POLYMORPHONUCLEAR LEUKOCYTES IN IMMUNOLOGIC REACTIONS

THE DESTRUCTION OF VASCULAR BASEMENT MEMBRANE IN VIVO AND IN VITRO*, ‡, §

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Several experimental immunologic diseases apparently share a common pathway of mediation. To date these include the Arthus phenomenon in which cutaneous vessels are injured, the arteritis in serum sickness (1), and the glomerulitis in acute nephrotoxic nephritis (2). Damage occurs in each of these following the interaction of many factors, several of which are apparently common to all the reactions. These common mediators include (*a*) antibody and antigen that combine at a focal point in a blood vessel wall (3–6), (*b*) complement (C′) that reacts with the immune complex (7–9, 1), and (*c*) polymorphonuclear leukocytes (PMN's) that accumulate at the site. Recent studies in this laboratory would suggest a complex of the 5th, 6th, and 7th components of C′ is responsible, at least in part, for this accumulation of PMN's (10, 11). A release of PMN constituents may in turn, also bring about accumulation of more PMN's (12–14). Each of these events has been shown to be essential for full expression of the reaction.

Considerable speculation exists as to the mechanism by which PMN's may injure blood vessel walls. In addition, little is known about the critical target in the vessel walls attacked by the PMN's. Several investigators have described permeability factors either released during incubation of PMN's in normal saline at 37°C (12) or contained within the cytoplasmic granules (13, 14). These factors have been shown to be cationic proteins (13, 14) and are capable of causing degranulation of rat mast cells (13). Other possible injurious agents within PMN's include the acid proteases or cathepsins (15). These are known to catabolize phagocytized immunologic reactants in Arthus reactions (16) or antigen and antibody in vitro (17). The possible roles played by these various

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agents in immunologic injury of tissue is not known and the target and mechanism of their attack remain uncertain. In order to identify and characterizd the agents within PMN's actually responsible for the injury of vessels it was essential to discover first the specific structures within the vessel walls that were altered during the reaction. Accordingly, the first portion of this study was undertaken in an attempt to analyze morphologically the critical structure in vessel walls that was altered by PMN's; the second part was to search for the substances within PMN's capable of causing the damage.

Materials and Methods

Preparation of Reagents.—

Glomerular basement membrane: Glomerular basement membrane was prepared by the method of Krakower and Greenspon (18). The membranes were suspended in veronal buffer containing at all times Ca (0.00015 M) and Mg (0.0005 M). The preparation was maintained in the frozen state except during purification. Special precaution was taken to remove all cellular constituents by sonic oscillation and verification of removal was performed by the electron microscopic and by light microscopic observations after staining the material with 20% trypan blue. Control studies regarding possible cellular contaminants of the basement membrane appear below. The sonicated membranes were washed twice in veronal buffer and once in distilled water prior to lyophilization.

Antigens.—Bovine serum albumin (BSA), 5 x crystallized, was purchased from Armour Co., Kankakee, Illinois. Rabbit gamma globulin (RGG), Cohn fraction II, was purchased from Pentex Inc., Kankakee, Illinois. When used for immunization, the RGG was passed through a column of DEAE-cellulose in Na phosphate buffer, 0.01 M, pH 8.0 to remove other serum proteins.

Antisera.—Antisera to BSA and RGG were prepared in rabbits and sheep respectively as described previously (16). When used for skin testing, these antisera were fractionated to obtain 7S gamma globulins (IGG), and each preparation was divided into small aliquots to avoid development of toxicity of the serum by repeated freezing and thawing.

Antisera to glomerular basement membrane was prepared by immunizing rabbits with purified basement membrane of mice, rats, and guinea pigs in incomplete Freund's adjuvant using monthly injections of approximately 5 to 10 mg of the material. Antisera to rabbit basement membrane was obtained after immunizing sheep with monthly injections of 20 mg purified rabbit basement membrane. These antisera were absorbed exhaustively with packed red and white blood cells obtained from buffy coats of peripheral cells, platelets, and lymph node cells and plasma. This absorption procedure eliminated precipitation, noted in agar diffusion, occurring between the unabsorbed antiserum and lysates of polymorphonuclear leukocytes. Gamma globulin fractions of each absorbed serum were then prepared by chromatography on DEAE-cellulose as noted above, and these globulin fractions were maintained frozen in small aliquots except when used. Similarly treated normal sera were employed at all times to verify a lack of toxicity. These precautions were found necessary to prevent development of factors in serum that bring about increases in permeability when injected into the skin. The specificity of the antibody was tested by two means. First it was conjugated with fluorescein and placed on sections of kidney and skin to find if antigens other than those in basement membrane could be visualized. Only the sharp linear fluorescence of vascular, glomerular, and peritubular membranes was observed. Endothelial and epithelial cells were devoid of fluorescence. Secondly, attempts were made to find basement membrane antigens in the most likely source of contamination, the renal cells, by two means: (*a*) Renal cells were cultured in vitro

from the kidneys of six 1.5 kg rabbits.[1] The cells were harvested after 6 days, lyophilized, and 10 mg of these cells and 10 mg of the preparation of basement membrane each were injected into 400-g guinea pigs in incomplete Freund's adjuvant over a 2 wk period. The guinea pigs were bled 3 wk after the final injection and their sera tested by double diffusion in agar using trypsin-digested basement membrane and a saline extract of the renal cells after sonic oscillation. Three out of four of the guinea pigs immunized with purified basement membrane responded with precipitins to the trypsinized basement membrane, and none of the four produced detectable precipitins to extracts of the cultured renal cells. By contrast, three out of four guinea pigs injected with cultured renal cells yielded precipitins to extracts of these cells but none responded to the basement membrane. (*b*) In addition to the above, the sheep anti-rabbit basement membrane employed in double diffusion precipitation tests failed to form precipitin bands with the antigens in the extracts of renal cells. Thus, antigens in the preparation of basement membrane were distinct from those in the renal cells, at least within the limits of sensitivity of the test.

Antisera to polymorphonuclear leukocytes (PMN's): These were prepared by immunizing sheep with rabbit PMN's contained in Freund's adjuvant and purified by absorbing with cells and plasma as noted previously (1). Anti-rat and guinea pig PMN's were obtained from rabbits and were purified as noted previously (2). PMN's were obtained from the peritoneal cavities of rabbits, rats, and guinea pigs by the method of Cohn and Hirsch (19).

Depletion of Circulating PMN's.—PMN's were depleted from the circulation of rabbits and mice by an injection of nitrogen mustard (1.75 mg/kg). PMN's of rats and guinea pigs were depleted as reported in a previous communication by use of specific anti-PMN serum (2). The methods employed and tests for specificity were the same as reported previously (2).

Skin Reactions to Immunologic Reactants.—Rabbits (1 to 2 kg), Sprague-Dawley rats (150 to 600 g), Hartley guinea pigs (300 to 400 g), and Swiss mice(25 to 30 g) were employed through out.

Skin reactions to injected antibody to basement membrane (ABM reactions) were performed by injecting 0.1 ml of the appropriate antibody IGG. Reversed passive Arthus (RPA) reactions were performed and graded as noted previously (3). Biopsy sections were removed at intervals for both fluorescent and routine histologic studies. Purified carbon (preparation C1431a, Gunther Wagner) was injected intravenously after dialysis against neutral 0.15 M NaCl, in the amount of 200 mg/kg. RPA reactions for electron microscopic reactions were elicited in the walls of urinary bladders of adult rabbits. Sections taken 4 to 6 hr after injection were fixed in OsO_4 and glucose and embedded in Vestopal. Tests of increased vascular permeability were performed by injecting 0.1 ml of a solution intradermally immediately after an injection of 1 ml 2.5% Evan's Blue/kg intravenously. The intensity of blued reactions was judged by measuring the large and smaller diameters of transilluminated skin, and then averaging the two measurements.

Fractionation of PMN Lysates.—PMN's were obtained from the peritoneal cavities of adult rabbits 4 or, at times, 16 hr after injection of 0.1% glycogen (19). The percentage of PMN's in the exudate averaged greater than 95% in the 4-hr preparations, and 80% in the 16-hr preparations, with a yield of 0.1 and 0.6×10^9 cells/rabbit respectively. The cytoplasmic granules were then obtained and washed, prior to lysis by freeze-thawing. On occasions, as noted, the whole PMN's were washed twice in 0.15 M NaCl and lysed by disruption for 5 min in a sonic oscillator (Raytheon Co., Waltham, Massachusetts). In experiments where fractionation of these PMN lysates was carried out, the lysate of 5×10^9 whole PMN's was dialyzed against 0.15 M sodium phosphate buffer, pH 8.0 for 1 to 2 hr. The lysate was then fractionated with 12 g DEAE-cellulose in a column of 2.05×25 cm at 4°C. Starting buffer was 0.01 M phosphate buffer pH 8.0 and a salt gradient was established after a 50 ml starting buffer had

[1] The author wishes to thank Dr. V. Bazely for preparing the cultures of renal cells.

passed through using as a terminal 0.5 M NaCl in the starting phosphate buffer. The effluent was tested first for protein by the Folin procedure and then for protease activity by adding 0.2 ml of each effluent tube to 0.4 ml denatured bovine hemoglobin (10 mg/ml) (Nutritional Biochemicals Corporation, Cleveland) at a pH of 2.5. The hemoglobin was previously passed through a column of Bio-Gel P60 to remove small protein fragments. Digestion was carried out at 37°C for 1 hr after which an equal volume of 5% trichloracetic acid (TCA) was added to the digestion mixture. 0.5 ml of this supernatant was tested for protein by the Folin procedure, the amount of nonprecipitated protein reflecting the quantity of proteolytic activity in each effluent sample. Appropriate fractions from the DEAE-cellulose column were then concentrated sixfold by negative pressure dialysis. The concentrates were analyzed both for the physicochemical properties of their components and for their ability to react with purified glomerular basement membrane.

A further purification of the proteolytic enzymes obtained from rabbit PMN's was effected by passage of the DEAE-cellulose eluates through Sephadex G-100, and then Sephadex G-200. Approximately 5 mg N in 3 ml of the DEAE-cellulose eluate containing either protease activity was passed through a column of Sephadex G-100 measuring 2.5 × 25 cm. Protease activity appeared in the void volume while much protein was retained by the Sephadex G-100. After concentration and testing, the protein in the void volume was passed through Sephadex G-200 (2.5 × 25 cm bed volume) resulting in the elution of a single protein peak. This peak contained the protease activity. The protein peak appeared shortly after the passage of the void volume.

In certain experiments, PMN granules were extracted with 0.2 N H_2SO_4 and fractionated with ethanol at 20 and 45% concentration as described previously (14).

Influence of Enzyme Concentration on Proteolysis.—To test the effect of enzyme concentration on its protease activity, increasing amounts of each of the two PMN proteolytic enzymes obtained from the DEAE column were incubated with 4.6 mg denatured hemoglobin. The conditions and method of analysis, were the same as those noted above. A plot was then made of the amount of enzyme employed versus the Folin absorption readings of the TCA supernatant. From this an enzyme unit was obtained as the volume of enzyme required to increase the Folin absorption reading by 0.001/hr. This definition was employed for its convenience in these experiments and should not be equated with the unit as defined originally for cathepsin E by Lapresle and Webb (60).

Influence of Substrate Concentration on Enzyme Activity.—To test the influence of substrate concentration on the proteolytic activity of each enzyme, human serum albumin (HSA, obtained through the courtesy of Dr. J. Pert of the American Red Cross) was substituted for denatured hemoglobin owing to the high background values associated with large volumes of hemoglobin. Increasing amounts of HSA were added to a constant amount of enzyme as noted in the text, and conditions were otherwise the same as above.

Tests of Possible Inhibition of Enzyme Activity by p-Chloromercuribenzoate (PCBM) and Iodoacetate and Activation by Cysteine.—These tests were carried out as described by Lapresle and Webb (20) using the following concentrations: PCBM 2 mM, iodoacetate 1 mM, cystein 5 mM. 1 unit of the first enzyme in the DEAE eluate was employed and 5.5 units of the second.

Tests of Fractions of PMN Lysate Permeability of Vessels, Lysis of Rat Mast Cells, and Pyrogenicity.—Fractions obtained from chromatographic separation of PMN lysates were injected into the skin of 2.0 kg rabbits prepared with 1.0 ml 2.5% Evan's Blue intravenously. 0.1 ml was injected intradermally after the solutions were brought to isotonicity by addition of 1.5 M NaCl. Lysis of rat mast cells was carried out as in reference 13, and tests of pyrogenicity in endotoxin-tolerant rabbits as in reference 12.

The Effect of PMN Lysates and Their Subfractions on Purified Glomerular Basement Membrane.—25 mg lyophilized basement membrane were accurately weighed and placed in tissue

culture roller tubes. PMN lysate or fractions of the lysate were then added, using 0.5 ml whole lysate or 1.0 ml of the separated fractions. The pH of the reaction was adjusted to 2.5 with 1 N HCl, and volumes of various tubes were made equal with 0.15 M NaCl. 0.1 M sodium acetate or 0.1 M sodium phosphate were employed to obtain other pH values noted in the text. The digestion mixture was allowed to proceed 16 hr at 37°C on a mechanical shaker, and the tubes were then cooled to 0°C, centrifuged, and the supernatants analyzed immediately.

Physiochemical and Immunologic Analysis of the PMN Lysates and the Supernatants of the Digestion Reactions.—

Vertical starch gel electrophoresis: This technique was performed according to the procedures of Smithies (21). Electrophoresis was carried out at pH 8.6 using EDTA tris buffer or at pH 4.0 with 0.1 M sodium acetate buffer. A voltage of 12.5 v/cm was employed for 2 hr at 4°C. At the termination of the separation, the gels were cut and stained with amido black.

Paper electrophoresis was performed using 20 μl of digestion supernatant on a Beckman apparatus, Beckman Instruments, Inc., Fullerton, California, with veronal buffer of ionic strength 0.075, pH 8.6 for 4 hr at 2.5 ma per 8 paper strips at room temperature. At the termination of electrophoresis, the paper strips were dried 30 min at 115°C and stained for protein and peptides with ninhydrin spray.

Double diffusion precipitation in agar: In order to achieve high sensitivity templates were made of Plexiglas, containing wells and troughs for immunologic reactants. These were placed on top of microscope slides, being supported above the slide by fine strips of plastic tape. Agar was then infused between the template and the microscope slide and allowed to gel. Immunologic reactants were then added to the wells or troughs in the template and the slides were placed in humidity chambers at 4°C for 2 to 3 days. The sensitivity of the technique was such that at 0.1 μg antibody N (anti-BSA) could be detected.

RESULTS

Studies in Vivo of the Effect of Polymorphonuclear Leukocytes on Venule Walls during Immunologic Reactions

Cutaneous reversed passive Arthus (RPA) and antibasement membrane (ABM) reactions were induced in rabbits, rats, and mice. More than 5 animals in each group were employed. After 1 to 6 hr, purified carbon (150 mg/kg) was injected intravenously and the lesions were excised for histologic study. The results were identical in each of the species and will be discussed together. As shown in Fig. 1, in these inflammatory vascular reactions PMN's accumulated in the vessel walls and the carbon passed through into the surrounding interstitial spaces. There was little or no barrier to this passage. Red blood cells also were noted to pass through the normal confines of the vessel wall. On the other hand, during increased permeability in Arthus and ABM sites taken *before* the accumulation of PMN's (< 1 hr after injection), or when 2-γ histamine was injected intradermally rather than immune reactants, the circulating carbon became lodged in vessel walls (Fig. 2). By electron microscopy, in sections taken prior to infiltration of PMN's or where histamine was injected, the carbon became lodged up against the basement membrane as noted previously (22–24, 7). The basement membrane apparently served as a structural barrier to the passage of the carbon during a state of increased permeability. By contrast, sections of RPA reactions taken from the urinary bladder of rabbits and examined

electron microscopically, confirmed that the carbon passed through the vascular basement membrane of RPA reactions in the presence of PMN infiltration. Histologic sections of control sites in which 7S gamma globulin of *normal serum* had been injected did not show deposition of carbon.

Observations with fluorescent antibodies of the RPA sites taken 1.5 hr after injection revealed the presence of BSA antigen and heterologous gamma globulin (antibody) along with host C'3 (beta 1C globulin) in vessel walls. In the ABM sites, heterologous gamma globulin and host C'3 were observed along the basement membrane of vessels and also of nerves and hair follicles.

Evidence of a loss or marked derangement of the antigenic constitution of the basement membranes of affected vessels was revealed in fluorescent antibody studies. Sections of 6- and 24-hr RPA, ABM reactions, and normal control skin in rabbits were stained with fluorescent antibody to basement membrane. Disruption of the fluorescent pattern was apparent when PMN infiltration had occurred (Figs. 3 and 4). This finding was more noticeable in 24-hr-old reactions, after considerable vascular damage had taken place.

Further electron microscopic examination of RPA reactions revealed actual gaps in the basement membrane of vessels in which PMN's had accumulated (Figs. 5 to 7). This was noted in reactions of 4 to 6 hr duration in rabbits not given carbon. The appearance varied from irregularities in the density of the membrane to its frank obliteration in portions of the damaged vessel wall. In areas where rupture of the membrane occurred, fibrin was frequently but not invariably deposited peripherally in the interstitial space. These changes were noted only in vessels in which PMN's had infiltrated. The PMN's themselves were most frequently intact although occasional free granules were found in the interstitial spaces and between pericytes of the vessel wall. The endothelial cells were frequently elongated, conforming to the dilated vessel wall, and spaces were not infrequently found between these cells and the intact portions of the basement membrane. Enlarged cytoplasmic vacuoles were commonly seen in the endothelial cells and cytoplasmic processes extended into the lumens of affected vessels.

Effects of PMN Depletion on the Localization of Carbon in Vessel Walls during Immunologic Reactions.—Further tests of the role of PMN's in damage to the vascular basement membrane during immunologic injury were performed in rabbits, rats, guinea pigs, and mice depleted of circulating PMN's. RPA and ABM sites were placed on members of each species of animal, employing 100 and 20 μg N antiMBSA for the former reaction and sufficient antibasement membrane globulin to yield 4+ and 2+ cutaneous reactions at 5 hr. Carbon was injected intravenously as before. After 5 hr the intensity of each lesion was evaluated and biopsies obtained for histologic localization of carbon and for fluorescent antibody studies. The results appear in Table I. As noted, passage of carbon through the confines of the vessel wall occurred only when PMN's

were present. The relationship of the carbon to vessel walls in the presence or absence of PMN's was identical to that shown in Figs. 1 and 2. Greater amounts of carbon appeared in vessels of ABM than RPA reactions in PMN-depleted animals. This observation is compatible with previous unpublished findings indicating that greater vascular permeability exists in the former lesions. Fluorescent antibody studies confirmed the deposition of abundant BSA in vessels of the RPA reactions, of heterologous gamma globulin along basement membranes in the ABM sites, and of host C'3 localized in a position identical

TABLE I

*Effect of PMN Depletion on the Integrity of Blood Vessel Walls in RPA and ABM Reactions**

Animal species	No. tested	PMN	Carbon localization	PMN accumulation in vessel wall
		per mm³ blood		
Guinea pig	3	150–250	Lim. to ves. wall*	±
Guinea pig	5	2250–6300	Pass. through ves. wall.	3+
Rat	3	50–300	Lim. to ves. wall‡	0 to 1+
Rat	2	2550–3500	Pass. through ves. wall.	3+
Rabbit	4	0–250	Lim. to ves. wall§	0
Rabbit	4	3500–6350	Pass. through ves. wall	2+

* RPA, reversed passive Arthus reaction; ABM, cutaneous reaction to injected antibody to the recipient's vascular basement membrane. Localization of carbon the same in each except where noted. Lim. to ves. wall, carbon localization limited to vessel walls; Pass. through ves. wall, carbon found in vessel wall with spread into interstitial space.

‡ Where a few PMN's entered vessel walls, mild carbon passage into the interstitial space was noted at that point.

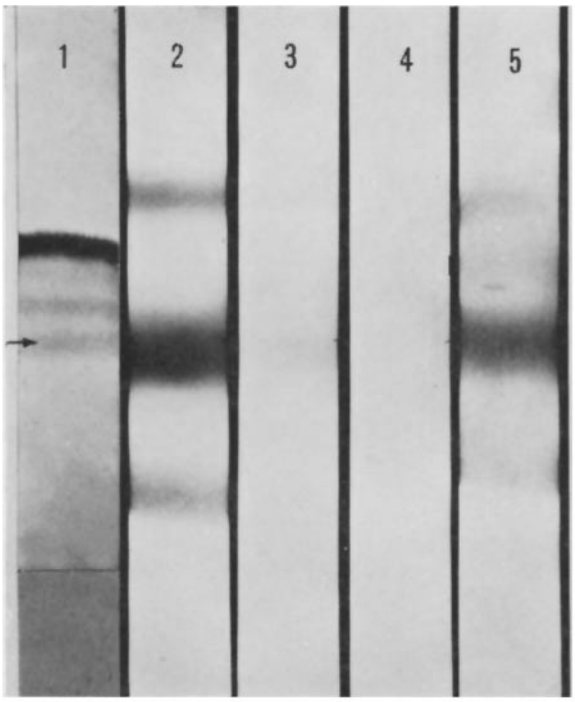
§ Few vessels in RPA reaction found with carbon in walls.

to that of the previous proteins in the vessel walls. This was true of PMN-depleted and normal animals. Depletion of PMN's was highly specific, the effect of the depleting methods on platelets, C' levels, and other blood elements being the same as reported previously (1, 2). As determined by electron microscopy and by studies using fluorescent antibody to the basement membrane, the vessels in ABM and RPA sites of two rabbits depleted of PMN's showed none of the disruptive effects noted above in the case of normal rabbits.

In Vitro Studies on the Effect of PMN Granule Lysates on Basement Membrane

In order to study a possible direct effect of PMN's on basement membrane, preparations of the two materials were combined in vitro. Glomerular basement membrane was incubated at 37°C for 18 hr with PMN granule lysates. After incubation, analysis of the supernatants by paper electrophoresis revealed four peptide and protein bands by ninhydrin staining, as shown in Text-fig. 1. One

of these formed a diffuse zone on the cathodal side of the origin, a second, heavy band remained at the origin, while two distinct bands migrated toward the anode. Trypsinization of the basement membrane produced three heavy bands as shown in Text-fig. 1. Control tubes containing PMN granule lysate or basement membrane alone, incubated under conditions of pH and temperature

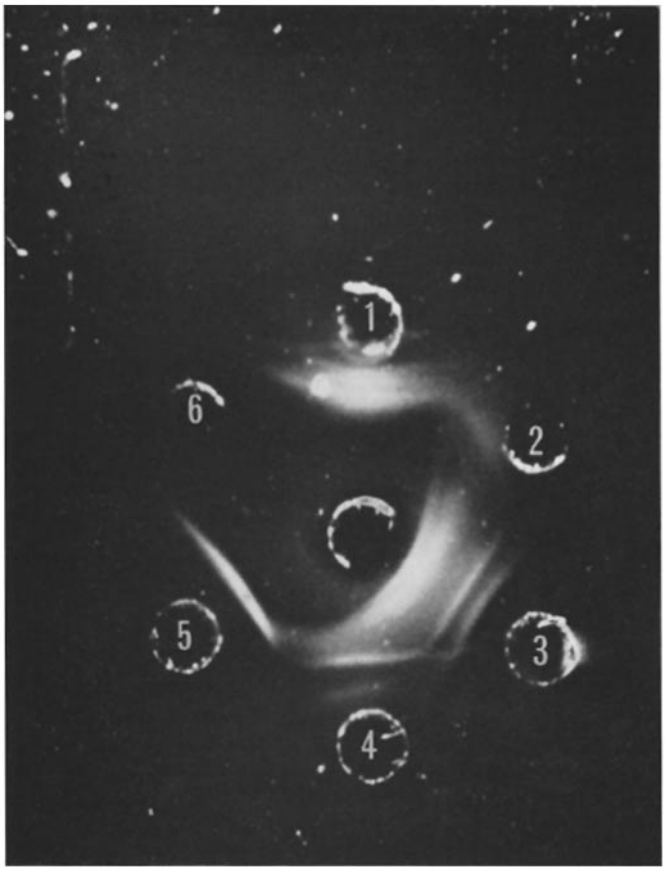


TEXT-FIG. 1. Paper electrophoresis of supernatants of reaction mixture containing basement membrane and PMN lysate. 1, migration of normal serum; 2, supernatant of trypsin-treated basement membrane; 3, supernatant of basement membrane plus distilled H_2O, at pH 2.5; 4, supernatant of PMN lysate incubated at pH 2.5; and 5, supernatant of PMN lysate incubated with basement membrane, pH 2.5.

identical to the experimental tubes, produced at most a trace protein stain at the origin on paper electrophoresis.

Samples of the supernatants of the digestion tubes were also tested by double diffusion precipitation in agar using specific anti-rabbit basement membrane serum. As shown in Text-fig. 2, several precipitin bands appeared when the antiserum reacted with either the supernatants of PMN granule lysate–basement membrane digestion or with trypsin–basement membrane digestion. Four precipitin bands could be distinguished in the first instance and six in the second. Supernatants of tubes containing basement membrane alone revealed one,

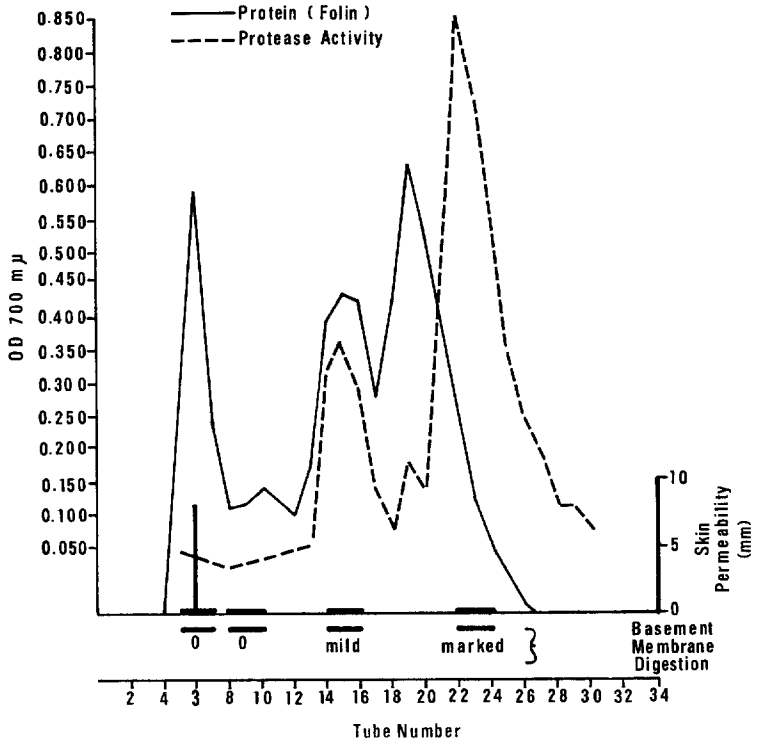
or at times, two weak bands. The stronger of these was noted to coalesce with four of the bands of the trypsin–basement membrane tubes and three from the PMN granule lysate–basement membrane tube.



TEXT-FIG. 2. Double diffusion precipitation analyses of supernatants of incubated basement membrane. Center well, sheep anti-rabbit glomerular basement membrane; well 1, supernatant of PMN lysate-incubated basement membrane; well 3, supernatant of trypsin-incubated basement membrane; well 2, well 4, and well 5, saline extract of sonicated basement membrane; and well 6, PMN lysate.

The PMN granule lysate was found to contain protease activity when incubated with denatured bovine hemoglobin as was previously described (19). The pH optimum of the total protease activity was 2.5 to 3.0 and no activity was found at pH 6.0, 7.0, 7.5, and 8.0 under the conditions of assay. When PMN granule lysate was placed on basement membrane at pH 2.5 and 7.0 and in-

cubated and tested as above, only the tube of pH 2.5 showed degradation of basement membrane, the tube containing granule lysate and basement membrane at pH 7.0 yielding nothing more than did the control tubes.

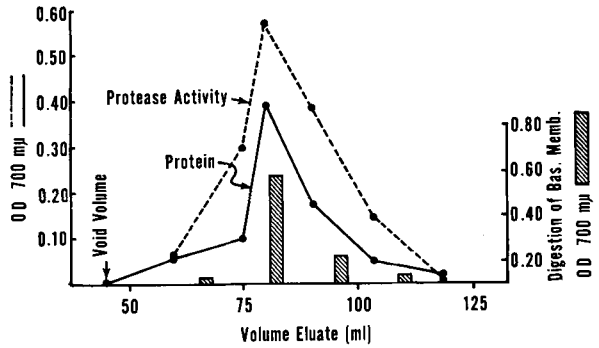


TEXT-FIG. 3. Chromatographic separation of PMN lysate on DEAE-cellulose. Protease activity measured by hydrolysis of hemoglobin at pH 3.0. Skin permeability tested by injection of 0.1 ml of eluate in isotonic medium intradermally in rabbits previously given Evan's blue intravenously. Digestion of basement membrane carried out at pH 2.5–3.0, 37°C, as noted in text.

Studies on the Identification of the Active Material in PMN's Responsible for Basement Membrane Alteration

Spearation of whole PMN lysate was then carried out on DEAE-cellulose, using the clear supernatant from 5×10^9 sonicated PMN's (97% PMN's, 3% mononuclear cells). Whole PMN lysates were employed at first since there is no indication that the granules exclusively act in tissue reactions and since in confirmation of the studies of Cohn and Hirsch (19), protease activity was found to exist in both granule and cytoplasmic fractions of PMN's. An example of such a separation is shown in Text-fig. 3. A large protein peak eluted from DEAE-

cellulose with the starting buffer, followed by two other protein peaks appearing as the concentration of NaCl passed from approximately 0.019 to 0.125M. Protease activity was found in various eluted fractions as is noted in Text-fig. 3, forming two distinct peaks. Fractions from tubes 5 to 7, 8 to 10, 14 to 16, and 22 to 24 were pooled and concentrated equally by negative pressure dialysis. These were then incubated with basement membrane at pH 2.5 and 37°C for 15 hr with shaking, and the supernatants were tested for evidence of basement membrane alteration. Paper electrophoresis revealed the presence of four bands, similar in all regards to those observed previously, only in the tube containing fractions 22 to 24 and basement membrane, although in the tube containing



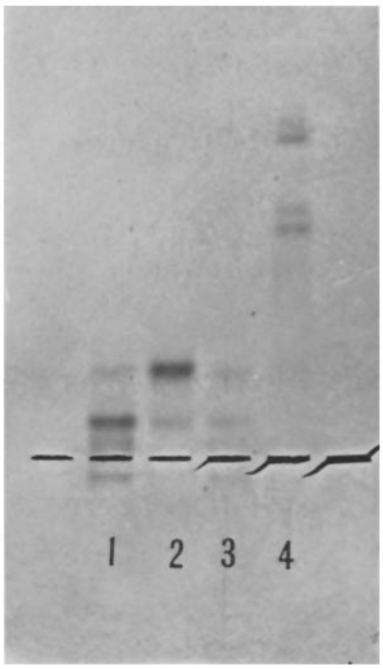
TEXT-FIG. 4. Tests of purified PMN protease after elution from Sephadex G-200. Background values of protease protein and protein released from basement membrane (Bas. Memb.) incubated without protease were previously subtracted from values given in vertical bar plots. Protease activity determined with bovine hemoglobin substrate pH 2.5.

fractions 14 to 16 and basement membrane, a smear of protein was noted adjacent to the origin extending 1 cm toward the cathode.

Further purification of the second, more active protease was obtained by chromatographic separation first on Sephadex G-100 and then Sephadex G-200. A peak of protease activity obtained in the void volume of the Sephadex G-100 separation induced release of peptides and antigenic fragments from isolated basement membrane in vitro much in the same way as the active fractions from DEAE-cellulose chromatography. The elution spectrum from Sephadex G-200 is presented in Text-fig. 4. Protein and protease activity eluted simultaneously from the column shortly after the void volume. Eluates incubated at pH 2.5 with isolated renal vascular basement membrane indicated a release of peptides had occurred only in fractions showing protease activity. This was evidence by Folin analysis (Text-fig. 4, vertical bars) as well as by paper strip electrophoresis and double diffusion precipitation in agar.

Tests for Cutaneous Reactions to PMN Fractions.—When tested for permeability activity in skin of rabbits (after dialysis against sterile 0.15 M NaCl),

fraction 5 to 7 obtained from DEAE-cellulose caused areas of blueing 8 to 10 mm in diameter within 15 min when tested whole and at a 1:2 dilution. Dilution to 1:16 yielded reactions measuring 4 mm in average diameter. When carbon was injected intravenously; it became localized in the vessel walls but failed to pass the confining basement membrane. No other fraction was active in inducing permeability, including fraction 22 to 24. The highly purified protease eluted from Sephadex G-200 was injected in large concentration into the skin of



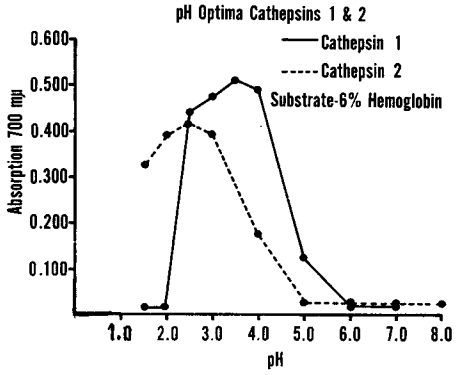
TEXT-FIG. 5. Vertical starch gel electrophoresis of eluates from DEAE chromatography shown in Text-fig. 3, in 0.1 M phosphate buffer, pH 8.0. Amino-Schwartz stain. Well 1, fractions 5 to 7; well 2, fractions 8 to 10; well 3, fractions 14 to 16; and well 4, fractions 22 to 24.

two rabbits that were previously given Evan's Blue intravenously. Neither blueing of the injection site nor detectable induration and hemorrhage resulted. Microscopically, sections of these sites taken 4 hr after injection revealed no apparent vascular abnormalities. This was not surprising in view of the acid pH requirement of the PMN proteases.

In similar chromatographic separations of PMN lysates, lipases were found to elute from the DEAE column at points roughly equivalent to tubes 8 to 10 and 26 to 28, while acid phosphatase eluted in tubes corresponding to fractions 8 to 10 and 20 to 24. Neither enzyme was present in the fractions rich in protease activity eluted from Sephadex G-200.

Starch gel electrophoresis of the PMN granule lysate fractions eluted from the DEAE-cellulose showed that the proteins all migrated toward the cathode at pH 4.0, with 5 distinct bands appearing in the run-off fraction of the DEAE column (fractions 5 to 7). In fractions 22 to 24, i.e. the fractions exhibiting protease activity and causing alteration of basement membrane, one principle spot and three minor components were noted on the cathodal side of the point of origin. When starch gel electrophoresis was carried out at pH 8.6, a change in migration of several protein components occurred as shown in Text-fig. 5, with only the run-off fractions 5 to 7 containing cationic proteins.

Properties of the Two Proteolytic Enzyme Fractions.—The pH optimum of the two proteases eluted from DEAE-cellulose was determined after adjusting the



TEXT-FIG. 6. pH optima of the two proteolytic enzymes obtained from DEAE chromatograph similar to that shown in Text-fig. 3. Cathepsin 1 and cathepsin 2 eluted in that order from the column. Cathepsin 1 is identical to cathepsin D, while cathepsin 2 is identical to cathepsin E, described by Lapresle and Webb (20).

digestion mixture to the desired pH with 1 N HCl. The results are shown in Text-fig. 6. Test of inhibition by *p*-chloromercuribenzoate (0.002 M) and iodoacetate (0.001 M) revealed that neither was inhibitory. Cysteine (5 mM) failed to activate the enzyme first eluted from the column (< 1% change in activity) but augmented the activity of the second 24%. Heating to 60°C for 60 min caused 98% inactivation of the enzyme eluting from the column (corresponding to fractions 14 to 16), while only 50% of the activity of the second enzyme (corresponding to fractions 22 to 24) was lost. Heating to 80°C for 10 min destroyed totally the activity of both enzymes.

Separation of PMN granule lysate by precipitation in ethyl alcohol was also carried out. 20 and 45% alcohol fractions were prepared and tested and both were found to release peptides from the isolated basement membrane at pH 2.5. Only the 20% fraction exhibited permeability properties when tested intradermally in rabbits. However, both 20 and 45% fractions contained protease activity at pH 2.5, a total of 16.6 and 24.6 units, being measured respectively. Similar

results were obtained on five different occasions. It should be added that two distinct precipitates formed during fractionation, the first appearing just prior to the point of 20% alcohol concentration and the second (after removal of the first) developing slowly when the 45% level had been reached.

Properties of the Basic Protein Released from PMN's.—The material eluted from the DEAE column in the void fraction, i.e. eluting without addition of NaCl, was found capable of increasing vascular permeability in rabbits, causing elevation of temperature in endotoxin-tolerant rabbits, and lysis of rat mesenteric mast cells. The results are shown in Table II. The DEAE eluate was also found to have strong anticomplementary activity. 0.1 ml containing 20 μg N

TABLE II

Activities of Fractions of PMN Lysates Excluded from DEAE-Cellulose

μg N employed	36	18	9	4.5	2.2	1.1
Permeability*	8.5	8.5	6.4	5.3	4.0	Trace
Mastocytolysis‡, %		67	55			
Pyrogenicity§	(108 μg N)	Average temperature elevation 0.76°C				

* Average diameter of cutaneous blueing 30 min following an intradermal injection of 0.1 ml into rabbits previously administered 1.0 ml 2.5% Evan's Blue/kg intravenously.

‡ Over 300 cells counted in each group. Figure represents per cent of rat mesenteric cells showing degranulation. <15% lysis of mast cells noted in controls.

§ Average of 3 endotoxin tolerant rabbits. Maximum elevation of temperature noted between 30 and 90 min.

completely inactivated 43.1 $C'H_{50}$ units (rabbit C') within 30 min at 37°C. The anticomplementary and permeability activity were not precipitated in 5% trichloracetic acid.

DISCUSSION

The Effect of Polymorphonuclear Leukocytes on Vascular Basement Membrane

These studies provide evidence that in immunologic tissue damage mediated by polymorphonuclear leukocytes (PMN's) one of the important and perhaps critical substrates of the reaction is the vascular basement membrane. It was found in Arthus reactions and in the vascular inflammation produced by the cutaneous injection of antibody to vascular basement membrane, that circulating carbon particles were able to pass through the normally retentive basement membrane of venules when PMN's entered the site (Figs. 1 and 2). Similar findings were noted in separate studies in nonimmunologic inflammation (25, 26). To a lesser degree, with time alone or when PMN's pass through the vessel wall, carbon (27, 28), may, like red cells (29), leak into the surrounding space. In the present studies, interruptions in the membrane occurred as noted by fluorescent microscopy and, in addition, frank disruption of the vascular base-

ment membrane was observed by electron microscopy (Figs. 5 to 7). In the absence of PMN's, that is in sites taken either before PMN entrance into the reaction site, or in sites placed on animals depleted of PMN's, disruption of the basement membrane was not observed.

In other studies reported from this laboratory, a second substrate of PMN injury in immunologic reactions was found to be the internal elastic lamina in the arteritis of serum sickness (1). In both this arteritis and the vasculitis of the Arthus phenomenon the PMN disruption of the barrier membrane appears to allow the spread of inflammation into the vessel wall and free passage of red blood cells beyond its confines. In permeability reactions induced by other agents such as vasoactive amines, bradykinin, etc., no significant diapedesis of red cells or formation of fibrin in the extravascular space occurs. It is not until aggregation of PMN's occurs with the attendant disruption of membrane structure that the severe sequellae of inflammation take place.

The studies carried out in which isolated fractions of PMN's and glomerular basement membrane were incubated together in vitro showed a fragmentation of the protein structure in the basement membrane. Four protein or peptide bands were observable in the digestion supernatants in paper electrophoresis (Text-fig. 1). In addition, in double diffusion in agar, using heterologous antiserum to rabbit glomerular basement membrane, at least four distinct precipitin bands were observed in the digestion supernatant (Text-fig. 2). When a saline extract of basement membrane was tested, it was noted that a single precipitin band developed that coalesced with 3 of 4 bands of PMN-digested basement membrane, and at least 4 of 6 bands of trypsinized basement membrane. This indicates separation of the basement membrane antigens had occurred during incubation with PMN extracts. This presents a possible explanation for the destruction of basement membrane as observed electron microscopically in Arthus reactions and vascular lesions produced by local injection of antibasement membrane antibodies.

Identity of the Substances in PMN's Responsible for Basement Membrane Alteration

The evidence obtained from studies in vitro strongly suggests that the major substances in PMN's responsible for the basement membrane alteration are cathepsins. Their characteristics closely resemble those of cathepsin D and E identified in rabbit PMN's by Lapresle and Webb (20): they eluted from DEAE-cellulose similarly to cathepsins D and E, and they were active in digesting basement membrane at pH 3.4 and 2.5, the pH optima of these two proteases respectively. Both were inactive on hemoglobin substrate at pH 7.0, 7.5, and 8.0 as was the whole PMN lysate. Their inactivation by heat and lack of dependence on sulfhydryl groups were in keeping with this conclusion. These findings clearly separate these enzymes from the enzyme described by Hayashi

et al. (30) which was extracted from 12- to 24-hr-old Arthus reactions. The latter enzyme, being unrelated to any found in PMN's, probably does not play a significant role in the development of the Arthus reaction. This subject was recently reviewed (31). In addition to the possible hydrolysis of basement membrane proteins in vivo, cathepsins D and E probably account for the proteolytic digestion of phagocytized immune precipitates that occurs within PMN's removed from Arthus sites (16) and for the catabolism of immune precipitates in vitro (17). This mechanism is apparently fundamental in ridding the site of an immunologic reaction of the offending antigen (32).

Direct measurements of pH in the affected vessels, i.e. observations that would indicate whether the pH of the reaction favored activity of these proteases, have not been reported. There are, however, indications that considerable lactic acid would be produced by PMN's after they become concentrated, phagocytize immune complexes, and metabolize carbohydrates by anaerobic glycolysis (33–36). This is in keeping with the often observed rapid fall of pH in the medium of a PMN suspension maintained at 37°C in vitro.

Other PMN cytoplasmic enzymes including lipase, acid and alkaline phosphatase, and lysozyme would not likely be responsible for the alteration in the structure of vascular basement membrane. Lipase and acid phosphatase were found in eluted fractions of the DEAE column that lacked activity toward hemoglobin or basement membrane. In addition, lipase, lysozymes, and alkaline phosphatase have pH optima far above the range in which PMN lysates cause disruption of the basement membrane.

The *cationic proteins* that caused an increase in vascular permeability were found in eluates of the DEAE chromatograph separate from the proteases that elicited hydrolysis of basement membrane (Text-fig. 3). In addition, the cationic proteins, while eliciting immediate permeability upon injection, did not cause basement membrane damage in vivo severe enough to allow diapedesis of circulating carbon. In Arthus reactions, passage of carbon is invariably found. The cationic proteins eluted from DEAE contained activities similar to those obtained by different extraction methods, i.e. permeability, and mastocytolytic and pyrogenic activities (12–14). Similar basic proteins have been studied by Zeya and Spitznagel (37, 38). These workers found a bactericidal component that could be eluted from paper after electrophoresis. The material migrated far in the cationic region. This migrational characteristic may be explained by the high content of arginine as shown by amino acid analysis of the crude extract. Amino acid analysis of a purer similar preparation that induced lysis of rat mast cells by Janoff et al. (39) has revealed a considerable content of arginine and relatively little lysine. In addition, the cationic proteins, in the present studies, were found to inactivate complement. Work done in collaboration with Dr. N. Ranadive has indicated the molecules responsible for permeability

changes and mast cell lysis have a molecular weight of between 3500 and 5000. This contrasts with a value of 1500 to 2500 mol wt of the mast cell lytic material studied by Janoff et al. (39). Activity is lost upon trypsinization. After trichloracetic acid precipitation (5%), permeability, pyrogenic, mastocytolytic, and anticomplementary activity remains in the supernatant. Further studies of these PMN factors are in progress.

The Arthus reaction serves as a prototype of several immunologic diseases as noted in the introduction. Evidence suggests the following sequence of events in its pathogenesis as understood to date: antigen and antibody interact and precipitate in and around the walls of small vessels. Complement is fixed, leading to the activation of the C'5-6-7 complex. This is released from the immune reactants and C'1, 4, 2, 3, and in great part brings about the accumulation of polymorphs. The polymorphs in the process of phagocytosis of immune complexes release the proteolytic enzymes, cathepsins D and E, and certain cationic proteins. The cathepsins attack the vascular basement membrane and the integrity of the vessel wall is lost. Hemorrhage and severe edema result. Fibrin formation and thrombosis produce a superimposed aggravation of these changes which may lead to necrosis of the center of the over all reaction site. The released cationic proteins also induce an increase in vascular permeability probably via release of histamine. Another permeability factor, released by antigen-antibody interaction in Arthus reactions and acute nephrotoxic nephritis, has been noted that acts independently of PMN's (2, 40).

SUMMARY

Vascular basement membrane was disrupted in the presence of polymorphonuclear leukocytes (PMN's) during two immunologic reactions: The Arthus phenomenon and the reaction to locally injected antibody to vascular basement membrane. This disruption was evidenced by (*a*) the inability of the basement membrane to retain circulating carbon, by (*b*) loss of antigenic constituents, and by (*c*) electron microscopic observation showing actual gaps in the structure of the vascular basement membrane.

The factors within PMN's responsible for damage to isolated glomerular basement membrane in vitro were found by isolation procedures to be cathepsins D and E. Cationic proteins of PMN's were separable from the cathepsins. While inducing vascular permeability upon injection, these basic proteins failed to inflict the severe damage to the basement membrane observed in Arthus and antibasement membrane reactions. It is concluded that the full expression of these immunologic lesions requires destruction of the basement membrane possibly brought about by cathepsins D and E.

Some of the physicochemical properties of these pathologically active leukocytic factors are given.

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EXPLANATION OF PLATES

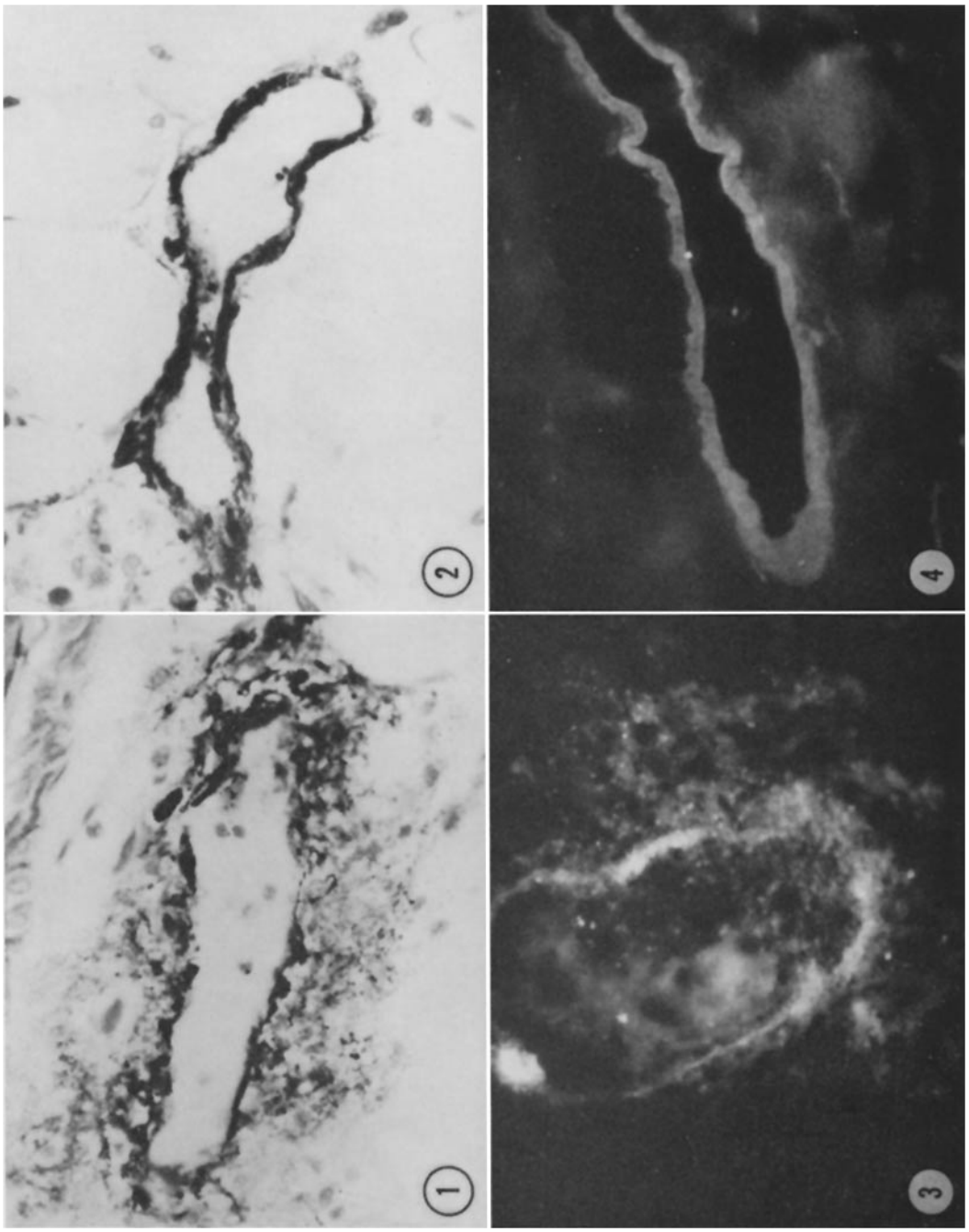
PLATE 82

FIG. 1. Microscopic section of a cutaneous vessel in a 4 hr Arthus reaction in a rabbit. Carbon was injected intravenously 20 min prior to biopsy, and is noted to lie across the wall and confines of the basement membrane. PMN's poorly stained with toluidine blue. × 500.

FIG. 2. Microscopic section of a vessel from the same rabbit shown in Fig. 1. Section taken from a 1 hr Arthus reaction prior to the infiltration of PMN's. Carbon fails to pass the basement membrane although permeability of the vessel exists. A small number of PMN's had emigrated at the left side of the vessel causing some passage of carbon outside the vessel wall. Toluidine blue. × 500.

FIG. 3. Photomicrograph of a tissue section from an 8 hr Arthus reaction, stained with fluorescent anti-rabbit vascular basement membrane. Note disruption of the basement membrane in the lower part of the vessel. Fluorescence within the lumen represents nonspecific staining. Fluorescence photomicrograph. × 350.

FIG. 4. Photomicrograph of a vessel taken from a 1 hr Arthus reaction in a rabbit. Section treated with fluorescent anti-rabbit vascular basement membrane. No disruption of the basement membrane has occurred prior to PMN infiltration. × 400.



(Cochrane and Aikin: Polymorphonuclear leukocytes in immunologic reactions)

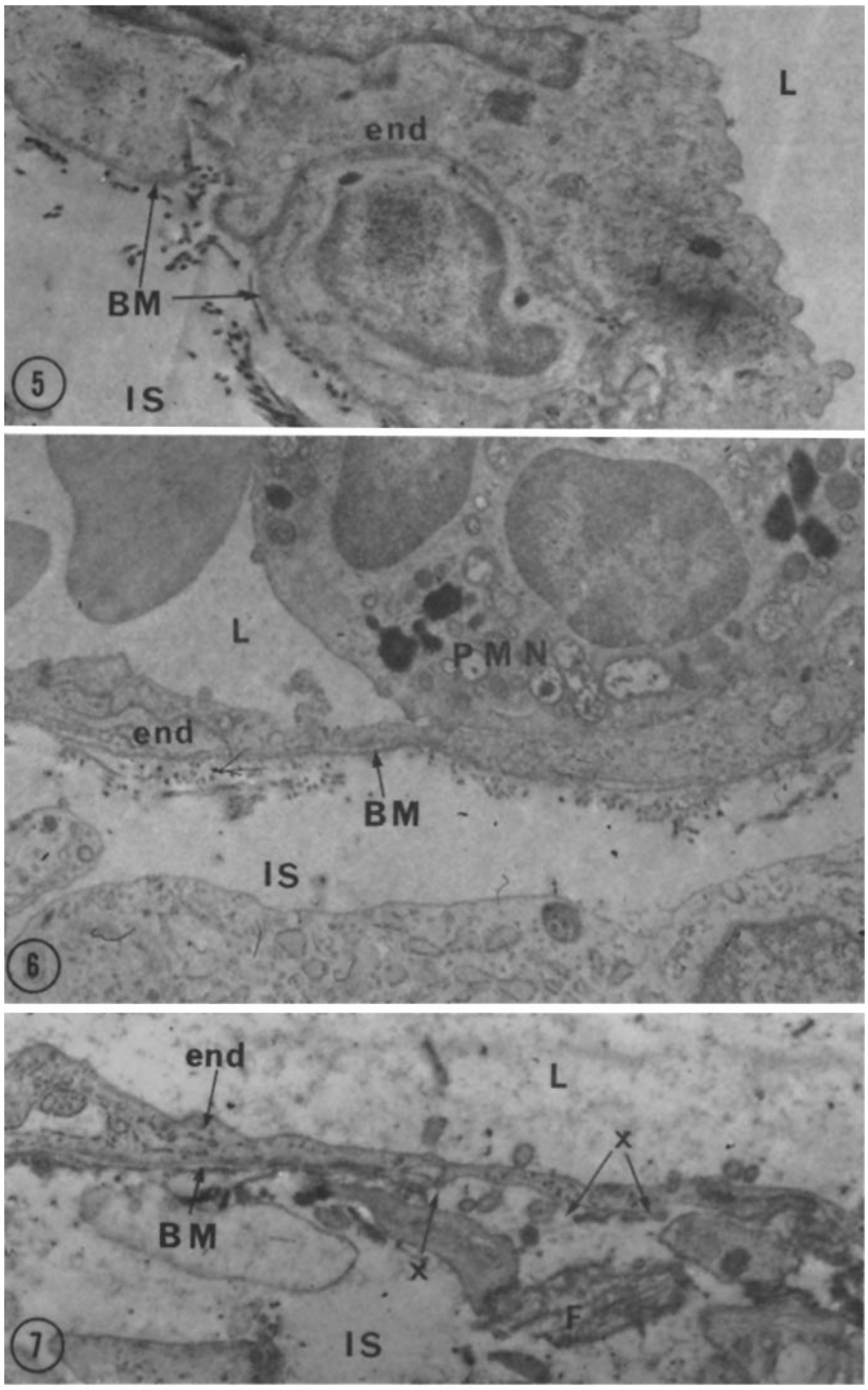
PLATE 83

BM, basement membrane
end, endothelial cell
F, fibrin
L, lumen
IS, interstitial space
PMN, polymorphonuclear leukocyte

FIG. 5. Electron photomicrograph of a normal venule from rabbit urinary bladder. A normal intact basement membrane may be observed along the external surface of the endothelial cell. × 11,400.

FIG. 6. Electron photomicrograph of a venule taken from a 2 hr Arthus reaction in a rabbit. PMN accumulation has begun in this affected vessel, but no damage to the basement membrane is observable. × 16,500.

FIG. 7. Electron photomicrograph of a 6 hr Arthus reaction in rabbit urinary bladder. Marked disruption of the basement membrane has occurred (*x*). While PMN's are not apparent in the photograph, several were seen in the interstitial spaces immediately adjacent. Fibrin deposition may be seen and the endothelial cell shows multiple evaginations. × 11,400.



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