTG averaged 103 $C'H_{50}$ units in 13 C′ depleted rabbits and 121.3 in 10 normals. Considerable overlap existed between the two groups, 4 of the polymorph-depleted rabbits having $C'H_{50}$ values well above the average of the normal group.

Experiments were conducted to find if exposure of the kidneys or other organs to HN_2 had rendered the glomeruli incapable of developing increased permeability in a manner unrelated to polymorph depletion. Rabbits were injected with HN_2 in the ear vein while the aorta was clamped below the renal arteries. Since the HN_2 is inactivated in less than 10 minutes, the femoral bone marrow was preserved if the clamp was held on for this period. Under these conditions, the HN_2 contacted all the tissues of the body above the point of clamping and yet polymorph production from the femoral marrow continued. After 3 days, polymorph counts in 3 rabbits so treated were 600, 1980, and 11,400/mm^3 instead of the expected 150 or less/mm^3. As noted, one of the rabbits developed moderate depletion despite clamping of the aorta. Levels of mononuclear cells were diminished to the same degree as those in the usual polymorph depleted rabbits without aorta clamping. When injected with a dose of NTG that yielded an average of 920 mg proteinuria in the first 24 hours in normal rabbits, the rabbits with aortic clamp treatment gave 236, 1150, and 790 mg protein (average 725 mg) in the urine respectively in 24 hours. In other HN_2 treated, polymorph depleted rabbits (without clamped aorta) the proteinuria was completely inhibited.

In tests to find if the peripheral vascular bed was susceptible to direct stimulation following treatment with HN_2, 2 μg histamine was injected intradermally in polymorph depleted and normal rabbits following the intravenous injection of 1 ml 5 per cent Evan's Blue. No differences in blueing in the histamine site in the two groups was noted, with reactions of 12 mm average diameter developing.

Attempts to replete polymorphs in the depleted animals by injections of fresh polymorphs were not made owing to the known stickiness of these cells in *in vitro* conditions and to previous failures in this regard (2). However, granules from polymorphs were obtained as determined in Materials and Methods and injected into polymorph depleted rabbits in the left cardiac ventricle 20 minutes after the administration of a dose of NTG calculated to be ½ the amount needed to induce minimal proteinuria in intact rabbits. In 5 rabbits so treated, using granules from 1.2×10^9 polymorphs, a range from 9 to 105 mg protein was noted in catheterized urine specimens at 10 hours. In only 2 rabbits could granules be identified histologically in glomeruli. In these, single clumps of

granules measuring less than 10 microns were observed in an average of 1 out of 20 glomeruli. Little change was otherwise noted histologically.

The Relationship of Complement Binding and the Accumulation of Polymorphs in Early Nephrotoxic Nephritis.—The relationship of C′ binding to polymorph accumulation in glomeruli in rats was assayed. This was accomplished by (*a*) counting polymorphs in glomeruli of rats injected with rabbit NTG after depletion of circulating C′; and (*b*) by injecting duck NTG, that fixes rat C′ poorly, into normal rats.

In the first experiments, 7 rats were injected with heat aggregated human gamma globulin (agg HGG) in order to deplete their levels of circulating hemolytic C′ and then challenged with 265 μg of kidney-fixing antibody. As

TABLE VI

*The Effect of Complement Depletion on the Accumulation of Polymorphs in Glomeruli and Proteinuria Following Intravenous Injection of Nephrotoxic Globulin in Rats**

	No. rats	Polymorphs in glomeruli‡	Polymorphs in blood/mm³ 0 hr.	$C'H_{50}$ 0 hr.	Fluor. ab. of glomeruli		Proteinuria at 9 hrs.	
					C′	RGG	No. rats	mg
C′ depleted§.....	7	4.0	10,960	<7.5	±	4+	3	2.1
Control.....	6	21.0	4200	38	3+	4+	5	41.7

* Each control and C′ depleted rat received 265 μg of kidney-fixing antibody.

‡ Average counts of polymorphs per 5 micron section of a single glomerulus in rats sacrificed 2.5 hours after NTG injection.

§ C′ depletion brought about with agg HGG (see Materials and Methods).

noted in Table VI rabbit NTG injected into these rats led to only a modest accumulation of polymorphs in the glomeruli. An average of 4 polymorphs were found per glomerular section as contrasted to 21 in NTG injected normal rats. $C'H_{50}$ levels were markedly depleted at the start of the experiment and fluorescent antibody observations on the glomeruli of treated rats at 2.5 hours showed a marked diminution of bound C′ although small amounts could still be found. Occasional focal granules of C′ were observed in the glomeruli that appeared to be in mononuclear cells on the capillary side of the basement membrane. These fluorescent granules were similar in appearance to granules of agg HGG also found in the glomeruli by the fluorescent antibody technique. In 3 rats treated as above with agg HGG and NTG, proteinuria of only 2.1 mg (average) developed in the first 9 hours while 41.7 mg were noted in controls for the same period (Table VI). Of special note, the circulating polymorphs were not depleted by the injection of agg HGG and indeed were elevated to over twice the values of control rats. In studies reported separately, but done in conjunc-

tion with these (5), the polymorphs of C′ depleted animals were found to exhibit a normal phagocytic capacity and normal motility. In addition, the polymorphs reacted normally to a chemotactic stimulus unrelated to C′.

In the second series of experiments, 4 normal rats were injected with 224 μg of duck kidney-fixing antibody, a dose sufficient to produce over 200 mg protein in the urine in the first 24 hours. However, sections of kidneys taken 2.5 and 6 hours after injection of the duck NTG revealed little or no rat C′ bound along the basement membrane and an average of only 1 polymorph per section of glomerulus. It was also found that the proteinuria induced by duck NTG was not inhibited in polymorph depleted rats.

DISCUSSION

Glomerular Injury in Nephrotoxic Nephritis that is Dependent upon Polymorphonuclear Leukocytes.—The data presented indicate that polymorphonuclear leukocytes (polymorphs) play a significant role in the glomerular damage of acute nephrotoxic nephritis (NTN) induced by mammalian antiserum. This was noted in both rats and rabbits. When moderate doses of nephrotoxic globulin (NTG) were injected into test animals depleted of their polymorphs, complete or nearly complete abrogation of proteinuria was noted (Tables I and IV). This was particularly apparent in experiments in which intact rabbits developed over 1800 mg of protein per kg in the urine in the first 24 hours after injection of nephrotoxic globulin. Other rabbits, depleted of polymorphs, and given the same amount of nephrotoxic globulin, failed to show any proteinuria. In rats (150 gm) the doses totally inhibited by polymorph depletion ranged from those that induced threshold proteinuria to those giving at least 100 mg protein in the first 24 hours. Several control studies indicated that the procedures of depleting polymorphs did not render the vascular bed unreactive, block glomerular localization of the nephrotoxic globulin and C′, or markedly affect other blood elements. That the polymorphs participated directly in the glomerular injury was suggested by the direct relationship between polymorph count in the glomeruli 2.5 hours after injection of NTG and the degree of associated proteinuria (Text-fig. 2). When a dose of NTG just sufficient for the accumulation of polymorphs was given, proteinuria was first observed. It was apparent, however, that by increasing the dose of NTG in rats to levels that saturated approximately 75 per cent (calculated) of the available renal antigens, *i.e.*, to 289 μg of KFAb, proteinuria occurred even in the absence of polymorphs. The significance of this polymorph-independent permeability will be discussed below.

In rats and rabbits protected from glomerular damage in the first 24 hours by polymorph depletion, proteinuria did not appear until the second stage of NTN, *i.e.*, when the animal produced antibody to the NTG (Tables I and IV). This suggested that the glomerular damage of the first stage of NTN was de-

pendent upon the polymorph injury occurring in the first hours after injection of NTG. The reason that polymorphs did not accumulate in large numbers in the glomeruli during the first few days after their return to the circulation is not certain. Any role of polymorphs in the second stage of NTN is not clear but there was no detectable accumulation of polymorphs in the glomeruli accompanying this phase of glomerular injury.

In addition to these observations in experimental animals, in acute glomerulonephritis of human beings, an accumulation of polymorphs in glomeruli beneath endothelial cells and immediately adjacent to basement membrane has also been noted (J. D. Feldman, personal communication).

The actual target of polymorph action that would account for the leakage of protein into the urinary space was not ascertained from these studies. However, in view of the suggested role of the glomerular basement membrane as a barrier to protein passage (21) and because of the intimate association of NTG, host complement and the host's polymorphs (Figs. 3 to 5) with the inner surface of the basement membrane this may well be the site of action. The polymorph effect could be manifested alone or in concert with the antibody and complement already present on the basement membrane. While an attack on the basement membrane seems a likely possibility, no direct evidence such as loss of morphologic integrity of the basement membrane is available. The most striking visable alteration of the glomerulus was the apparent displacement of the endothelial cells away from the basement membrane by the intruding polymorphs. While this might account for the increased glomerular permeability, there is certain evidence to the contrary: (*a*) since the endothelial cells do not appear to prevent protein passage in the normal state (21) their displacement *per se* should not alter filtration greatly; (*b*) proteinuria can be brought about by antisera, *e.g.*, of duck origin, or by other agents that did not cause such marked dislodgement of the glomerular endothelial cells; and (*c*) the endothelial cells returned to a reasonably normal state within 12 to 24 hours and yet proteinuria continued. The epithelial cells appeared normal at a time when proteinuria was well advanced indicating that significant morphologic alterations of these cells is not an early event in the development of proteinuria.

Lysis of polymorph granules or the cytoplasmic membrane was not observed suggesting that the action of polymorphs does not require the ultrastructural changes in cytoplasmic organelles that accompany some other reactions. In Arthus reactions (3, 22, 23) or in phagocytosis *in vitro* (24–26) digestion vacuoles form during phagocytosis (of antigen-antibody complexes in the former) and the lysosomes then fuse with these vacuoles, releasing into them their content of hydrolytic enzymes, etc. Whether these events have anything to do with the destruction of vascular structures in the Arthus phenomenon is not known. The present observations, however, demonstrate that polymorphs are able to inflict considerable change to the glomerulus without themselves showing any great extent of granule lysis.

NTN Glomerular Injury Occurring Independently of Polymorphs.—While the development of proteinuria was dependent upon the presence of polymorphs when moderate doses of NTG were used, large doses of NTG could induce proteinuria without polymorphs. Under these circumstances, mediators other than polymorphs apparently brought about the glomerular damage. The question of what mediators might be responsible for this damage, is of great interest. Unless the damage is brought about by the physical deposition of large amounts of immune complexes themselves, a search among the kinins, hemolytic complement, angiotensin, and acetylcholine, vasoactive amines, anaphylatoxin, the serum permeability factors, and blood coagulation factors would seem indicated. The mediation of this permeability may be similar to that in the second stage of NTN in which small amounts of NTG and β1C-globulin have been associated with proteinuria (27). This damage apparently involved a continuous interaction of mediators and glomerular capillary membranes.

A Role of Complement in Attracting Polymorphs to the Basement Membrane.—Two studies strongly suggested a role of complement (C′) in attracting polymorphs to the glomerular basement membrane. First, when rats were depleted of circulating C′, the polymorphs in the circulation failed to accumulate in glomeruli even though present in the blood stream in normal or increased numbers. Second, normal rats, when injected with duck nephrotoxic globulin that fixed rat C′ poorly, did not show a glomerular accumulation of polymorphs (although, as noted previously, they did develop proteinuria). Thus, at least one (indirect) role of C′ in early NTN would seem apparent, namely the attraction of polymorphs. These cells in turn bring about damage of the glomerulus. While the possibility exists that C′ plays a direct role in the glomerular injury of NTN independent of polymorphs, evidence for this is lacking at present. These results parallel the finding that in Arthus reactions when C′ is not bound by antigen and antibody in vessel walls, polymorphs are not attracted and vascular damage does not ensue (5). For this attraction of polymorphs in guinea pigs, at least the first three reacting components are required (C′1, 4, 2). Further studies have strongly suggested that when components of rabbit C′ through C′6 are activated, chemotaxis of polymorphs *in vitro* occurs (28). The actual substance(s) responsible in greatest part appears to be a complex of C′5 and 6 that rapidly interacts with and is liberated from the immunologic reactants.

Thus in both the Arthus reaction and acute nephrotoxic nephritis, produced with moderate dosages of nephrotoxic globulin, a common chain of events occurs. Antibody complexes with antigen rapidly fixing abundant complement. Active chemotactic agents are released that attract and cause the accumulation of polymorphs. With the accumulation of polymorphs damage of the blood vessels occurs leading to edema and hemorrhage in the one case, and proteinuria in the other. In the Arthus phenomenon and perhaps in early NTN the basement membrane appears to be the main target of the polymorph attack (29).

However, this chain of mediators may be by-passed when large amounts of nephrotoxic antibody are employed, glomerular damage then apparently occurring without the contribution of polymorphs.

SUMMARY

In acute nephrotoxic nephritis, polymorphonuclear leukocytes (polymorphs) accumulated in large numbers in the glomeruli in the first 12 hours. The endothelial cells were dislodged by the polymorphs which then came to lie immediately adjacent to the glomerular basement membranes. Ultrastructural changes in neither polymorphs nor basement membranes were observed.

Depletion of polymorphs in both rats and rabbits prevented the development of proteinuria. This occurred when doses of nephrotoxic globulin were employed that produced proteinurias of as much as 1800 mg/kg/24 hours in intact rabbits, or enough to yield near maximal immediate proteinuria in intact rats. In addition, measurable glomerular damage was frequently averted until the onset of the secondary stage of NTN. Controls indicated that the polymorph depleted animals exhibited minimal non-specific changes in the blood, that the ability of their vascular beds to react to stimuli was not affected, and that deposition of nephrotoxic antibody and C′ in the glomeruli was not inhibited. Elimination of polymorphs from the circulation was only partially effective in preventing glomerular damage when large doses of nephrotoxic globulin were used. This indicated that under these circumstances, a polymorph independent glomerular injury may also take place in first stage nephrotoxic nephritis.

An indirect role of C′, *i.e.*, the accumulation of polymorphs, in bringing about glomerular injury in first stage nephrotoxic nephritis was apparent. When rabbit nephrotoxic globulin was injected into rats depleted of C′, or when duck nephrotoxic globulin that fixed C′ poorly was injected into normal rats, C′ failed to bind with the antibody along glomerular basement membranes and polymorphs did not accumulate.

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EXPLANATION OF PLATES

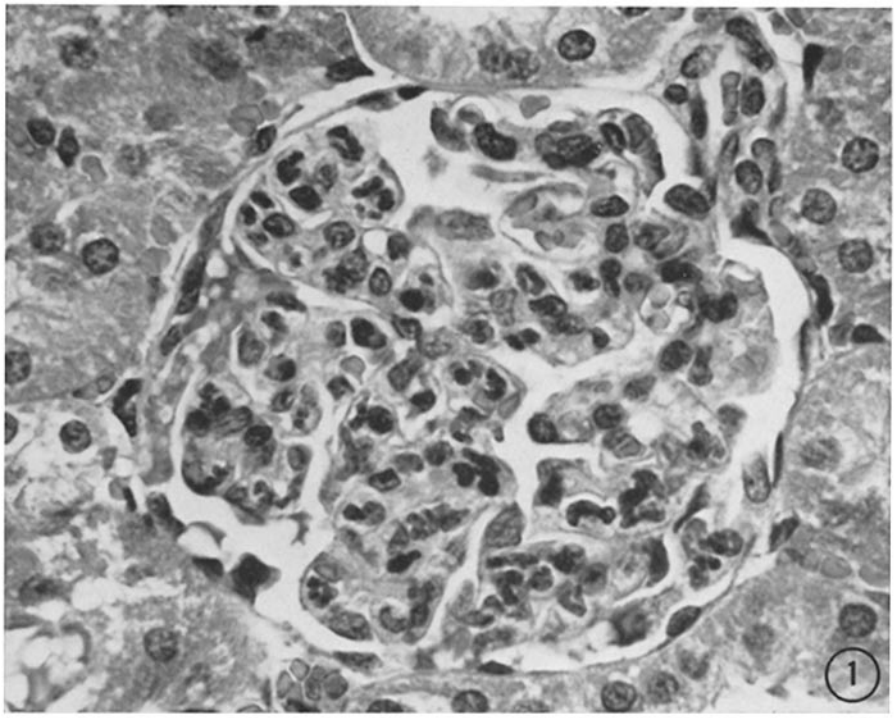
Abbreviations for Figures

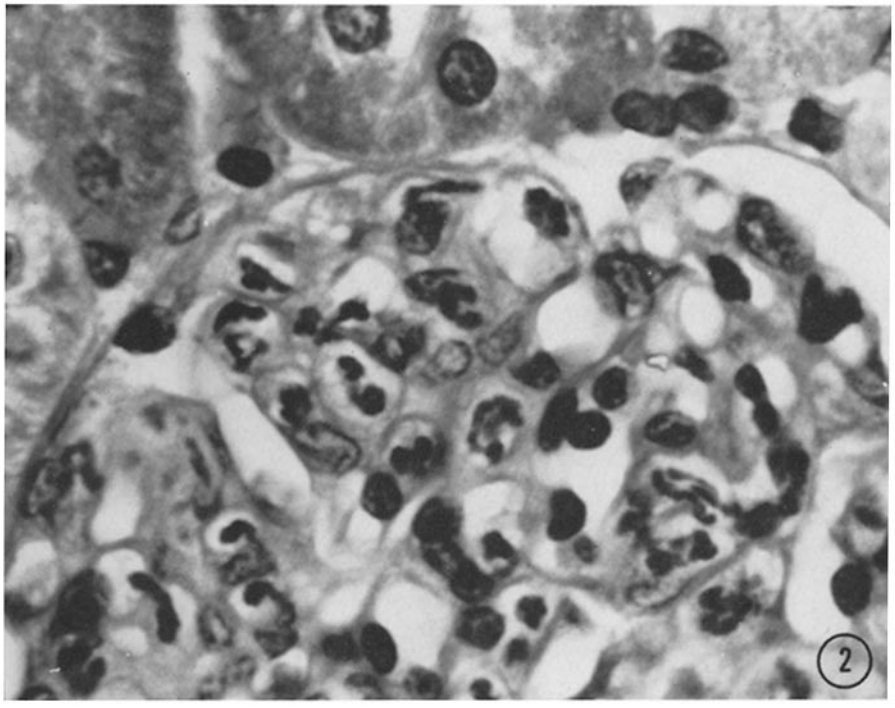
BM, basement membrane
end, endothelial cell
ep, epithelial cell
CL, capillary lumen
n, nucleus
PMN, polymorphonuclear leukocyte
RBC, red blood cell

PLATE 15

FIG. 1. Microscopic section of a glomerulus taken from a rat 2.5 hours after the injection of nephrotoxic globulin. Note the influx of polymorphs. Fluorescent antibody studies revealed the presence of nephrotoxic globulin and rat C′ along the basement membranes in similar sections of the same kidney. Hematoxylin-eosin. × 600.

FIG. 2. Higher power photomicrograph of a glomerulus, similar to that in Fig. 1, showing the crowding of polymorphs in capillary loops. Hematoxylin-eosin. × 900.





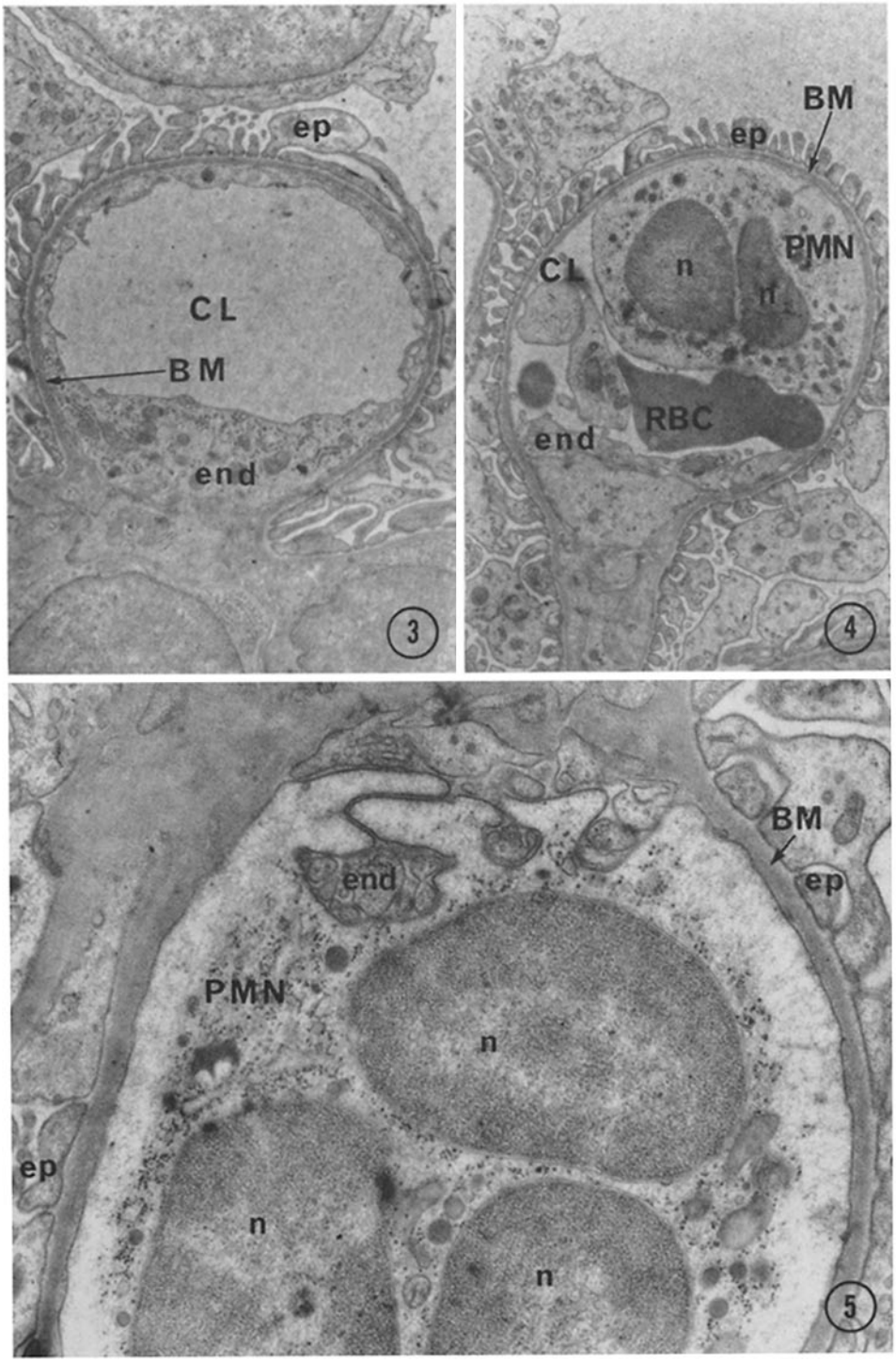
(Cochrane *et al.*: Polymorphonuclear leukocytes)

PLATE 16

FIG. 3. Electron photomicrograph of a glomerular capillary loop, shown for comparison with Fig. 4, taken from a rat 2.5 hours after an intravenous injection of nephrotoxic globulin. Polymorph infiltration has not occurred and the endothelial cell (*end*) cytoplasm is distributed normally over the surface of the basement membrane (*BM*). × 7600.

FIG. 4. Electron photomicrograph of another glomerular capillary loop from a rat 2.5 hours after an intravenous injection of nephrotoxic globulin. The endothelial cell (*end*) has been swept aside by the polymorph (*PMN*), leaving a denuded basement membrane (*BM*). The polymorph has thus gained intimate contact with the basement membrane. By 6 hours, most polymorphs have left the glomerular loops, and between that time and 24 hours, the endothelial cells largely return to a position along the basement membrane although proteinuria continues. Note that the density of the basement membrane is not altered. × 7600.

FIG. 5. Electron photomicrograph of another capillary loop from a rat similar to that shown in Fig. 4. The pseudopods of the polymorph are seen extending beneath the endothelial cell (*end*) cytoplasm, forcing the endothelial cell away from the basement membrane (*BM*). Again, note the absence of visible alteration of the basement membrane. In addition, neither granules lysis nor rupture of the polymorph cytoplasmic membrane exists. × 23,000.



(Cochrane *et al.*: Polymorphonuclear leukocytes)