BOUND COMPLEMENT AND IMMUNOLOGIC INJURY OF BLOOD VESSELS*,‡

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Plates 15 and 16

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The interaction of antigen and antibody in walls of blood vessels resulting in an intense inflammatory reaction is the central feature of the Arthus reaction. The antibody involved must be precipitating in quality (1), since physical precipitation in the walls of blood vessels is apparently essential to bring about the subsequent events in the inflammatory cycle (2). Associated with the interaction of antigen and precipitating antibody in tissues, complement (C′) is fixed (3), polymorphonuclear leukocytes (PMN's) are attracted, and tissue damage ensues. Recently, it has been suggested that the contents of cytoplasmic granules in PMN's may act as mediators of this damage (4, 5). In the absence of PMN's, the damaging effects of antigen-antibody interaction in tissue are abrogated (6–9).

The role of C′ in the pathogenesis of the Arthus reaction has been questioned. Bier and Siqueira inhibited Arthus reactions in rats by treatment with C′-depleting agents but found a poor correlation between levels of serum C′ and the degree of inhibition (10). Of interest, however, is the recent demonstration that attraction of PMN's to immune reactants *in vitro* requires the presence of a heat-labile substance, possibly C′ (11).

The present experiments were designed to determine if C′ plays a role in the development of immunologic vasculitis. Arthus activity was studied in normal rats and guinea pigs and in animals depleted of C′ by various agents. Also, antibodies which varied in ability to fix C′ *in vitro* were studied for their vasculitis-inducing capacities in normal animals, and the degree of reaction was compared with the presence or absence of C′ binding in tissues.

Materials and Methods

Antigens.—Crystallized bovine plasma albumin (BSA, Armour Pharmaceutical Co., Kankakee, Illinois), and five times crystallized hen egg albumin (EA, Pentex, Inc., Kankakee,

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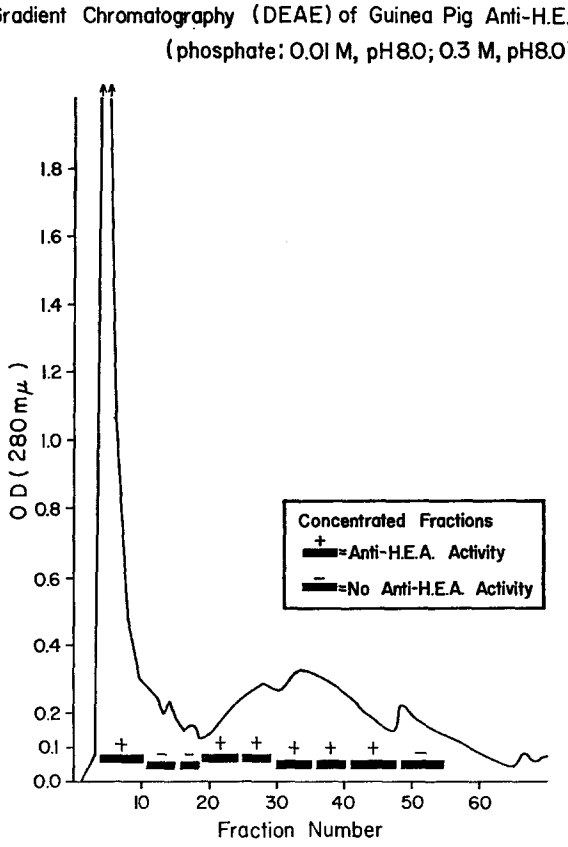
Illinois), were used as antigens in reversed passive Arthus reactions. Rabbit γ-globulin (RGG), fraction II, was obtained from Pentex, Inc.

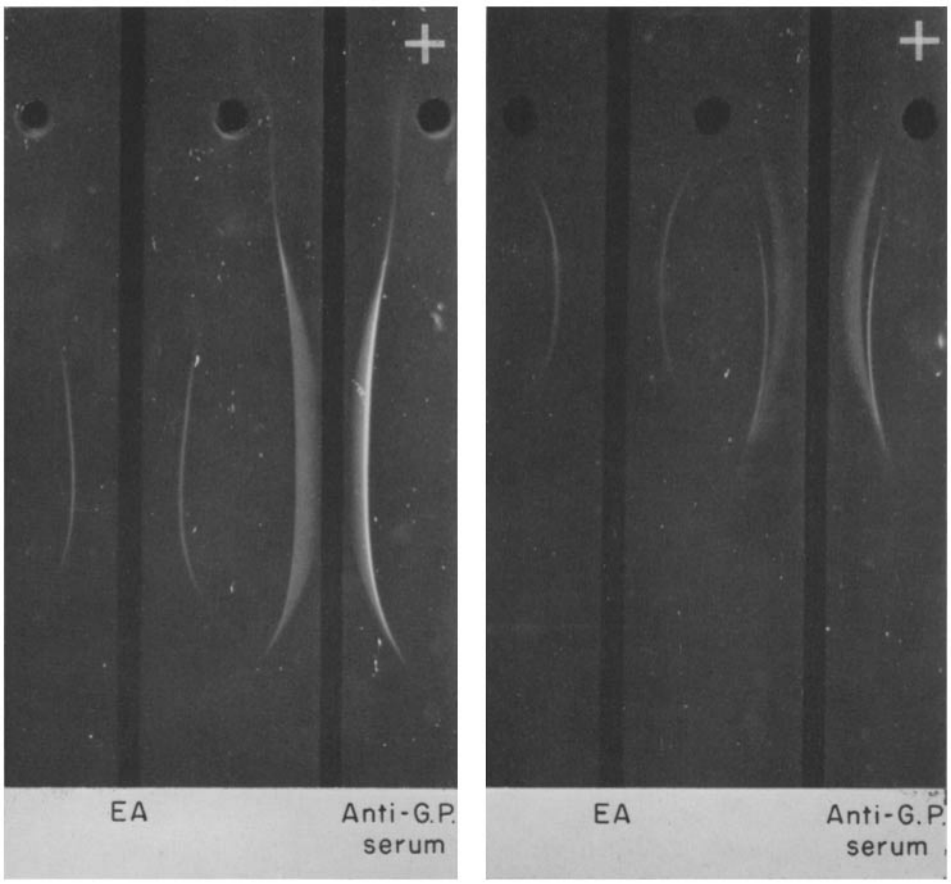
Antibodies.—Rabbit anti-BSA (precipitating antibody to BSA) was obtained by immunizing rabbits with BSA in 0.15 M NaCl in repeated courses. The rabbit serum was then



TEXT-FIG. 1.—Chromatogram of DEAE fractions of guinea pig anti-hen egg albumin (40 per cent ammonium sulfate fraction of pooled serum), obtained by a constantly increasing salt gradient, reveals two major areas of antibody activity, determined by quantitative precipitin assay on each fraction. Each horizontal block refers to pooled, concentrated fractions (10 ml/fraction). Plus and minus signs refer to presence or absence of antibody activity. Two general areas of antibody activity are present. See text for details of isolation.

fractionated with 40 per cent ammonium sulfate and the γ-globulin isolated by elution from diethylaminoethyl cellulose (DEAE) with 0.01 M phosphate buffer, pH 8.0.

Pepsin degraded anti-BSA was prepared according to the method of Nisonoff *et al.* (12), using DEAE-fractionated rabbit anti-BSA. Ultracentrifugation of the digested anti-BSA revealed a single peak lying between known 7S and 4S peaks in agreement with previously reported results (12).



TEXT-FIG. 2.—Immunoelectrophoresis (250 volts, 100 minutes) of "slow" and "fast γ" anti-EA fractions (obtained by elution of guinea pig anti-EA from DEAE cellulose) shows characteristic differences in mobilities. Each well received the respective antibody fraction, while the left trough in each case (EA) contains antigen (egg ablumin, 11 γ N/ml). Each right-sided trough contains rabbit anti-whole guinea pig serum. The "fast γ" fraction shows two protein bands which were also inseparable by electrophoresis in pevikon.

Duck anti-BSA[1] was prepared as recently reported (13). Antibody activity in each preparation was measured by the technique of quantitative precipitation (14).

Precipitating antibody to EA (*anti-EA*), was obtained by immunizing 300 gm male guinea pigs (Hartley strain) with EA in complete Freund's adjuvant, as recently described (15). Pools of serum, each from approximately five guinea pigs, were fractionated with 40 per cent ammonium sulfate. The resulting globulins were further fractionated either by block electrophoresis in pevikon (16) or by elution from DEAE cellulose employing a salt gradient as

[1] The authors wish to thank Dr. F. J. Dixon for this preparation.

recently described (17). Two types of antibody, differentiated by electrophoretic characteristics as "fast γ" and "slow γ" anti-EA (15, 17), were obtained by each method of isolation. The chromatographic separation is illustrated in Text-fig. 1. Horizontal bars represent pools of fractions (10 ml each) which were concentrated by negative pressure dialysis. The first pool, from tubes 2 to 10, had precipitating antibody activity and migrated as a "slow γ" protein (Text-fig. 2). After a zone in which no antibody activity was demonstrable (the next two pools) a broad zone of antibody activity was found. Concentrated pools from tubes 19 to 24 and 25 to 29 were each analyzed and found to contain antibody activity that migrated as "fast γ" anti-EA (Text-fig. 2). The *in vitro* C′ fixing properties of these two "fast γ" pools as well as their Arthus-inducing activities were similar and the preparations could be used interchangeably. To minimize the possibility of contamination of "fast γ" anti-EA with "slow γ" antibody, the "fast γ" preparation (obtained from DEAE) was submitted to electrophoresis in Pevikon and the rapidly moving material isolated.

Rabbit antibody to guinea pig β1C-globulin, anti-β1C-globulin (anti-C′3c), which has recently been reported to be closely related or identical with the 3c component of guinea pig C′ (18), was prepared as described elsewhere (18). When the anti-β1C-globulin preparation also contained antibody to γ-globulin, this was removed by absorption with guinea pig γ-globulin isolated from whole serum by chromatography in DEAE cellulose.

Rabbit antibody to rat β1C-globulin was obtained in a similar manner, utilizing fresh rat serum and zymosan. The antibody reacted with a single band in the β-globulin region, designated β1C-globulin. When present, antibody to rat γ-globulin was removed by addition of purified rat-γ-globulin (obtained by preparative electrophoresis in pevikon). Rat β1C-globulin had electrophoretic characteristics in agar similar to those of guinea pig C′3c. When fresh rat serum was aged several days at 0°C, or treated with 15 mg zymosan per ml serum (20°C, 2 hours), or immune complexes (100 μg N rabbit anti-BSA with antigen added at equivalence), the β1C-band converted to a slower migrating band, termed β1A-globulin. When neutralized 0.01 M ethylenedinitrilotetraacetic acid, disodium salt (EDTA) was added to fresh rat serum prior to incubation, no changes in electrophoretic behavior occurred after similar treatment. After conjugation with fluorescein isothiocyanate, this anti-rat β1C-globulin imparted bright fluorescence to dermal vessels taken 1 to 2 hours after induction of Arthus reactions in normal rats. This fluorescence in Arthus sites was lost if the antibody was previously absorbed with washed zymosan or immune complexes which had first been incubated in fresh rat serum. Absorption of the fluorescence was not possible if the serum, to be incubated with zymosan or immune complexes, had been previously heated (56°C, 30 minutes) or treated with 0.01 M EDTA.

Antibody to rat PMN's was obtained by immunizing rabbits with rat PMN's (approximately 20×10^6 PMN's/rabbit) in incomplete Freund's adjuvant. The rabbits were bled 3 weeks later. The PMN preparation was obtained by instilling 0.1 per cent glycogen intraperitoneally into rats, followed by peritoneal lavage with sterile saline 4 hours later. The rabbit antiserum was absorbed with rat erythrocytes and lymphocytes (from lymph nodes) before use. The dose and schedule of treatment of rats with antiserum was: 1 ml intraperitoneally 24 hours before and again at 2 hours before Arthus testing. The same results were obtained if a single dose (1.5 ml) of antiserum was given intraperitoneally 18 hours before Arthus testing.

In Vitro Studies of Leukocytes, Motility and Phagocytosis.—PMN's for studies *in vitro* were obtained by collecting 4 ml peripheral blood from individual rats that had previously been injected with heparin intravenously (400 units/250 gm rat). The blood was centrifuged and the resulting buffy coat mixed with fresh autologous rat plasma and medium 199 (Microbiological Associates, Washington, D. C.) in a 1:1 volume. Two or three drops of cell suspension were placed on cover slips and left at 37°C in a humidified chamber for 60 minutes. After gentle

washing, PMN's and monocytes, adherent to the coverslip, could be observed for motility by phase microscopy at 37°C.

In the studies of phagocytosis, buffy coat cells were suspended in autologous or homologous plasma and medium 199 (1:1 volume) in siliconized tubes. Three-tenths ml of the leukocyte suspension (approximately 4×10^6 cells) was added to each tube. One ml of the mixture of fresh rat serum and medium 199 was then added, followed by 0.1 ml boiled zymosan suspension (10, 2.5, and 0.6 mg/ml) in medium 199. Following incubation at 37°C for 1 hour, smears were made from each tube and treated with Wright's stain. The per cent of PMN's containing zymosan particles and the average number of particles per cell were determined.

Chemotaxis of PMN's in C′-Depleted Rats with Lysates of PMN Granules.—Because of the possibility that C′-depleting agents may alter PMN's, lysates from cytoplasmic granules of normal rat PMN's, which have recently been shown to exert a chemotactic effect on PMN's (19), were tested in normal rats as well as rats depleted of C′ by heat-aggregated human γ-globulin (agg HGG) in doses described below. For the preparation of the PMN extract, PMN's were obtained from peritoneal exudates of rats after the instillation of 0.1 per cent glycogen (as noted above). The cells were disrupted by hypotonic sucrose and the granules isolated by centrifugation (20). The granules were then disrupted by freeze-thawing (19, 20), and the supernatant was dialyzed overnight against medium 199. For the skin tests, 0.3 ml (approximately 1.2 μg N) of the PMN-granule extract was injected intradermally into rats. Three hours later, the sites were graded for edema and biopsies obtained for histologic assessment of PMN infiltrates (grading as described below).

Arthus Reactions.—Two hundred and fifty gm male Sprague-Dawley outbred rats and 300 to 400 gm random bred male albino guinea pigs (Hartley strain) were used. Reversed passive Arthus reactions were produced by the injection of 50 μg N anti-BSA or anti-EA intradermally followed by 1.0 mg N of the appropriate antigen intravenously. When pepsin-degraded anti-BSA was used, the antibody activity following digestion was measured by quantitative precipitin technique so that 50 μg N precipitating antibody was employed in biological testing. Within 2 to 4 hours after injection, the reactions were characterized by intense edema and erythema and/or hemorrhage. Arthus sites were graded 0 to 4+ accordingly: 1+, PMN's limited to walls of dermal vessels; 2+, PMN infiltrates that also involved perivascular connective tissue; 3+, extensive perivascular and interstitial accumulations of PMN's; 4+, diffuse dermal, subcutaneous, and subepidermal cellular infiltration. Sections of skin from normal control rats, as well as rats depleted of C′ by zymosan and agg HGG, were stained with toluidine blue in order to determine if mast cells were degranulated by procedures of C′ depletion.

Fluorescent Antibody Studies.—With minor modifications, the direct fluorescent antibody technique of Coons and Kaplan (21) was employed. Antibody fractionated with 40 per cent ammonium sulfate was conjugated with fluorescein isothiocyanate. Tissue sections from frozen skin sites were examined for the presence of BSA, RGG, and rat or guinea pig β1C-globulin. Grading of fluorescence was similar to that employed for assessment of cellular infiltrates. With serial sections from frozen tissues, it was possible to compare the localization of BSA and rat or guinea pig β1C-globulin in the same vascular wall. In normal control sites, C′ was fixed in a distribution identical with that of BSA and RGG.

C′-Depleting Procedures.—(*a*) Heat-aggregated (63°C, 20 minutes) human γ-globulin[2] was fractionated with sodium sulfate, according to the method of Christian (22). Three mg N intraperitoneally plus 3 mg N intravenously were injected 30 minutes before preparation of

[2] Human γ-globulin, Cohn fraction II of human plasma, was obtained through the courtesy of Dr. J. H. Pert of the American Red Cross.

Arthus sites (time 0), followed by 400 μg N intravenously and intraperitoneally at time 0, and 400 μg N intravenously 1 and 2 hours thereafter (+1 and +2 hours). (*b*) Boiled zymosan suspended in buffered saline (10 to 15 mg/ml) was injected as follows: 15 mg intraperitoneally + 10 mg intravenously 30 minutes before preparation of Arthus sites, followed by 10 mg intravenously and intraperitoneally at time 0 and +1 hour. (*c*) Rabbit anti-β1C-globulin (rat or guinea pig), was injected accordingly: 1 ml intravenously + 1 ml intraperitoneally 60 minutes before preparation of Arthus sites, followed by 1 ml intravenously at 0 and +1 hour. (*d*) Carrageenan (kindly supplied by the Marine Colloids, Inc., Springfield, New Jersey) was suspended in phosphate-buffered saline and injected in a dose of 20 mg intraperitoneally + 15 mg intravenously 30 minutes before Arthus testing, followed by 10 mg intravenously + 10 mg intraperitoneally at times 0, +1 and +2 hours. Treatment by any of the C′-depleting agents caused animals to become lethargic, but acute anaphylactic symptoms and deaths were rare with the doses described.

Complement Assays.—

C′H$_{50}$: C′ levels in serum or plasma, expressed as C′H$_{50}$ units, were determined according to the method of Osler *et al.* (23). Samples were collected in 0.01 M ethylenedinitrilotetraacetic acid (EDTA) and then reconstituted with Ca^{++} and Mg^{++} to 0.00015 M and 0.0005 M respectively in the cold, immediately prior to performing the C′H$_{50}$ assay.

C′1,4 assay: As a measure of the relative amounts of the first and fourth components of guinea pig C′(C′1 and 4), the T_{max} values for sensitized sheep cells, alexinated with the C′1 and 4 of the various sera to be tested, were determined as outlined by Mayer (24). The following modifications were employed: (*a*) sheep cells were sensitized with rabbit amboceptor (1:100 dilution) in the presence of isotonic veronal buffer containing 0.1 per cent gelatin and 0.01 M EDTA (EDTA-GVB) in order to obviate the uptake of small amounts of C′ components other than 11S protein of the first component of rabbit C′ (25); (*b*) incubation of the sensitized cells with serum was carried out for 7.50 minutes at 0°C, employing 0.10 ml serum (measured with lambda pipettes) per 4.0 ml sensitized cells (2×10^9/ml); and (*c*) the buffer used for uptake of C′1 and 4 onto sensitized erythrocytes was isotonic veronal buffer with 0.00015 M Ca^{++} in 0.1 per cent gelatin (CaGVB). The T_{max} values of sera from individual guinea pigs prior to treatment with C′-depleting agents were between 6 and 12 minutes, indicating an abundance of sites (26) containing C′1 and 4 on sensitized erythrocytes (EAC′1a,4).

In preliminary experiments, it was found that minor variations in time of incubation as well as volume of serum employed in the formation of EAC′1a,4 could not account for the differences in T_{max} values in the experiments to be reported.

C′2 assay: The activity of the second component of guinea pig serum (C′2) was determined by a procedure similar to that reported by Austen and Beer for human C′2 (27). Differences in technique included: (*a*) Guinea pig serum or plasma to be tested was diluted (2 fold) from 2,000 to 64,000 times. (*b*) 0.5 ml diluted serum was added to 0.5 ml EAC′1a,4 (1.5×10^8 cells/ml). (*c*) The activity of C′2 was expressed by that dilution of serum which altered EAC′1a,4 cells such that 50 per cent lysis eventuated. This was called the *C′2$_{50}$ value*; it ranged between 15,000 and 20,000 for normal guinea pig serum. In dilution >4,000 the T_{max} value of EAC′1a,4 was not altered, suggesting that there was no significant addition of C′1 and 4 to these cells during the assay. Lambda pipettes were employed for all procedures requiring dilutions.

C′3 assay: The modification of the immune adherence technique (28) to measure the third component of guinea pig C′ was employed (29).

Complement Fixation in Vitro.—Each antiserum was tested in amounts of 5, 10, and 20 μg N of antibody (Ab N) with an equivalent amount of antigen (determined by quantitative precipitation). Control tubes included: C′ alone (guinea pig serum), C′ plus 20 μg N antibody,

and C′ plus antigen at the highest dose employed. The control tube giving the lowest $C'H_{50}$ value was used as the reference blank, designated the control C′ value. The test system involved 1.0 ml of fresh guinea pig serum in a total volume of 1.30 ml. Reactants were incubated at 37°C for 1 hour, followed by 5°C for 18 hours. Residual C′ levels were determined according to the $C'H_{50}$ assay described above and expressed as per cent of control values.

RESULTS

Development of Immunologic Vasculitis before and after C′ Depletion.—

Arthus reactions in normal controls: In normal rats and guinea pigs 2 to 3 hours after the intradermal injection of anti-BSA and intravenous injection of

TABLE I

Effect of C′ Depletion in Rats and Guinea Pigs on PMN Attraction and Development of Arthus Reactions after 2 to 3 Hours

C′-depleting agent*	Skin sites					
	Macroscopic‡	Microscopic§ PMN infiltrate	Fluorescent studies§		$C'H_{50}$ units/ml plasma	
			BSA	Rat or guinea pig C′	Rats	Guinea pigs
None	4+	4+	4+	3+	38–60	177–250
Aggregated HGG	0	0 to ±‖	3+	±	<8	<15
Non-aggregated HGG	4+	4+	4+	3+	45–60	—¶
Zymosan	0	±	4+	±	16–27	100–139
Anti-β1C-globulin	±	± to 1+	4+	±	18–25	11–105
Carrageenan	0	±	3+	0	<10	<10

* See Materials and Methods for doses and method of injection. At least 5 rats and guinea pigs were used per group.

‡ Edema and erythema, see text for grading.

§ See Materials and Methods for explanation of grading.

‖ ±, trace.

¶ Not done.

antigen, skin sites were edematous, indurated, and erythematous (macroscopically graded 4+, Table I). Microscopically intense perivascular and interstitial infiltration of PMN's was found, although vascular thrombi were rarely seen (Fig. 1). Fluorescent studies revealed the presence of bright deposits of BSA, as well as RGG, in walls of vessels and in interstitial areas (Fig. 3). Rat β1C-globulin and guinea pig β1C-globulin (C′3c) were found in a distribution identical with that of antibody and antigen (Fig. 4) as shown by serial frozen sections in which the same vessels could be assessed for all three reactants. Plasma levels of C′ in rats ranged from 38 to 60 $C'H_{50}$ units/ml while the values for guinea pigs varied from 177 to 250 units at the time of maximal Arthus activity (Table I).

Arthus reactions in C′-depleted animals: When animals were pretreated with agg HGG, zymosan, anti-β1C-globulin, or carrageenan, Arthus reactions were uniformly inhibited, as summarized in Table I. The skin sites remained unchanged in the gross and microscopically infiltration of PMN's was greatly inhibited or totally absent (Fig. 2). Toluidine blue stains revealed the presence of normal numbers of intact mast cells in the skins of rats depleted of C′ with agg HGG when compared with normal control rats. With fluorescent antibody techniques, RGG (antibody) and BSA were found fixed to walls of vessels but *little or no C′* was present in association with these immune reactants (Table I, Figs. 5 and 6). The antigen and antibody formed a smooth band corresponding to the walls of vessels, whereas in normal controls, the pattern was granular and irregular owing to the accumulation of PMN's with resulting phagocytosis and scattering of immune complexes (Figs. 3, 4). That the action of the agg HGG was a function of its altered physical state was confirmed by the finding that rats treated with equivalent amounts of non-heated human γ-globulin developed Arthus reactions that macroscopically and microscopically were similar to normal controls (Table I).

The degree of reduction of plasma hemolytic C′ levels varied according to the method of C′ depletion. Whereas agg HGG and carrageenan caused nearly complete loss of activity, treatment with zymosan and anti-β1C-globulin, at best, caused slightly more than a 50 per cent loss of $C'H_{50}$ in rats and guinea pigs (Table I). (Further analysis of these findings is discussed below.)

Development of PMN infiltrates and vasculitis after return of C′ to plasma: In order to determine the eventual fate of skin sites prepared for Arthus reactions in C′ depleted animals after the return of C′ activity to the circulation, rats and guinea pigs were followed for 6 hours (4 hours after cessation of treatment with agg HGG or zymosan). At 2 hours, as noted above, injected skin sites showed little or no reaction, and PMN infiltration and C′ binding in the tissues were not observed, even though antigen and RGG were present (Table II). At this time levels of C′ in plasma were unmeasurable or reduced, depending on the type of treatment. By 6 hours, however, the sites had become edematous and erythematous. Binding of C′ to immune reactants and extensive PMN infiltrates were now found microscopically (Table II). At the same time, rising levels of plasma C′ were noted. There was an apparent temporal association of local C′ binding, infiltration of PMN's and development of Arthus reactions in all animals.

Multiple attempts to replete C′ by parenteral injections of 3 ml homologous or heterologous (rat or guinea pig) serum, as well as by injection of 0.2 ml fresh homologous serum into skin sites of both rats and guinea pigs, failed both in bringing about binding of detectable β1C-globulin in tissues and in restoring hemolytic activity of the plasma. Attraction of PMN's to immune deposits in vessels did not occur, and Arthus activity was not seen.

Effect of C′ depletion on circulating blood elements: The possibility was con-

sidered that the C′-depleting procedures interferred with the development of immunologic vasculitis by causing reductions in numbers of circulating PMN's and/or platelets. As noted in Table III, treatment of rats with agg HGG, zymosan and anti-β1C-globulin, at the doses described above, was generally associated with significant *increases* in absolute PMN counts, while platelet

TABLE II

Development of Arthus Reactions in C′-Depleted Rats and Guinea Pigs after 6 Hours

Animal No. and treatment*	Skin sites								$C'H_{50}$ serum		
	Macroscopic		Microscopic PMN infiltrate		Fluorescent studies						
					BSA		Rat or guinea pig C′				
	2 hrs.	6 hrs.	2 hrs.	6 hrs.	2 hrs.	6 hrs.	2 hrs.	6 hrs.	2 hrs.	6 hrs.	12 hrs.
A. Rats											
1 agg HGG	0	±	0	2+	2+	3+‡	±	2+	<6	<6	—§
2 agg HGG	0	1+	1+	3+	2+	3+	0	±	<6	7	21
3 agg HGG	1+	1+	±	3+	3+	3+	0	1+	<6	7	36
4 agg HGG	0	1+	0	4+	3+	4+	0	4+	<6	7	17
5 agg HGG	0	1+	0	3+	3+	4+	0	2+	<6	7	17
6 control	3+	4+	3+	4+	4+	4+	3+	2+	44	54	46
B. Guinea pigs											
51 agg HGG	±	2+	±	2+	2+	4+	±	2+	<10	15	—
52 agg HGG	±	2+	0	2+	2+	4+	0	3+	<10	47	—
53 agg HGG	1+	3+	±	4+	2+	4+	0	3+	<10	86	—
54 zymosan	1+	2+	1+	1+	2+	3+	±	2+	134	200	—
55 zymosan	0	2+	±	3+	2+	3+	0	3+	126	212	—
56 control	3+	3+	3+	3+	3+	4+	3+	4+	215	177	—

* Different animals used at 2- and 6-hour intervals; see text for doses of C′ depleting agents employed.

‡ Increase in amount of fluorescence caused by phagocytosis by PMN's and spreading of complexes into areas surrounding vessels.

§ Not done.

levels were relatively uneffected. Circulating numbers of mononuclear cells were not altered. Although not included in Table III, alterations of blood counts in guinea pigs following the same C′-depleting procedures yielded substantially similar results.

Motility and phagocytic capacity of leukocytes from C′ depleted rats: PMN's obtained from C′-depleted rats when mixed with zymosan particles phagocyted the particles to the same degree as did PMN's from normal animals. In addi-

tion, leukocytes from the peripheral blood of rats depleted of C′ by agg HGG or zymosan showed active motility at 37° C similar to PMN's from normal, untreated rats (Table IV).

TABLE III

Effect of C′-Depleting Agents on Blood Counts in Rats

Treatment*	Before C′ depletion		After C′ depletion‡	
	PMN/mm³	Platelets × 10^{-3}/mm³	PMN/mm³	Platelets × 10^{-3}/mm³
Normal Arthus controls	4,100	945	8,100	770
	6,500	585	7,100	615
	2,150	—§	7,200	665
Agg HGG	3,300	725	6,050	490
	3,100	660	4,400	351
	3,800	550	5,400	580
	3,210	510	8,400	700
	—§	—	8,050	685
	—	—	8,500	738
	—	—	11,000	584
Zymosan	—	—	3,100	567
	—	—	5,420	760
	—	—	3,200	635
	—	—	4,300	600
Anti-βIC	—	—	6,900	495
	—	—	9,760	330
	—	—	4,600	526
	—	—	8,250	193
	—	—	2,160	210

* See text for doses of agents injected.

‡ Each animal was tested for Arthus activity as outlined in Table I and in Materials and Methods.

§ Not done.

Chemotaxis of PMN's in C′-depleted rats with lysates of PMN granules: Lysates from granules of rat PMN's have been shown to exert a chemotactic effect on circulating PMN's when placed on rabbit or rat mesentary (19). Similar lysates from rat PMN granules were obtained, as described above, and 1.2 μg N (0.3 ml) injected intradermally into rat skin. Two normal controls as well as two rats depleted of C′ with agg HGG were tested. In both groups of rats moderate edema developed in the skin sites, accompanied by perivascular and interstitial infiltrates of PMN's. No quantitative differences were apparent

between the two groups. At sites injected with 0.3 ml medium 199, no PMN infiltration occurred.

The Effects of Various C′-Depleting Agents on Specific C′ Components in Guinea Pigs.—Studies were carried out to determine the effect that various C′-depleting procedures had on certain components of C′ in guinea pigs. C′1,4 activity, C′2, and C′3c levels were measured utilizing the assay procedures described in the section on Methods and Materials. The results are summarized in Table V. In normal control guinea pigs developing typical Arthus reactions, the C′1,4 and C′2 activities were virtually unchanged 3 hours after preparation of the skin

TABLE IV

Motility and Phagocytic Capacities of Leukocytes from C′-Depleted Rats

Treatment of PMN donor*	Animal No.	Phagocytosis—positive cells (particles/cell)			Leukocyte motility
		Dilution of zymosan			
		None‡	1:4	1:16	
		per cent	*per cent*	*per cent*	
Normal controls	1	88 (3)	84 (3)	44 (1)	+§
	2	98 (3)	90 (3)	75 (1)	+
	3	94 (3)	92 (3)	35 (1)	+
Agg HGG	4	98 (4)	97 (3)	36 (1)	+
	5	100 (4)	90 (3)	40 (1)	+
	6	100 (4)	89 (3)	52 (1)	+
Zymosan	7–9	—‖	—	—	+

* See Materials and Methods section for dose of C′-depleting agent.

‡ 10 mg zymosan/ml; each tube contains: 0.1 ml zymosan solution + 0.3 ml cell suspension (approximately 4 × 10^6 cells) + 1.0 ml fresh serum—medium 199 mixture.

§ Movement and pseudopod formation in cytoplasm.

‖ Not done.

sites, while C′3c and C′H_{50} values were not altered by more than 25 per cent. In contrast, after treatment with agg HGG, C′1,4 activity was not measurable, and C′2 and C′3c activities were almost totally lost, as was the level of hemolytic C′. On the other hand, treatment with zymosan had little effect on levels of C′1,4 and C′2, whereas an average of 77 per cent of C′3c activity was lost. At the same time the C′H_{50} value for zymosan-treated guinea pigs fell only 43 per cent. Depending upon the particular batch of anti-C′3c used, C′1,4 activity was slightly to markedly depleted (Table V), the C′2 level was reduced by 51 per cent, and the C′3c level almost totally lost, as was the C′H_{50} activity in the plasma. Treatment with carrageenan affected predominately the C′1,4 activity and caused nearly complete loss of C′H_{50} activity. Changes in activities

of C'2 and C'3c were considerably less effected. It should be noted that regardless of the method of C' depletion, the inhibition of the Arthus reactions in all animals was associated with the lack of binding of C'3c to immune reactants in tissues of the guinea pigs (Table I).

Vasculitis-Inducing Properties of Antibodies with Different C'-Fixing Capacities.—The foregoing data have dealt with the ability of antibodies of potent C'-fixing capacities *in vitro* to induce immunologic vasculitis in normal and C'-depleted animals. It was of interest to determine in *normal animals* the vasculitis-inducing properties of antibodies which fix C' poorly *in vitro*. Rabbit anti-BSA obtained as a single peak by elution from DEAE cellulose (described

TABLE V

Effect of C'-Depleting Agents in Vivo on Components of Guinea Pig C'

C'-depleting agent employed*	No. animals	Component measured			Plasma
		C'1 and/or 4 (T_{max}, min.)	C'2 reduction‡	C'3c reduction‡	$C'H_{60}$ reduction‡
			per cent	*per cent*	*per cent*
Controls	4	6–12	3	25	18
Aggregated HGG	6	α§	85	89	93
Zymosan	5	6–14	1	77	43
Anti-β1C-globulin (anti-C'3c)	6	18-α	51	96	91
Carrageenan	6	α	22	30	93

* See text for doses.

‡ All measurements on plasma, obtained during biopsy of Arthus skin sites, represent averages of figures determined by comparing plasma activities for C' components before and after C' depletion.

§ Less than 5 per cent lysis after addition of excess C'2 and incubation for 10 to 15 minutes; see text for assay procedure.

above) had potent C'-fixing capacity *in vitro*. 20 μg N anti-BSA with antigen added at equivalence fixed 87 per cent of approximately 200 units of guinea pig C' (Table VI). However, after digestion with pepsin, only 11 per cent of the C'-fixing capacity remained. With the preparations of anti-EA the "slow γ" fraction fixed 94 per cent of the C' whereas "fast γ" fractions fixed no more than 6 per cent of the total C' (Table VI).

In *rats* a direct correlation existed between C' fixation *in vitro* and C' binding in tissues, the latter appearing concomitantly with infiltration of PMN's and acute vasculitis. When rabbit anti-BSA was digested with pepsin, such that nearly 90 per cent of the C'-fixing capacity *in vitro* was lost, little or no PMN attracting and Arthus activity remained, in spite of the fact that the dose of antibody was adjusted so that 50 μg N preciptating anti-BSA was used.

Similarly, with duck anti-BSA, which did not detectably fix guinea pig C′ *in vitro*, rat C′ was not bound in tissues and PMN infiltrates did not occur (Table VI).

TABLE VI

Association of Complement Fixing Capacities of Antibodies and Arthus Activity

Animals	Antibody preparations*	No. of animals	C.F.‡ *in vitro*	Arthus reactions			
				Macroscopic	Microscopic (PMN infiltrates)	Fluorescent studies	
						BSA or EA	Guinea pig or rat C′
			per cent				
Rats	Rabbit anti-BSA (DEAE)						
	Untreated	4		4+	3+	3+	3+
	Pepsin digested	5		2+	±	3+	±
	Duck anti-BSA	10		3+§	1+	4+	0
Guinea pigs	Rabbit anti-BSA (DEAE)						
	Untreated	4	87	2 to 3+	4+	3+	2 to 3+
	Pepsin digested	4	5–11	2+	2+	3+	2+
	Duck anti-BSA	12	0	2+	3+	3+	2+
	Guinea pig anti-EA‖						
	Slow γ	10	94	3+	3+	3+	3+
	Fast γ	10	6	3+	3+	3+	3+

* See text for description of antibody preparations.

‡ Per cent of approximately 200 $C'H_{50}$ units guinea pig C′ fixed by 20 μg antibody N with antigen added at equivalence.

§ Edema only; no erythema or hemorrhage in skin sites of rats injected with duck anti-BSA.

‖ Prepared by Pevikon block electrophoresis or by separation on DEAE cellulose (see text).

In *guinea pigs* no such correlation was found between C′-fixing capacities *in vitro* of various antibodies and their vasculitis-inducing properties (Table VI). Pepsin-digested rabbit anti-BSA, duck anti-BSA, and guinea pig anti-EA of the "fast γ" type fixed no more than 11 per cent of 200 units of C′ *in vitro*. Nevertheless, each antibody caused the binding of C′3c in tissues, infiltration of PMN's and development of typical Arthus reactions. No difference could be detected between the "fast γ" preparations of guinea pig anti-EA, whether separated by pevikon block electrophoresis, by chromatography on DEAE cellulose, or a combination of the two. Thus, in the guinea pigs no significant

quantitative or qualitative differences in biological activity could be related to the C′-fixing capacities of the various antisera *in vitro*.

C′ Fixation to Immune Reactants in the Absence of PMN's.—While information about the role of PMN's in the development of immunologic vasculitis is available in several species (6–9), it was considered important to determine if the attraction of PMN's to immnologic deposits in tissues was vital to the development of vascular injury in rats. Therefore, rats were depleted of circulating PMN's and Arthus sites were prepared in the usual manner with rabbit anti-BSA. In animals with PMN counts of less than 100/mm³ blood, little or no edema developed, and, although abundant deposits of BSA and rat β1C-globulin were found fixed in the walls of dermal vessels, PMN infiltration was not

TABLE VII

*Arthus Activity in Rats after Depletion of PMN's**

No. of animals	PMN/mm³ blood‡	Macroscopic	Microscopic PMN infiltrate	Fuorescent studies§		C H_{50}/ml serum
				BSA	Rat C′	
3	<100	± to 1+	0	+	+	<34, 40, 49
5	100–600	± to 2+	0 to 2+	+	+	34–55
1	1067	2+	3+	+	+	39
2	2000–6000	1 and 4+	3+	+	+	29, 44
4‖	2000–5000	4+	4+	+	+	44–63

* See text for dose and schedule of injection of anti-PMN serum.

‡ Counts made before preparation of Arthus sites.

§ + denotes presence of protein in walls of blood vessels.

‖ Normal controls.

observed and evidence of tissue damage was lacking (Table VII). When PMN counts exceeded 100/mm³, edema, erythema, and PMN infiltration were present in varying amounts, roughly proportional to the number of PMN's in the circulation, and Arthus vasculitis developed. Plasma levels of C′ in PMN-depleted animals varied, but, with few exceptions, were not reduced by more than 20 per cent (Table VII).

DISCUSSION

The data presented above demonstrate that a correlation exists between the presence of concentrations of C′ in vascular structures, in association with immune deposits, and the accumulation of polymorphonuclear leukocytes (PMN's). The evidence for this is derived by two experimental approaches: (*a*) in animals *depleted* of C′ by injection of heat-aggregated HGG, zymosan, anti-β1C-globulin, or carrageenan, C′ was not bound to immune reactants in

the skin, PMN infiltration did not occur, and no significant tissue damage ensued; (*b*) in *normal* rats and guinea pigs the accumulation of PMN's at Arthus sites, prepared with antibodies of different C′-fixing capacities, varied with the degree of binding of host C′ to immune reactants in the skin. In rats antibodies that fixed C′ poorly in tissues failed to bring about accumulation of PMN's (Table VI). In guinea pigs, on the other hand, the various antibody preparations all fixed C′ *in vivo*, and PMN's accumulated in tissue sites. The reason for the poor fixation of C′ to vascular structures in rats by certain antibodies, in contrast to the relatively good fixation with the same antibodies in guinea pigs, is not apparent. Tests to determine the amount of C′ fixed systemically *in vivo* using antisera that fix C′ poorly *in vitro* have confirmed the observations reported above; *i.e.*, that rats and guinea pigs react differently to antibodies in terms of C′ fixation *in vivo* (30).

Because of the possibility that the procedures of C′ depletion might adversely effect numbers and function of circulating leukocytes, thus, indirectly influencing the development of Arthus lesions, the formed elements of the blood in C′-depleted animals were scrutinized. Animals injected with C′-depleting agents had little or no reduction in numbers of circulating leukocytes and platelets, contrasted to the reports of Bier and Siqueira (10). In fact, absolute PMN counts frequently doubled in the course of treatment. It was not possible, therefore, to ascribe inhibition of the Arthus reactions to reductions in numbers of circulating PMN's. Furthermore, since leukocytes from C′-depleted rats behaved similarly to those from normal controls with respect to motility and phagocytic capacities, any functional abnormality of the PMN's obtained from C′-depleted rats was not apparent. Furthermore, upon injection of a known chemotactic agent into the skin of C′-depleted rats, PMN infiltration ensued.

Further evidence of a correlation between attraction of PMN's and the binding of C′ in tissues was provided by the finding that when animals were depleted of C′ and followed over a period of 6 hours (*viz.* 4 hours after cessation of treatment with C′-depleting agents), Arthus reactions developed in the usual manner. By 6 hours C′ binding with antigen and antibody in dermal vessels was apparent, and this was associated with an influx of PMN's as well as the development of edema, erythema, and hemorrhage, all signs of vascular damage (Table II). By this time levels of plasma C′ were beginning to rise.

Attempts at local and systemic repletion of C′ were not successful, presumably because of the persistence of circulating C′-depleting agents. β1C-globulin could not be found in skin sites and serum C′ activity was unaltered by the injections of fresh serum. Thus, the correlation between tissue-bound C′ and the infiltration of PMN's cannot yet be considered causal. In addition, the possibility must be considered that other factors may act in concert with C′ to cause attraction of PMN's to the antigen-antibody deposits *in vivo*.

Analysis of the components of C′ that may be important in the promotion of PMN attraction to immune deposits *in vivo* drew attention to the various components of C′ reacting after C′2, namely the C′3 group. Assuming that the availability, and indeed fixation, of C′1,4 and 2 at tissue sites is reflected by their levels in plasma, the data from guinea pigs treated with zymosan suggest that the first three reacting components are not involved in the chemotaxis of PMN's, in view of the fact that levels of these C′ components were little altered by treatment with zymosan. Since antigen and antibody were fixed in vascular walls of the skin in these animals, it would appear likely that their proximity to plasma would allow for fixation of C′1,4 and 2 in such areas. If this is so, then C′3c or some later reacting component of C′ may be involved in the attraction of PMN's. Possibly pertinent to these speculations are the results of Arthus testing in C′-deficient B10·D2 *old line* mice (18). These mice have been shown to have a deficiency of a component in the C′3 group (31). However, Arthus lesions were readily induced and were associated with the local binding of β1C-globulin and influx of PMN's (18). The reactions were equal in intensity to those of C′ sufficient B10·D2 *new line* mice. These results suggest the importance of β1C-globulin or some component other than C′1,4 and 2 in the development of the Arthus reactions. Recently reported studies of genetically C′-deficient rabbits indicates a lack of macroscopic Arthus activity although the nature of the deficiency in serum C′ has not yet been elucidated (32).

Apparent from the data is the lack of a correlation between $C'H_{50}$ levels and the binding of C′3c in tissues. In zymosan-treated animals, although plasma levels of C′ were not reduced much more than 50 per cent, no C′3c was bound in tissues. The assay procedures for sera from these animals revealed relatively little alteration of C′1,4 and 2 levels in plasma, whereas much of the C′3c activity was lost. It may well be that, in the assays for hemoltyic C′ employing plasma from zymosan treated animals, abundant sites containing C′1,4 and 2 were formed on sensitized erythrocytes; then the few C′3c molecules present might be sufficient to contact such active sites and, along with the subsequent components of the C′3 group, bring about lysis of the cells. The amount of C′3c would not be sufficient, however, either to allow for visualization in skin sites or initiation of the chain of events resulting in PMN attraction and the Arthus reaction. On the other hand, plasma of guinea pigs treated with aggregated HGG did not exhibit hemolytic C′ activity probably, in part at least, because the C′1,4 and 2 levels were markedly depleted along with C′3c levels. The data suggest that the $C'H_{50}$ assay is a relatively sensitive indicator of C′3c activity, when abundant C′1,4 and 2 are present, whereas the development of immunologic vasculitis requires relatively more C′3c.

Finally, these data underscore the requirement of PMN's as a prerequisite to the development of extensive vascular injury. The lack of significant tissue

damage in rats depleted of PMN's, even though C′ was found associated with antigen and antibody in vascular structures, suggests that these cells are required for the degree of vascular alteration noted in Arthus reactions. In addition, it was apparent that antigen, antibody and bound C′ (detected as β1C-globulin) by themselves did not account for significant damage to the vessel walls.

SUMMARY

Rats and guinea pigs were depleted of complement (C′) by treatment with heat aggregated human γ-globulin (agg HGG), zymosan, anti-β1C globulin, and carrageenan. Although antigen and antibody were bound to vascular structures, Arthus reactions were inhibited. This inhibition was characterized by the lack of C′ binding to walls of vessels, the lack of polymorphonuclear (PMN's) cellular infiltrates, and the lack of significant vascular damage. When the same animals were followed for several hours thereafter, levels of serum C′ began to rise, C′ was bound in tissues, PMN infiltrates appeared, and immunologic vasculitis developed. Blood counts, chemotaxis of PMN's induced by lysates of PMN granules, together with studies on motility and phagocytosis by PMN's obtained from C′ depleted rats, failed to establish any abnormality in these cells which would account for inhibition of Arthus reactions.

The specificity of C′ depletion in terms of effects in the first four reacting components of guinea pig C′ was studied. Treatment with agg HGG led to loss of activity in all components, whereas zymosan and anti-β1C globulin predominately affected the third component (C′3c). Carrageenan mainly affected the first two reacting components of C′. Thus, the availability of the 3c component, or a subsequently reacting component, correlated with the attraction of PMN's to immune reactants *in vivo*.

Various antibodies with different C′ fixing capacities *in vitro* were tested for their ability to induce immunologic vasculitis in *normal* animals. In rats, only those antibodies which fixed C′ *in vitro* possessed biological activity, whereas in guinea pigs, all antibodies tested, regardless of C′ fixation *in vitro*, induced Arthus reactions. For a given antibody in rats the vasculitis-inducing property was reflected in its ability to bind C′ in vascular structures.

Rats depleted of circulating PMN's by specific antibody were tested for Arthus activity. Although concentrations of immune reactants and C′ were readily detected in vascular structures, no PMN infiltration occurred and significant vascular damage was averted.

Addendum.—Recent studies in this laboratory on *in vitro* chemotaxis of PMN's indicate that serum C′ is involved in the generation of a chemotactic factor following the interaction of normal rabbit or guinea pig serum with antigen-antibody complexes. Similar treatment of serum from genetically C′-deficient rabbits (kindly supplied by Dr. K.

Rother) does not result in the release of the chemotactic factor unless purified human C'6 is added (kindly supplied by Dr. U. Nilsson and Dr. H. Müller-Eberhard.)

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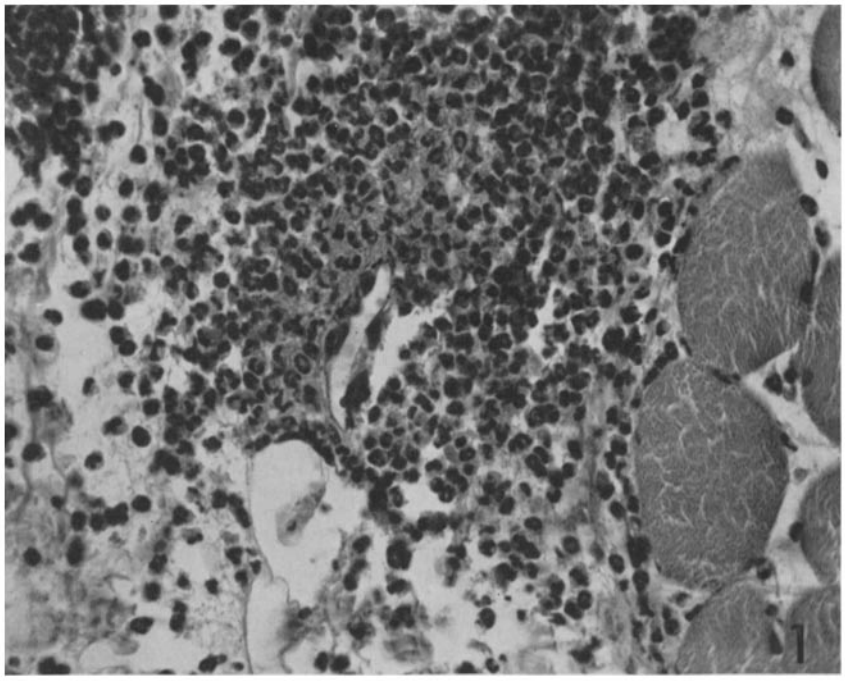
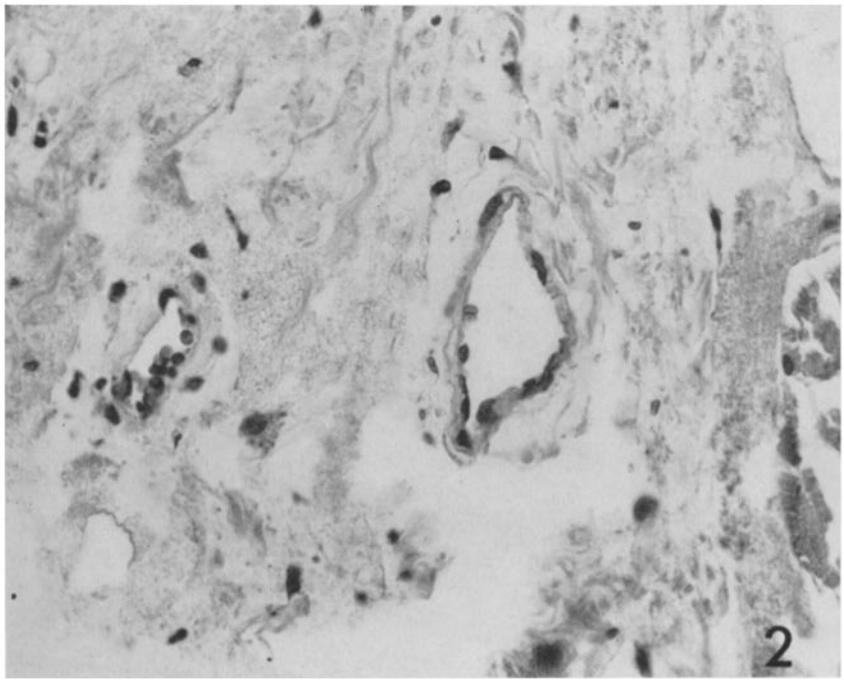
EXPLANATION OF PLATES

PLATE 15

FIG. 1. Dermal vessels adjacent to skeletal muscle in an Arthus site of normal control rat 2 hours after injection of antigen and antibody. There is extensive infiltration of PMN's in and around walls of vessels. Hematoxylin and eosin stain. × 350.

FIG. 2. Dermal vessels adjacent to skeletal muscle in an Arthus site of zymosan-treated rat 2 hours after preparation of Arthus site. No cellular infiltrate is present. Hematoxylin and eosin stain. × 350.





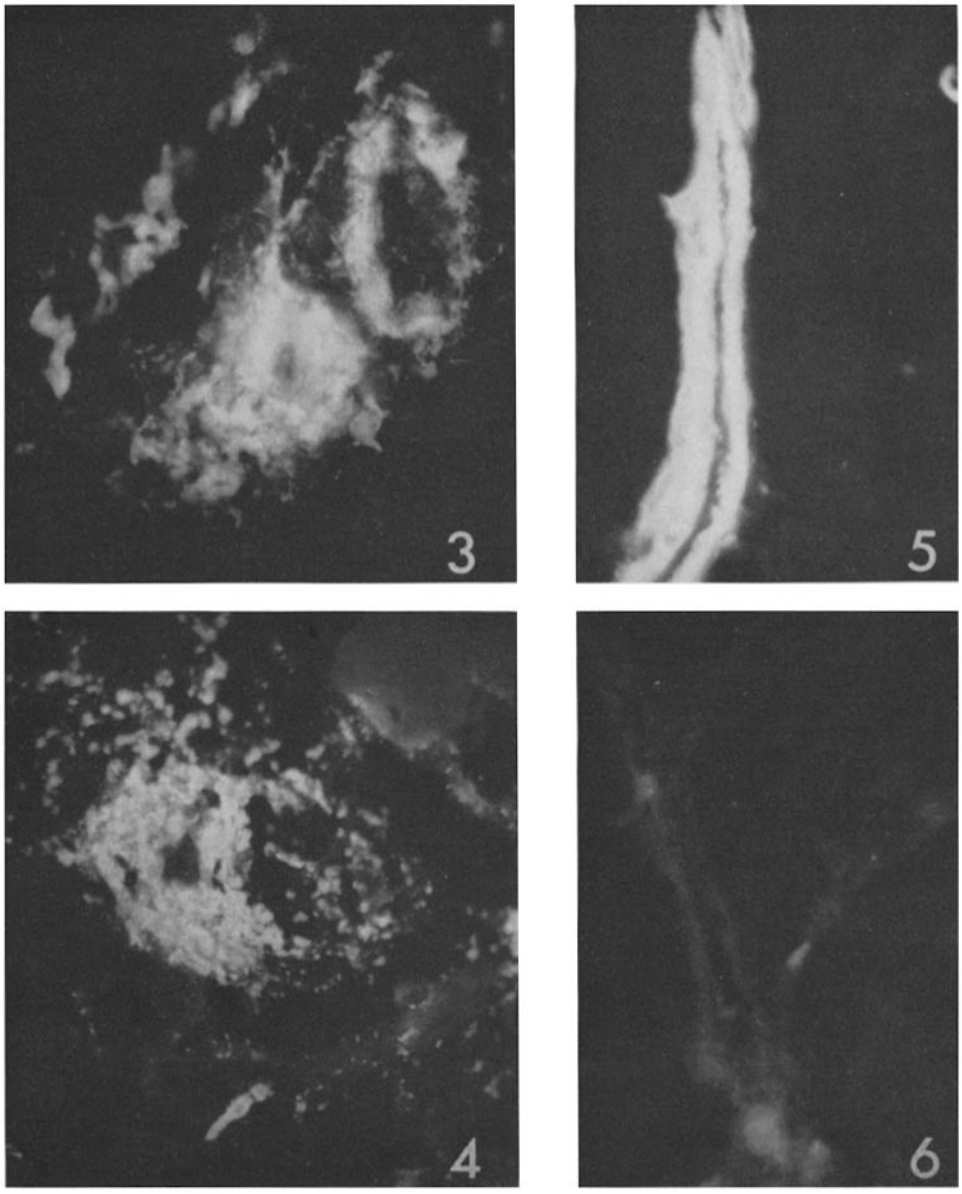
(Ward and Cochrane: Bound complement and immunologic injury)

PLATE 16

FIG. 3. Fluorescent photomicrograph showing BSA (antigen) in walls of vessels of a reversed passive Arthus site in normal control rat. BSA is present in walls of vessels and in perivascular connective tissue. Rabbit γ-globulin was found in an identical distribution. $\times$ 200.

FIG. 4. Fluorescent photomicrograph of an Arthus skin site from animal similar to Fig. 3 stained for rat complement (β1C-globulin). Rat β1C-globulin is present in walls of vessels and within PMN's in adjacent areas in a distribution identical with that of the BSA. $\times$ 200.

FIGS. 5 and 6. Fluorescent photomicrographs of serial sections from an Arthus site of a zymosan-treated rat 2 hours after injection of antigen and antibody. In Fig. 5, BSA is sharply localized in the wall of an intermuscular vessel (in longitudinal section). However, in Fig. 6, virtually no rat β1C-globulin is apparent. $\times$ 250.



(Ward and Cochrane: Bound complement and immunologic injury)