THE ROLE OF SERUM COMPLEMENT IN CHEMOTAXIS OF LEUKOCYTES IN VITRO*, ‡

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Plate 22

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The necessity of serum complement (C′) for the development of immunologic vasculitis and certain forms of experimental nephritis has recently been emphasized (1, 2). It appears from these studies that the local binding of C′ to immunologic reactants, whether in walls of vessels in the skin or renal glomerular capillary loops, is related to an influx of polymorphonuclear leukocytes (PMN's) and subsequent vascular damage. If serum C′ is depleted by any one of several agents, C′ binding in vascular walls does not occur and infiltrates of PMN's do not appear. It has been postulated that the local fixation of C′ leads to the appearance of chemotactic agents that cause accumulation of PMN's, which, in turn, play a significant role in the damage of vascular structures (1). The end result of such events has been defined in terms of the edema, erythema, and hemorrhage associated with the rupture of the basement membranes in the damage of venules, necrosis and fibrinoid deposition in injury of arteries (3), and by immediate proteinuria in early glomerulonephritis (2).

The specific components of C′ associated with the influx of PMN's *in vivo* are not known. Recently, data have been obtained which suggest that the first three reacting components of C′ are not, by themselves, capable of bringing about chemotaxis of PMN's, but that later reacting components are required (1).

The study of chemotaxis has been facilitated by the application of *in vitro* techniques, and the data of Boyden (4) have indicated that a heat labile factor in serum is indeed necessary for generation of a factor that causes attraction of PMN's. The present report deals with the identification and characterization of components of C′ involved in the chemotaxis of PMN's *in vitro*.

Materials and Methods

Chambers Used in Chemotaxis Studies.—Stainless steel rings with an internal diameter of 25 mm and a height of 10 mm were utilized. Each chamber consisted of the steel ring and a bottom

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glass coverslip, on top of which rested two rubber gasket rings with an interposed micropore filter having a pore size of 650 mμ (Millipore Filter Corporation, Bedford, Massachusetts). The micropore filter thus separated the chamber into an upper and lower compartment. A steel ring was then screwed downward to cause pressure sealing of the filter between the gasket rings. A glass coverslip was loosely placed on the top of the apparatus. Small holes in the steel ring allowed for the injection of 1 ml solution to be tested into the lower compartment with a syringe and needle by perforating the rubber gasket. Air was vented by the similar insertion of a needle into the lower compartment at some point opposite the injection site. The upper compartment was then filled with 1 ml cell suspension by lifting off the upper coverslip. Prepared chambers were incubated in air at 37°C for 3 hours. Following aspiration of contents from the upper compartment, each chamber was carefully disassembled and the micropore filter fixed and stained by routine histologic methods. The filter cleared when immersed in xylene.

Quantitation of Chemotaxis.—By light microscopy each filter contained two readily distinguishable planes of focus: an upper level was identified as the upper surface of the micropore filter containing PMN's and occasional mesothelial cells, monocytes, and lymphocytes; the lower level was found to be the lower surface of the filter which contained PMN's which had migrated through the interstices of the filter. The chemotactic value of a test sample was determined by counting under high power magnification (utilizing a micrometer) five fields of the lower surface of a filter. Cells remaining on the upper surface of the filter were not counted.

In all cases, the material being tested for chemotactic activity, as well as the leukocytes, was suspended in medium 199 (Microbiological Associates, Albany, California). Ten per cent autologous serum was used in the lower compartments in some experiments; in others no serum was added to medium 199. In most experiments the cell suspension of each upper compartment contained 10 per cent fresh autologous serum (with 1.5×10^6 PMN's in 1.0 ml). It was found that cell migration *did* occur when the serum was omitted from the cell suspension, if the lower compartment contained chemotactically active material. However, in most instances a cell suspension with 10 per cent serum was employed in the upper compartment, since more consistent results were obtained.

PMN Preparations.—Rabbit PMN's were obtained from the peritoneal cavity according to the method of Cohn and Hirsch (5). The suspension obtained was diluted twofold with medium 199 and centrifuged at low speed in sterile siliconized glassware. The resulting layer of cells was resuspended in medium 199 and the count adjusted to 1.5×10^6 PMN's/ml. As mentioned above, in most instances autologous serum was added as a 10 per cent solution in medium 199. When guinea pig and mouse cells were used, similar techniques were employed. Human buffy coat and rat peritoneal PMN's were also found to respond to chemotactic agents, but the results were somewhat difficult to assess due to the high background counts.

Antibody Preparations and C′-Fixing Agents Employed in Chemotaxis Experiments.—Chromatographically separated 7S rabbit antibody (IgG) to bovine serum albumin (anti-BSA) was utilized before and after digestion with pepsin. Immune precipitates made up with duck anti-BSA were also tested for ability to generate this chemotactic factor in fresh rabbit serum. Details regarding these preparations are listed elsewhere (1). In terms of C′ fixation, 20 μg N duck anti-BSA or pepsin-digested rabbit anti-BSA fixed less than 6 per cent of 200 units guinea pig C′ when antigen was added at equivalence (1). On the other hand, 20 μg N rabbit anti-BSA plus antigen, 40 μg N heat-aggregated human gamma globulin (HGG) (Agg HGG, see reference 1) and 10 mg zymosan were all tested for ability to generate chemotactic activity. It was found that doses of these preparations caused fixation of more than 90 per cent of 200 units guinea pig C′ (1). Additionally, it was found that immune complexes of rabbit anti-BSA fixed rabbit C′ whereas duck antibody complexes failed to do so. In some experiments serum was heated at 56°C for 30 minutes before the addition of immune complexes in order to determine the heat lability of substances in serum required for generation of the chemotactic factor.

Complement Deficient (C'def.) Rabbit Serum.—Serum from rabbits genetically deficient in serum C' was used for testing, through the generosity of Dr. K. Rother (New York) and Dr. Carlos Biro (Mexico City). Such serum has been shown deficient in hemolytic C'. The defect is correctable by the addition of isolated human or rabbit C'6 (the sixth reacting complement component, see reference 6). In the present chemotaxis experiments, 5 lambda human C'6 (approximately 0.5 μg N) was added to 1 ml of the 10 per cent solution of C' deficient rabbit serum containing immune complexes.

C' Deficient Mouse Serum.—Male mice of the B10·D2 *old line* strain genetically deficient in hemolytic C' were used. The defect has been shown to be due to a lack of a protein analogous to the 5th component of human C' (7). C' sufficient serum from B10·D2 *new line*, as well as serum from Swiss-Webster mice, was used. Isologous cells (with respect to serum) were used in tests with B10·D2 mouse serum, and homologous cells with Swiss-Webster mouse serum.

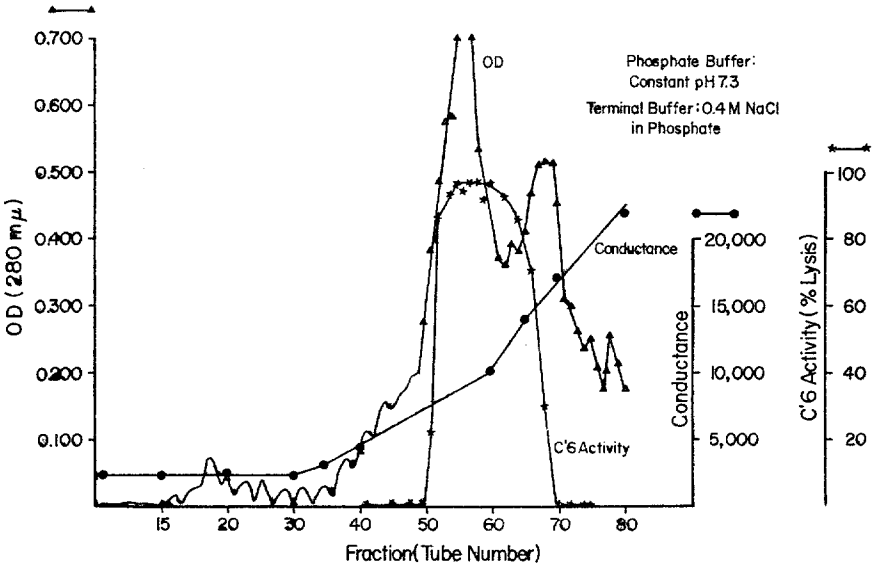
Intermediate Complexes of Guinea Pig C'.—In some studies sensitized sheep erythrocytes containing various intermediate complexes of C' were tested in the lower compartments of chambers for their ability to attract autologous PMN's. Washed sheep erythrocytes (E), erythrocytes heavily sensitized with rabbit amboceptor (EA), EA with the first two reacting components (EAC'1a,4) and first three components (EAC'1a,4,2a) were tested. In addition, the C' components in euglobulin were also added to the EAC'1a,4,2a cells before and after heating (56°C, 15 minutes) of the euglobulin. The details of the techniques employed to make up these intermediate complexes have been reported elsewhere (1). Controls consisted of EAC'1a,4 lysed by freeze-thawing (five times), euglobulin alone, and medium 199 alone. In all instances the lower compartments of the chambers each received 1×10^9 cells, or equivalent, in medium 199. When euglobulin was used, 0.1 ml was added to the cell suspension. No serum was present in the lower compartments of chambers when EAC' complexes were tested.

Preparation and Characterization of Components of Rabbit C'.—Partially purified components of rabbit C', as well as an intermediate complex with EA, were employed for study of chemotactic activity. The intermediate complex was made by the addition of heavily sensitized and washed EA (using amboceptor in a 1:100 dilution) to C' deficient (C'def.) rabbit serum (1×10^9 cells/1 ml C' def. serum diluted 1:10 in veronal buffer with 0.0005 M Mg^{++} and 0.00015 M Ca^{++}) followed by incubation at 32°C for 15 minutes. Under these conditions the cells did not lyse. They were diluted fivefold in veronal buffer, centrifuged, and resuspended in medium 199. Such cells which were positive in the test for immune adherence (8) were designated EAC'def. These cells apparently contain only the first four reacting components of C' (6).

In other experiments preparations of rabbit C' components were "activated" by the addition of stromata of EAC' def. cells. For this, 125 ml sensitized cells (1×10^9 EA/ml) were lysed by the addition of veronal buffer diluted 1:20 in distilled water and the stromata obtained by ultracentrifugation (27,000 RPM, 30 minutes) followed by washing × 3. The stromata were then incubated at 32°C for 10 minutes with 10 ml of C' def. rabbit serum diluted 1:4 in veronal buffer and then immediately separated in the ultracentrifuge at 4°C, as above, in order to obtain EAC' def. stromata.

Crude fractions of rabbit C' containing components analogous to human C'5 and C'6 were obtained by chromatographic separation of rabbit euglobulin (from 90 ml rabbit serum) on TEAE-cellulose. The resulting material termed the *TEAE fraction* was eluted with an increasing gradient of phosphate buffer at constant pH (7.3) as shown in Text-fig. 1. The eluted fraction contained material, referred to as *rabbit C'6*, that reconstituted hemolytic activity of C' def. rabbit serum. This fraction was also found to contain β1C-globulin, and a protein which cross-reacted with antibody produced in C' deficient mice after immunization with human β1F-globulin (C'5, see references 7 and 9). A similar antibody cross-reacting with human β1F-globulin and a protein in rabbit serum could also be obtained in the C' deficient B10·D2 "old"

line strain after immunization with serum from C'sufficient B10·D2 "new" line mice (7, 10). The rabbit protein which is antigenically related to human β1F-globulin appears analogous to human C'5 and will hereafter be designated *rabbit C'5* or β1F-globulin (β1F). The nomenclature for the rabbit C' components was derived from the observations that they behaved similar to human components (β1F-globulin or C'5, and C'6) in the following respects: (*a*) elution from TEAE-cellulose; (*b*) electrophoretic mobility; (*c*) interaction with EAC' def. cells; (*d*) cross-reacting antigenic characteristics (C'5) determined by double diffusion in agar; and (*e*) on the basis of similarities in ultracentrifugal properties (7, 9). The term "β1C-globu-

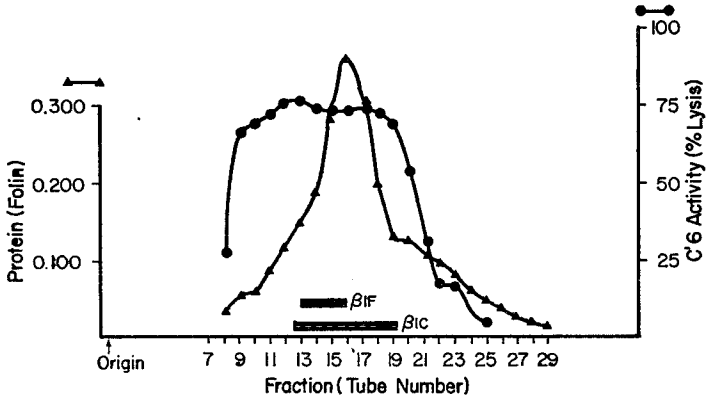


TEXT-FIG. 1. Chromatographic separation of rabbit euglobulin from TEAE-cellulose. Proteins were eluted by an increasing salt gradient of constant pH, with a starting phosphate buffer, pH 7.3, T/2 = 0.05, and a terminal buffer of phosphate in 0.4 M NaCl. Fractions 50 to 68 were pooled and concentrated and were termed the *TEAE fraction.*

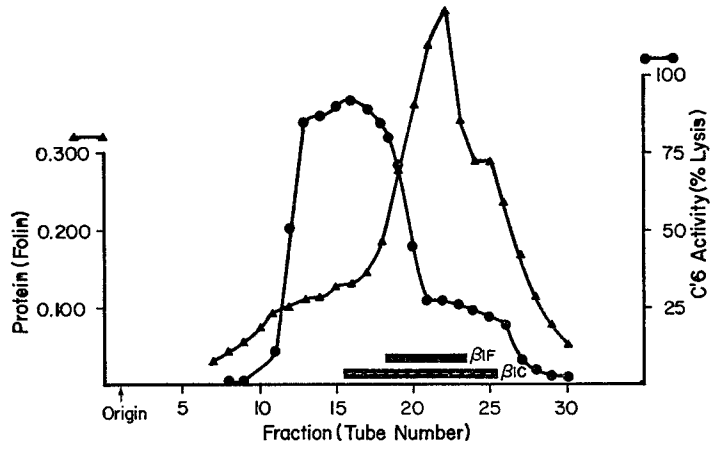
lin" was employed to indicate a protein, presumably the third component of C', present both in normal rabbit serum and in serum after incubation with antigen-antibody (AgAb) precipitates. Contrary to other species studied, no alteration in electrophoretic mobility of rabbit β1C-globulin occurred after such treatment (11).

Eluates from tubes containing C'6 activity (tubes 50 to 68, Text-fig. 1) were pooled and concentrated to 8 ml by ultrafiltration. Electrophoretic separation in pevikon of this pool, referred to as the *non-activated TEAE fraction* was carried out in barbital buffer, pH 8.6, T/2 = 0.05, for a period of 30 hours (12). A broad zone of C'6 activity (see below for details of assay procedure) was found, with a centrally located zone of β1F-globulin and overlapping zone of β1C-globulin (Text-fig. 2). The pH of each fraction was subsequently adjusted to 7.3 by dialysis against phosphate buffer (T/2 = 0.05). No chemotactic activity was found in any of these fractions when approximately 30 μg N from each fraction was tested in chambers. For purposes of later studies, *non-activated C'5 and C'6* were obtained by concentrating fractions 14 to 15 and 10 to 11, respectively. As can be seen in Text-fig. 2, the non-activated β1F or C'5 preparation also contained C'6 and β1C-globulin.

An *activated TEAE fraction* was obtained by incubation of 170 ml of fresh rabbit serum with an immune precipitate containing 10.9 mg N rabbit anti-BSA with antigen added at equivalence. After incubation for 2 hours at 37°C, the precipitate was removed by high speed centrifugation, followed by ultracentrifugation (30,000 RPM, 30 minutes). The TEAE fraction was ob-



TEXT-FIG. 2. Electrophoretic separation of *non-activated* (untreated) *TEAE fraction* of rabbit serum, pH 8.6. A wide zone of C'6 activity is present with smaller central zones of C'5 (β1F) and β1C-globulin. See text for details.

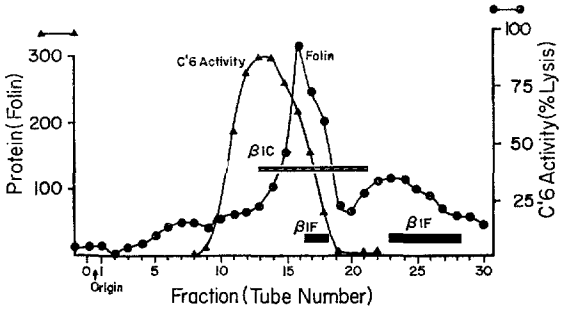


TEXT-FIG. 3. Electrophoretic separation (pH 8.6) of activated TEAE fraction from rabbit serum incubated with immune precipitate. The C'5 has a faster mobility relative to C'6. See text for details.

tained according to the procedure described above and eluates containing C'6 activity pooled and concentrated. Electrophoretic separation of this material at pH 8.6 (Text-fig. 3) resulted in separation of C'5 and C'6. The activated β1F-globulin had a faster mobility than the "non-activated" β1F-globulin (Text-fig. 2) thus allowing separation of C'5 and C'6. Pools from tubes 12 to 17 and 21 to 23 constituted sources of activated C'6 and C'5, respectively, for later studies. The pH of these fractions was adjusted to 7.3 by dialysis in phosphate buffer.

Activation of the TEAE fraction was also accomplished by addition of EAC′ def. stromata (prepared as described above) to the concentrated TEAE fraction at pH 7.3, followed by incubation at 37°C for 2 hours. The stromata were then separated by ultracentrifugation. This *activated TEAE fraction* contained chemotactic activity (Text-fig. 10) which was absent in the untreated TEAE fraction. When the material was separated electrophoretically at pH 8.6, the β1F-globulin had a considerably faster mobility (tubes 23 to 28, Text-fig. 4), thus allowing for separation of C′5 from C′6. A narrow zone of β1F-globulin was also noted in the region of slower mobility (tubes 17 to 18). For later studies pools of tubes 12 to 14 and 26 to 27 served as sources for "activated" C′6 and C′5 respectively.

Assay of Rabbit C′6 Activity.—0.1 ml EA cells, made up as previously described (see reference 1), consisting of 0.5×10^8 cells, was added to 0.1 ml test solution and 0.2 ml C′ def. serum (diluted 1:15 in veronal buffer with Ca^{++} and Mg^{++}, as above). After incubation at 32°C for



TEXT-FIG. 4. Electrophoretic separation (pH 8.6) of the TEAE fraction following activation by incubation with EAC′ def. complexes. Most of the β1F-globulin is fast moving and widely separated from C′6, although a small zone of slower moving C′5 is also present.

60 minutes, 2 ml veronal buffer was added and the supernates read at 412 mμ for lysis. The per cent of total lysis was then determined.

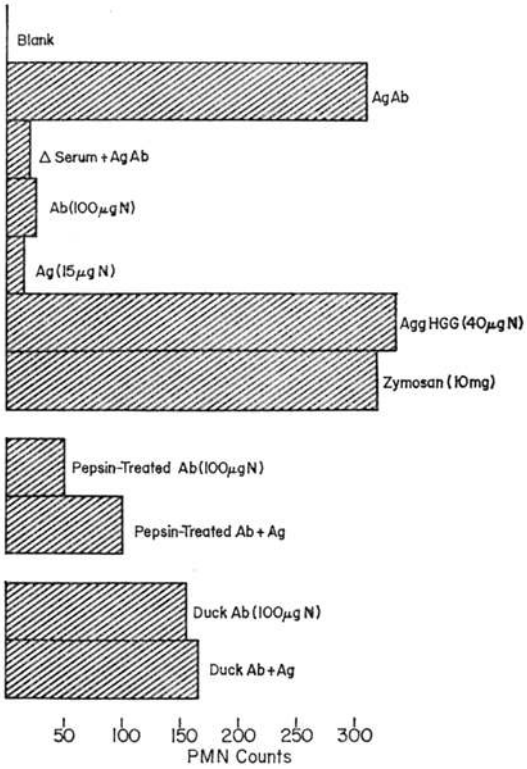
Fractionation by Density Gradient Ultracentrifugation.—Sucrose solutions of 10 and 40 per cent in phosphate buffer pH 7.3 (T/2 = 0.05) or barbital buffer pH 8.6 (T/2 = 0.05) were used to make up gradients according to the method of Kunkel (13). 0.1 to 0.2 ml test material was layered on the top of each gradient solution. Centrifugation was carried out at 35,000 RPM for 21 hours at 4°C in a Spinco L-2 preparative ultracentrifuge, utilizing a swinging bucket (SW-39) rotor.

RESULTS

Chemotaxis of Rabbit PMN's in Vitro.—

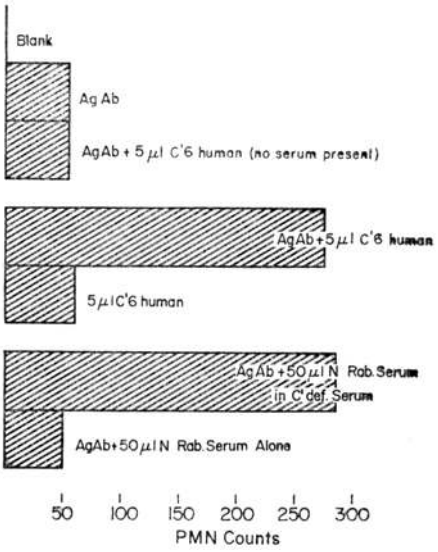
Relationship of C′ activity of normal rabbit serum and chemotaxis of PMN's: In a chamber not containing chemotactically active material in the lower compartment, the distribution of cells on the *upper* surface of the micropore filter after incubation was uniform (Fig. 1 *a*). In contrast, when the lower compartment contained chemotactically active material, cells tended to be clumped with a somewhat irregular distribution on the upper surface of the filter (Fig. 2 *a*). Cells migrating through the filter were readily observed (Figs. 1 *b* and 2 *b*). When antibody and an equivalent amount of antigen, Agg HGG, or zymosan

was added to a 10 per cent solution of fresh isologous rabbit serum in medium 199, significant chemotactic activity appeared. In contrast, little or no chemotaxis was noted in chambers whose lower compartments contained 100 μg N anti-BSA alone, 15μg N BSA alone, or medium 199 by itself (Text-fig. 5). The

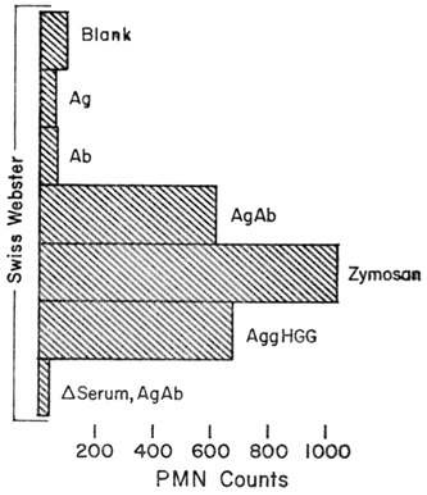


TEXT-FIG. 5. Chemotaxis of rabbit PMN's *in vitro* using normal rabbit serum. The agents noted were added to the lower compartments containing 10 per cent fresh rabbit serum in medium 199. Blank in this and subsequent 3 figures contained 10 per cent rabbit serum in medium 199.

chemotactic activity of the antigen-antibody complexes could be diminished by the pepsin digestion of rabbit antibody or by the use of duck antibody, each of which fixed little C′ *in vitro* (Text-fig. 5). Duck antiserum by itself had some chemotactic activity (0.05 ml duck serum containing 20 μg N anti-BSA fixed 50 units guinea pig C′ *in vitro*, see reference 1 for method), but little effect was noted when antigen was also present. (*In vitro*, immune precipitates made with duck antibody did not fix rabbit C′.) In additional experiments it was found that serum treated with antigen-antibody precipitates or zymosan (for 1 hour at 37°C), in the same proportions as in chambers, was chemotactically active,



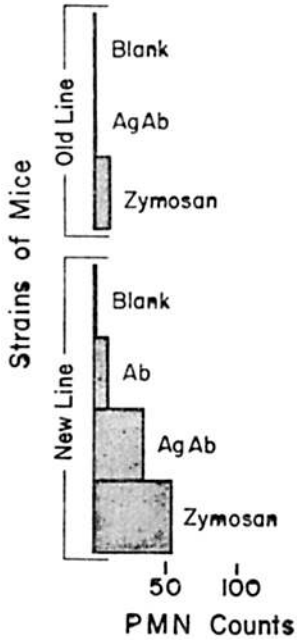
TEXT-FIG. 6. Chemotaxis of rabbit PMN's *in vitro* using genetically C′ deficient rabbit serum. Lower compartments contained 10 per cent C′ deficient rabbit serum with additional agents listed. Third bar from top indicates compartment with agents added in absence of any rabbit serum. In all other cases, except bottom bar, agents were added in presence of 10 per cent C′ deficient rabbit serum.



TEXT-FIG. 7. Chemotaxis of Swiss-Webster mouse PMN'S *in vitro*. Blank contained 10 per cent homologous serum in medium 199. Lower bar indicates compartment with serum heated 56°C for 30 minutes before addition of immune reactants.

after separation of particulate material by high speed centrifugation (14). These results indicated the release of chemotactically active material into supernatant fluid.

It was also found that the presence of 0.01 M ethylenedinitrilotetraacetic acid (EDTA, pH 7.1) in serum at the time of treatment with antigen-antibody precipitates or zymosan prevented the subsequent appearance of the chemo-



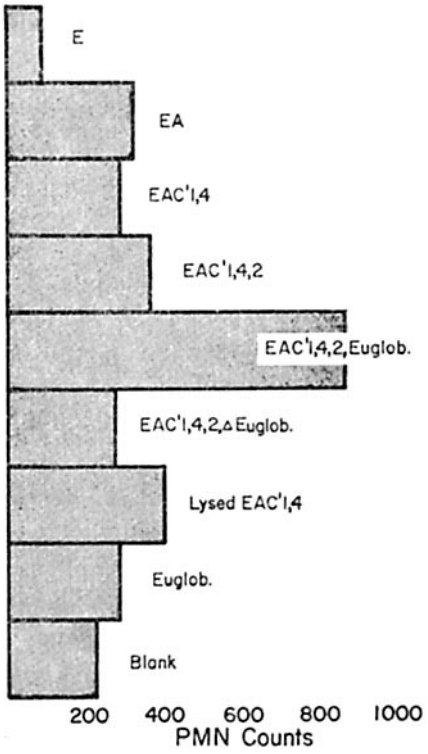
TEXT-FIG. 8. Chemotaxis of isologous mouse PMN's *in vitro* following addition of immune reactants to B10·D2 "old line" (deficient in C'5) and B10·D2 "new line" serum (sufficient in C'5).

tactically active material. (Serum was separated from the immune complexes or zymosan after incubation and then dialyzed against medium 199 with 0.01 M Ca^{++} and Mg^{++} present.) These findings indicated the requirement of cationic factors in the generation of the chemotactic factor.

Chemotaxis with C' def. rabbit serum: Under conditions described above, C' deficient rabbit serum together with immune complexes did not bring about chemotaxis of PMN's unless human C'6 was added (5 lambda, approximately 0.5 μg N) or 50 lambda of normal rabbit serum (Text-fig. 6). As controls, neither human C'6 alone nor immune complexes in the presence of 50 lambda normal rabbit serum brought about significant chemotaxis.

Chemotaxis of Mouse PMN's Utilizing Mouse Serum.—Employing Swiss-

Webster cells (in 10 per cent homologous serum), it was found that the addition of C′-fixing agents to 10 per cent homologous serum in the lower compartment led to substantial chemotactic activity (Text-fig. 7). However, when C′ deficient B10·D2 "old line" mouse serum was used, little to no chemotactic activity could be generated (Text-fig. 8). While use of cells and serum from B10·D2

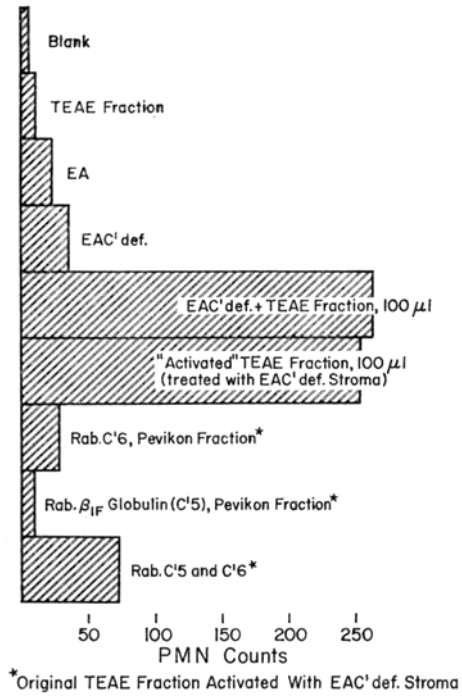


TEXT-FIG. 9. Chemotaxis of guinea pig PMN's *in vitro* by intermediate complexes of sensitized erythrocytes. Sheep erythrocytes (E), sensitized erythrocytes (EA) and intermediate complexes of guinea pig C′ (EAC′1a,4; EAC′1a,4,2a and EAC′1a,4,2a + normal or heated euglobulin) were suspended in medium 199 without serum. Controls included blank (medium 199 alone) and EAC′1a,4 lysed by freeze-thawing.

"new line" mice containing hemolytic C′ activity did not result in high chemotactic activity (Text-fig. 8), distinctly greater chemotactic values were obtained than when B10·D2 old line serum was used.

Chemotaxis of Guinea Pig PMN's with Intermediate Complexes of Guinea Pig C′.—The use of EA with intermediate C′ complexes indicated that cells through the EAC′1a,4,2a stage had little chemotactic activity, when compared with the control blank (medium 199 alone) or E alone (Text-fig. 9). However,

the addition of euglobulin containing some important heat labile factors led to a significant increase in counts. Under these conditions lysis of cells occurred. That released intracellular material did not account for the chemotactic activity was demonstrated by the fact that lysed (freeze-thawing) EAC'1a,4 cells resulted in counts that were only half of the peak counts with EAC'1a,4,2a plus euglobulin (Text-fig. 9). This experiment was repeated two more times with similar results.



TEXT-FIG. 10. Chemotaxis of rabbit PMN's *in vitro* employing various preparations of rabbit C'5 and C'6. Blank control consisted of medium 199 alone. Serum was not added to any of the lower compartments. For details, see text.

Tests for Chemotaxis of Rabbit PMN's using Activated Components of Rabbit C'.—Chromatographic separation of rabbit euglobulin with a continuous salt gradient resulted in a zone of C'6 activity in tubes 50 to 68 (Text-fig. 1). This also contained abundant β1F or C'5 after concentration. 16 μg N of this "non-activated TEAE fraction" had little chemotactic activity by itself (Text-fig. 10, second bar from top). However, when this fraction was incubated with EAC' def. cells (2×10^9 cells/ml TEAE fraction) at 32°C for 10 minutes or "activated" by treatment with EAC' def. stromata (see Materials and Methods), sub-

stantial chemotactic activity resulted (Text-fig. 10, bars 5 and 6 from top). Fractions containing activated C'5 (5.4 μg N) or C'6 (1.6 μg N, see Table I,

TABLE I

Chemotaxis Associated with Recombined Fractions of Rabbit C'5 and C'6

Experiment No.	Fractions tested (μg N)*				Chemotaxis value
	Non-activated		Activated		
	C'5	C'6	C'5	C'6	
1‡					
			54	8	104
			27	8	61
			14	8	35
			54	16	73
			54		9
				16	18
Blank control (medium 199 alone)					12
2§					
		7.5			0
	13.5				0
	13.5	7.5			0
			15.5		0
				11	0
			15.5	11	88
			15.5	5.5	70
	13.5			11	0
		7.5	15.5		24
Blank control					0
Antigen-antibody in normal rabbit serum‖					78

* Total protein per sample.

‡ In experiment 1, source of activated C'5 and C'6: tubes 26 to 27 and 12 to 14, respectively, see Text-Fig. 4.

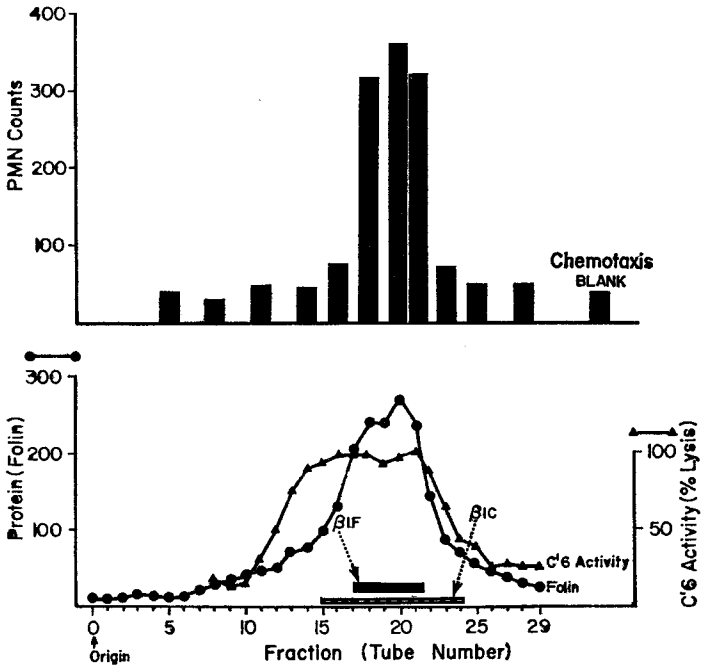
§ In Experiment 2, source of nonactivated C'5 and C'6: tubes 14 to 15 and 10 to 11, respectively, see Text-fig. 2. Source of activated C'5 and C'6: tubes 21 to 23 and 12 to 17, respectively, Text-fig. 3.

‖ 100 μg N rabbit anti-BSA + BSA at equivalence in 10 per cent normal rabbit serum.

experiment 1 for source) had little chemotactic activity by themselves (bars 7 and 8 from top, Text-fig. 10), whereas their combination led to significant chemotactic activity (bottom bar, Text-fig. 10). Further data regarding chem-

otaxis associated with combinations of fractions rich in rabbit C'5 and C'6 are presented below.

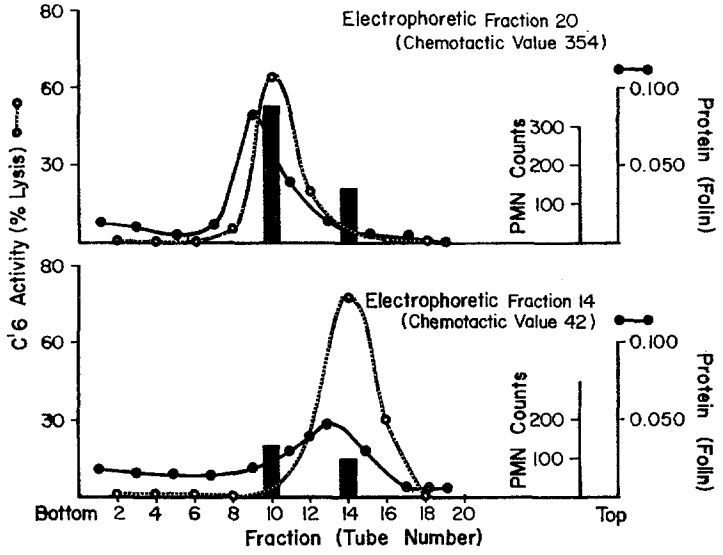
In attempts to define more clearly the possible role of C'5 and C'6 in chemotaxis, the activated TEAE fraction (after incubation with EAC' def. stromata) was separated by preparative electrophoresis in pevikon. When a pH 7.3 was



TEXT-FIG. 11. Chemotaxis of rabbit PMN's *in vitro* by electrophoretically separated (pH, 7.3) fractions of the TEAE fraction activated by EAC' def. stromata. Chemotactic activity is found in fractions containing both C'5 and C'6.

employed, a wide zone of C'6 activity was found in fractions 11 to 26 (Text-fig. 11). However, only fractions 17 to 21 from the faster region of this zone also contained C'5. Little or no chemotactic activity was present in individual fractions that did not contain both C'5 (β1F) and C'6, while when both components were present, chemotactic activity rose five- to sevenfold above background. No correlation between chemotaxis and the β1C-globulin distribution was found. No relationship between the quantity of protein contained in the fraction being tested and the degree of chemotaxis was noted. Fractions outside of the C'6-C'5 (β1F) zone were tested at concentrations containing greater amounts of protein than those within the C'5-C'6 rich zone and still yielded low chemotactic values. This experiment has been subsequently repeated with new starting material

and the end results in terms of chemotactically active fractions after electrophoretic separations are identical to those above. These results suggested that fractions containing C'5 and C'6 were both required for chemotactic activity. Supporting data were obtained by further testing of activated and non-activated fractions of C'5 and C'6 obtained by electrophoretic separation (Text-figs. 2 to 4). *Non-activated* C'5 and C'6 alone or together had no chemotactic

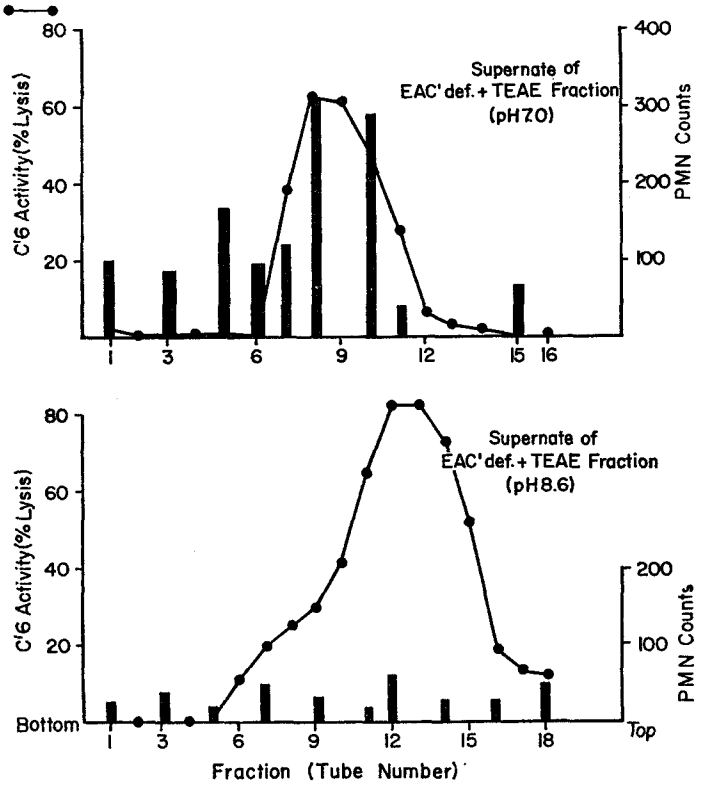


TEXT-FIG. 12. Density gradient ultracentrifugal studies on electrophoretic fractions 14 and 20 of Text-fig. 11. Chemotaxis assays were performed on various fractions obtained after ultracentrifugation. See text for details.

activity (Table I) whereas the combination of fractions containing *activated* C'5 and C'6 resulted in chemotactically active material. As noted in experiment 2 listed in Table I, when non-activated C'5 was combined with activated C'6 no chemotactic activity resulted, whereas the combination of activated C'5 and non-activated C'6 resulted in material with minimal activity. These results indicated that the combination of activated C'5 and C'6 resulted in a material which had chemotactic activity not present in either component alone or in mixtures containing fractions of either non-activated C'5 or C'6 with its activated homologous component.

Density Gradient Ultracentrifugation Studies of Fractions Containing Rabbit C'5 and C'6.—Density gradient ultracentrifugation studies were carried out on fractions isolated after preparative electrophoresis of the activated TEAE fraction at pH 7.3, as noted in the preceding paragraph. Fractions 14 and 20 (Text-fig. 11) with respective chemotactic values of 42 and 354 were investi-

gated. (Fraction 14 was first concentrated so that its protein value was similar to that of fraction 20.) As seen in Text-fig. 12, analysis of fraction 20 revealed that C'6 activity and the chemotactically active material appeared in a rapidly sedimenting fraction (tube No. 10), whereas peak C'6 activity in electrophoretic fraction 14 sedimented more slowly, appearing in tube 14. No chemotactic



TEXT-FIG. 13. Density gradient ultracentrifugal studies of "activated" TEAE fractions at pH 7.0 and 8.6. At the neutral pH, C'6 sediments more rapidly and is associated with chemotactically active material. See text.

activity was associated with this peak. The background values were approximately 100 with medium 199 and sucrose alone.

In order to study the chemotactic activities of C'5 and C'6-rich fractions under conditions favoring association (neutral pH) or dissociation (alkaline pH) of the two components, density gradient ultracentrifugation of chemotactically active TEAE fractions was carried out at two different pH values, 7.0 and 8.6. As noted in Text-fig. 13, the material centrifuged at pH 7.0 contained peak C'6 activity as well as maximal chemotactic activity in tubes 8 and 10. However,

when the same material was centrifuged at a pH 8.6, C'6 sedimented more slowly (tubes 12 and 14) and no chemotactic activity was observed. The presence of sucrose tended to raise background counts in chemotaxis assays, (upper portion of Text-fig. 13), and considerable chemotactic activity was noted in tube No. 5 which did not contain C'6 activity. Tests for C'5 by antigenic analysis were not possible owing to the dilution of the starting sample.

DISCUSSION

It is clear from the data presented that complement (C') is necessary for the generation, by immunologic reactants, of a factor that brings about chemotaxis of polymorphonuclear leukocytes (PMN's) *in vitro*. Furthermore, there is considerable evidence that rabbit C'5 and C'6, presumably functioning as a protein-protein complex, become altered in the process of "activation" by interaction with earlier C' components (*i.e.*, the first four reacting components) and that this activated C'5-C'6 complex is in great part responsible for this chemotaxis of PMN's. Several pieces of evidence support this hypothesis. Fractions rich in either C'5 or C'6 could be isolated by chromatographic separation from TEAE-cellulose followed by block electrophoresis at pH 8.6. Neither material by itself was chemotactically active (Table I). However, when C'5 and C'6 were recombined at pH 7.3, significant chemotactic activity appeared and ultracentrifugal studies indicated that combination of C'5 and C'6 had occurred as indicated by the increased velocity of sedimentation in the ultracentrifuge. In addition, when the electrophoretic isolation procedure was carried out at pH 7.3, the C'5 and C'6 apparently remained in bound form and migrated as a unit. Chemotaxis of the eluted material was then noted only in the zone containing the C'5-C'6 activity (Text-fig. 11). Thus chemotactic activity was associated with a complex of activated C'5-C'6 molecules, an activity that neither component alone possessed to any measurable degree. That C'5 and C'6 function in hemolytic activity as a complex was shown in the recent data of Nilsson and Müller-Eberhard (9).

Of interest is the fact that alterations in electrophoretic mobility (at pH 8.6) of C'5 and C'6 following activation by interaction with other C' components (Text-figs. 2 to 4) appear to indicate changes in the proteins concomitant with the acquisition of biological activity. The change in electrophoretic mobility of β1F-globulin following activation was not constant. The method of activation, either by EAC' def. stromata interacting with the TEAE fraction or by addition of immune precipitates to serum, probably explains the differences in increased electrophoretic mobilities. While altered mobilities, either the slowing of C'6 or the speeding up of C'5, do not necessarily *per se* reflect the specific changes in protein structure requisite for biological activity, they do indicate that structural alterations in the proteins have occurred. It is not possible at this time to determine if these alterations are directly associated with the acquisition of chemotactic activity.

It is apparent that the first four reacting components of guinea pig C′ are necessary for generation of a chemotactic factor. However, these components, by themselves, are capable of bringing about only limited chemotaxis (Text-fig. 9). In addition, the data derived from work with rabbit components fail to suggest that the first four reacting components of rabbit C′ are important *per se* in chemotactic activity. It seems reasonable to postulate that interaction of the first four reacting components leads to the subsequent activation of C′5 and C′6, which, *per se*, are transformed into a chemotactically active complex. This active agent affects PMN's, in a manner as yet unclear, such that they migrate in the direction of the higher concentration of the chemotactic agent.

Recently reported studies *in vivo* on immunologic vasculitis (Arthus reactions) have indicated that the first three reacting components of C′ in guinea pigs do not play a significant role in the accumulation of PMN's at the site of interaction of antigen and antibody (1). These data suggest that additional reacting components of C′ are required for chemotaxis of PMN's. The findings *in vitro* reported in this paper support and extend these interpretations.

Studies of the earliest reactions to antigen-antibody complexes *in vivo* carried out utilizing ear chambers in rabbits showed clumping of PMN's within the lumens of small vessels (15). Interestingly, in the present studies it was found that the addition of chemotactically active agents to lower compartments led to a clumping of PMN's on the upper surfaces of filters. This suggests that alterations on cell membranes occur during the initiation of chemotaxis. The relationship of this clumping to cell migration is, as yet, unclear.

The recent reports of David and coworkers have elucidated a phenomenon of cell migration with mononuclear cells and lymphocytes obtained from animals with delayed hypersensitivity to the immunizing antigen (16). Migration of cells was inhibited in the presence of antigen, contrasting to the cell migration described in the present studies. The phenomenon of chemotaxis of PMN's as described in the present experiments underlines the differences between the two systems. Chemotaxis of PMN's was brought about by the activation of C′ components which contrasts to the inhibitory effect of antigen on migration of mononuclear cells. In our studies migration of mononuclear cells and lymphocytes did not occur, although pore size of the filters may have been a limiting factor.

It has been suggested that lysates of PMN granules may act as mediators leading to the attraction of PMN's to tissue sites (17, 18). Such substances also cause increased vascular permeability (19). However, extracts of PMN's would not account for the initial attraction of leukocytes, although augmentation of subsequent PMN influx by these cell lysates is probable. It may well be that the activated C′5-C′6 complex is the primary stimulus for PMN attraction to deposits of antigen-antibody complexes *in vivo*. Then, once PMN's have migrated into a tissue site, subsequent breakdown of these cells could result in release of agents responsible for an additional influx of PMN's.

In addition to these exogeneous factors probably involved in chemotaxis of PMN's *in vivo*, intrinsic factors released from damaged tissues may be responsible for a successive wave of cellular infiltration. Extracts from burned tissues, as well as supernates from normal tissues incubated with serum and PMN's, were strongly chemotactic *in vivo* and *in vitro* (20). In the case of immunologic reactions (Arthus reaction, early nephrotoxic nephritis, arteritis of serum sickness, and the local vascular damage induced by injection of antibody to basement membrane, see reference 21), such factors most likely are secondary as mediators of chemotaxis. There is little question about the role of the PMN's, once in tissues, in the mediation of tissue damage.

Finally, it might be pointed out that in the chemotaxis studies *in vitro* of Boyden, filters of 3 μ pore size were used (22), while Hurley used filters with a pore size of 5 μ (20). We have used filters of similar size but have preferred the 650 mμ size since, in our experience, background counts are considerably increased when pore sizes exceed 650 mμ, making quantitation and interpretation most difficult.

SUMMARY

By the use of chambers containing two compartments with an interposed micropore filter, chemotaxis of polymorphonuclear leukocytes (PMN's) *in vitro* was studied employing various agents that fixed serum complement (C′). Antigen-antibody complexes, zymosan, and aggregated human gamma globulin, in the presence of fresh rabbit, guinea pig, or mouse serum resulted in the migration of PMN's through the micropore filter. Pepsin–degraded rabbit antibody or unaltered duck serum containing antibody did not exhibit such activity after addition of antigen. Heating of the serum before treatment or the presence of EDTA prevented the generation of the chemotactic factor. The chemotactic factor could not be generated in whole serum from rabbits genetically deficient in C′. However, the defect in this rabbit serum could be corrected by addition of rabbit or human C′6. Serum of B10·D2 mice deficient in hemolytic C′ also yielded poor chemotactic activity.

Interaction of the first four reacting components of guinea pig C′ did not result in significant chemotactic activity unless guinea pig euglobulin with heat labile components was also present. In rabbit serum, C′5 and C′6, when "activated" by interaction with the first four reacting components, behaved like a protein-protein complex and exhibited marked chemotactic activity. By employing conditions favoring dissociation of the complex, the individual components were isolated and shown to be chemotactically inactive. Upon recombination of the two components, however, activity reappeared. Using another approach, the C′5-C′6 complex was isolated intact, and shown to be chemotactically active while other fractions not containing these components were not active. It is postulated that the C′5-C′6 complex is the active chemotactic factor generated in serum after the addition of C′-fixing agents.

Note Added in Proof.—Chemotactic assays have recently been completed on the following vasoactive substances; bradykinin, kallidin, histamine, serotonin, and whole lysates of PMN cytoplasmic granules as well as the cationic protein extracted from this material. (We are greatly indebted to Dr. A. Janoff for supplying this extract.) None of these substances possessed significant chemotactic activity *in vitro*. Whereas leukocytic margination in mesenteric vessels of rabbits was induced with the cationic protein, none of the substances elicited significant leukocytic accumulations in the skin. On the other hand, antigen-antibody deposits with complement caused considerable perivascular accumulations of PMN's in cutaneous and mesenteric vessels.

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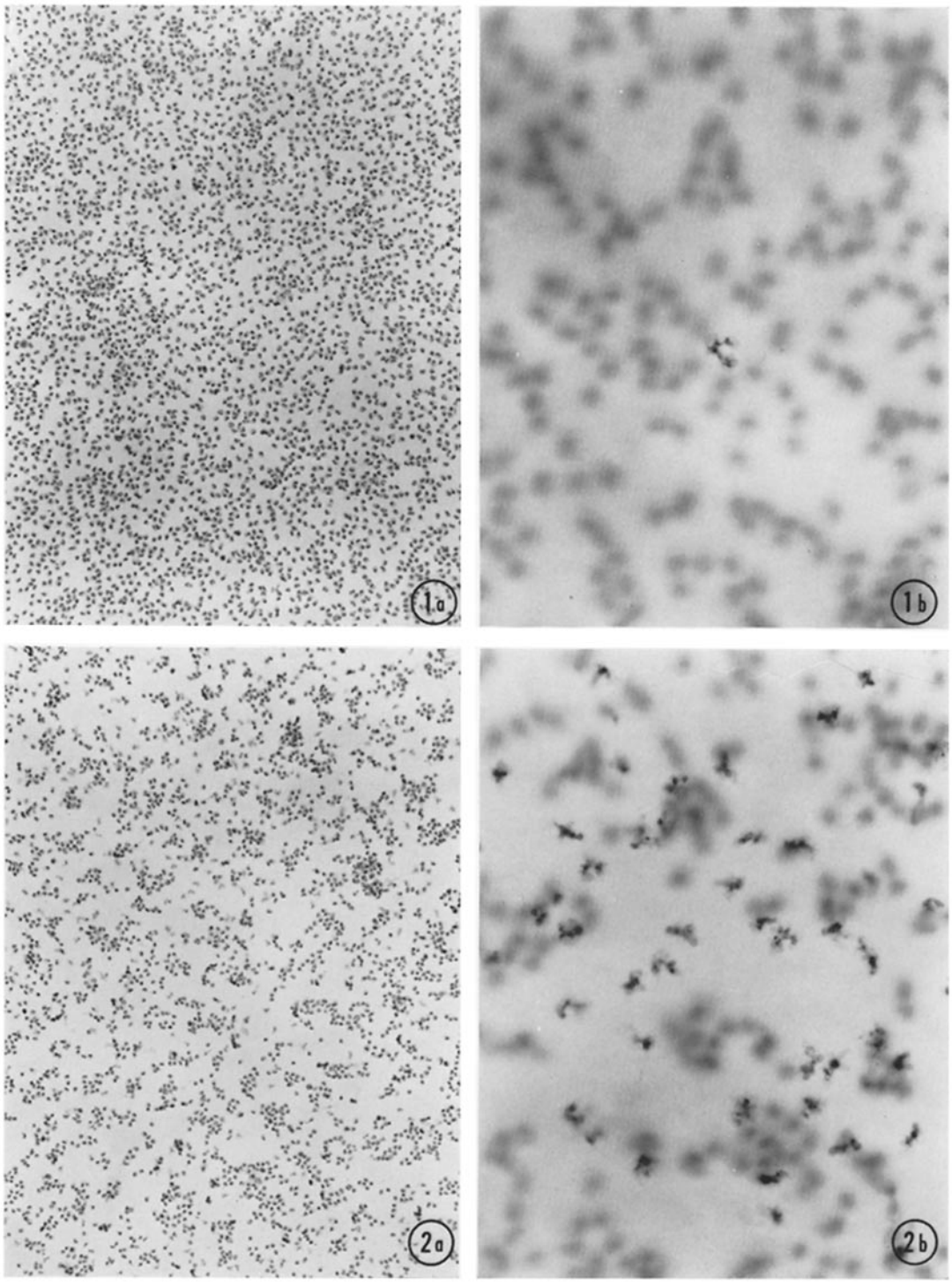
EXPLANATION OF PLATE 22

FIG. 1 *a*. Upper surface of micropore filter from chamber containing fresh rabbit serum in lower compartment. There is a rather uniform distribution of PMN's. × 85.

FIG. 1 *b*. Lower surface of filter in Fig. 1 *a*. A single PMN has moved through the filter. The upper surface containing a sheet of PMN's is out of the field of focus. × 400.

FIG. 2 *a*. Upper surface of filter from chamber containing immune complexes and fresh rabbit serum in lower compartment. Clumping of PMN's is a prominent feature. × 85.

FIG. 2 *b*. Lower surface of filter in Fig. 2 *a*. In the presence of chemotactically active agents, numerous PMN's in several planes of focus can be seen in the interstices of the micropore filter. × 400.



(Ward *et al.*: Serum complement in chemotaxis)