STUDIES ON THE PATHOGENESIS OF ACUTE INFLAMMATION

V. An Assessment of Factors That Influence in Vitro the Phagocytic and Adhesive Properties of Leukocytes Obtained from Rabbit Peritoneal Exudate

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The sticking of leukocytes to vascular endothelium during inflammation always occurs before these cells undergo diapedesis. Accordingly, cellular adhesion represents a key to better understanding of this critical phase of the defense against noxious stimuli. Despite considerable investigative effort, the mechanism responsible for margination and adhesion of white blood cells to endothelium in response to injury has not been discovered. Evidence of our own as well as from other laboratories indicates that the fibrinogen-fibrin system of the blood is not responsible for this event even though this area seemed for a time most promising.[1,2] From subsequent studies of the micro-circulation within ear chambers of rabbits, it was suggested that the sticking reaction might depend upon the release of matter from damaged tissue that modified the surface properties of endothelium of adjacent blood vessels.[3] Search for material with the capacity to evoke such a change *in vivo* was focused upon the constituents of connective tissue, principally chondroitin sulfate and hyaluronic acid, but neither of these substances nor their products of degradation could be causally related to the sticking of white cells.[4]

The consistent failure to make progress in this field with experiments based upon intact animals underlined the need for a less complex experimental model. In order to satisfy this requirement, a suitable *in vitro* system should not only be more exact but also lend itself to simple modification in order to expedite the systematic sorting out of facts bearing on cellular adhesion. Of particular relevance to this question, Garvin [2] devised an *in vitro* technique for estimating the adhesiveness

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of white cells contained in whole venous blood obtained from healthy human subjects. Under these circumstances, it was found that the sticking of human leukocytes and platelets to glass beads was not diminished by an anticoagulant such as heparin. On the other hand, deionization accomplished by passing whole blood through an ion exchange column almost completely inhibited cellular adhesion. Restoration of either calcium, magnesium, or both cations to the system restored the adhesive properties of cells. However, of several metabolic inhibitors examined, only high concentrations of iodoacetamide prevented leukocytes from sticking.[2] Under somewhat different conditions Nelson and colleagues [5,6] observed that an intact system of complement was required before bacterial particles adhered to either erythrocytes or platelets prior to their being phagocytosed. Further, both magnesium and calcium ions were required for completion of the immune adherence phenomenon. Circumstantial data of this sort suggests that an enzymatic mechanism may lead to the adhesion of leukocytes during inflammation.

In the experiments to be reported, the stickiness of leukocytes from the peritoneal exudate of rabbits was investigated, using an *in vitro* model. It was found that white cells mixed with rough strain pneumococcus in rotating tubes promptly exhibited phagocytosis and irreversible clumping, and agglutination of cells developed shortly thereafter. Separation of phagocytosis from cellular clumping proved difficult since modification of one condition almost always changed the other. There was evidence, however, to support the thesis that adhesion of cells depended upon an enzymatic mechanism. This may well represent a proteolytic system since the clumping of cells was blocked by epsilon aminocaproic acid, a known enzyme antagonist.

Material and Methods

Leukocyte Preparation

Adult male rabbits of Flemish-New Zealand stock obtained from Eldridge Rabbitry, St. Louis, Missouri, served as sources of peritoneal exudate. Animals were each given 400 ml. 0.1 per cent shell fish glycogen (Mann Research Labs, Inc., New York) intraperitoneally in pyrogen-free isotonic saline; 18 hours later exudate from the sterile inflammatory reaction was harvested after sacrifice by intravenous pentothal sodium.[7] The occasional clots of fibrin and cellular clumps encountered during recovery of exudate were removed by straining through sterile gauze. Heparin sodium (Upjohn Company, Kalamazoo, Michigan) was added to exudates in a final concentration of 4 units per ml. unless stated otherwise. Enumeration of exudate cells, in a Neubauer bright line counting chamber, revealed an average of 5.9×10^6 leukocytes per ml. Differential cell counts were made from Wright-stained smears. At least 90 per cent of cells were polymorphonuclear granulocytes (PMNG), and viability upon removal was greater than 99 per cent as determined by supravital preparations stained with trypan blue. Exudate for experiments was neither concentrated nor washed but used within 15 minutes after the donor rabbit was killed.

Bacterial Preparation

A rough strain pneumococcus (R36NC), obtained from Dr. W. B. Wood, Jr., Department of Microbiology, Johns Hopkins University School of Medicine, was maintained in defibrinated rabbit blood at 4° C. until used. Unless stated otherwise, a 15-hour culture of pneumococcus in brain heart infusion medium (Difco) was used. In several experiments, as described in the text, 4-hour cultures of rough pneumococcus were employed to insure high levels of bacterial viability. The cultures were concentrated by centrifugation at 2,000 r.p.m. for 30 minutes at 4° C. (PR2 model International Centrifuge, head no. 253) in sterile plastic tubes. After the supernatant was decanted, bacteria were washed in Na/K saline and resedimented as before. As soon as the growth pattern was found to yield consistent numbers of bacteria, measured in a Petroff-Hausser bacteria counting chamber, the procedural step of direct counts was eliminated in order to conserve time.

Cell-Bacterium Mixtures

In a modification of methods from Wood,[8] sufficient bacteria were added to the exudate to give a leukocyte-bacterium ratio of approximately 1 to 10. The mixtures in a volume of either 5 or 10 ml. were placed in leak-proof, screw-capped, siliconized glass tubes for incubation. Immediately after mixtures were prepared, tubes were placed horizontally in a circular rack for mixing by rotation at 9 r.p.m. for 30 minutes at specified temperatures. As described in some experiments, mixing by hand inversion every 30 seconds was also used. At predetermined intervals, tubes of the mixture were removed for macroscopic and microscopic study. Supravital preparations stained with trypan blue were examined to determine viability of PMNG. The intensity of phagocytosis was determined with methylene blue and Wright-stained smears. In general, phagocytosis was estimated from study of both unaggregated and clumped PMNG. An exact enumeration of phagocytosis was usually not possible since single cells were scarce and the cellular aggregates were too dense for accurate counting. The intensity of PMNG clumping was estimated macroscopically and from wet-mount cover-slip preparations.

Reagents and Chemicals

Deionization was accomplished with either disodium ethylenediaminetetraacetate (EDTA) prepared in sterile 1 per cent solution (J. T. Baker Chemical Company, Phillipsburg, New Jersey) or with a cation exchange resin, (Fenwal Laboratories, Inc., Framingham, Massachusetts). The pyrogen-free resin was supplied in sterile packets for aseptic handling. It was a Dowex sulfonated polystyrene divinyl benzene copolymer in the sodium cycle which sequestered calcium, magnesium and potassium ions. Approximately 98 per cent of calcium and magnesium ions and 80 per cent of potassium ions were said to be removed from blood plasma; whereas sodium was increased by 2 per cent.

All glassware was rendered sterile and pyrogen-free by heating to 170° C. for 2 hours. Likewise, the glassware, including slides, cover slips, test tubes, etc., and needles were siliconized with Dri-film SC-77 (General Electric Company) before use. Solutions were prepared in sterile, nonpyrogenic Na/K saline. Whenever possible, only pyrogen-free reagents were used, but a regular check for pyrogenicity was not performed. As described in the text, some solutions were buffered with Tris buffer, according to Gomori,[9] and with glycine-sodium hydroxide and also glycine-hydrochloric acid buffers, according to Sorenson.[9]

The proteolytic enzyme inhibitors were soybean trypsin inhibitor (SBTI) (Worthington Biochemicals Corporation, Freehold, New Jersey) and epsilon aminocaproic acid (EACA) (Aldrich Chemical Company, Milwaukee, Wisconsin). EACA for intravenous infusion, pyrogen-free, was in sterile ampuls (generously

provided by Dr. J. M. Ruegsegger, American Cyanamid Company, Pearl River, New York). Proteolytic enzymes employed included varidase with 100,000 units streptokinase and at least 25,000 units streptodornase per vial (American Cyanamid Company), lyophilized crystalline trypsin with 125,000 Armour units per vial (Tryptar, Armour Laboratories, Chicago, Illinois) and lyophilized hyaluronidase with 150 turbidity reducing units (TRU) per vial (Infiltrase, Armour Laboratories). Other reagents were sodium fluoride, magnesium chloride, nickel chloride (J. T. Baker Chemical Company), L-cysteine, L-lysine ethylester dihydrochloride, p-tosyl-L-arginine methylester hydrochloride (free base) (TAME), n-acetyl-L-tryptophan, n-acetyl-DL-tryptophan, L-canavanine sulfate, n-acetyl-L-tyrosine ethylester, p-chloromercuric benzoic acid sodium salt (PCMB), reduced glutathione (Mann Research Labs, Inc.), 2,4-dinitrophenol (Eastman Kodak, Rochester, New York), and sodium cyanide (Vaughan, Inc., Memphis, Tennessee).

Chemical Measurements

Calcium levels of exudate samples were performed either by the phosphate method of Roe and Kahn [10a] or by the Ferro and Ham chloranilate method,[11] using Dade Spectro Calcium kit reagents (Scientific Products, Evanston, Illinois). Magnesium contents of exudate samples were measured by either the phosphate method of Denis [10b] or the titan yellow method modified from Neill and Neely.[12] Hydrogen ion concentrations of exudate were measured with a Beckman Zeromatic pH meter.

RESULTS

In Vitro Experiments

Characteristics of Rabbit Peritoneal Exudate Cells. The prompt addition of heparin to exudate, 4 units per ml., prevented formation of fibrin strands that otherwise tended to sieve out many cells. Although not encountered commonly, exudate recovered from some animals contained more fibrin than that from others, but rarely was there an amount sufficient to interfere with experimentation. If allowed to stand for 2 hours without rotation at 25° C., the leukocytes settled to the surface of the glass tube, became firmly adherent, and could be dislodged only by direct scraping. After rotational mixing for 30 minutes, the PMNG remained individually dispersed, did not stick to surfaces of the tube, and only a few loose aggregates were encountered on completion of the process. Exudates from some animals tended to form these loose aggregates more than others, and this characteristic seemed directly related to the content of fibrin. Trypan blue stains of both individual and loose clumps of cells indicated that viability was unimpaired by the procedure.

Characteristic of Bacterium-Exudate Mixture. Addition of rough strain pneumococcus to heparinized exudate followed by rotation at 37° C. changed the behavior of the PMNG dramatically. Within 5 minutes, there was extensive phagocytosis of pneumococci, and the formation of tight clumps of cells containing organisms was already under way. This could be detected macroscopically as well as micro-

scopically. Adhesiveness of PMNG determined by the formation of tight clumps of cells was graded from 1 to 4 plus, depending upon the approximate numbers incorporated into the agglutinated masses. Thus, involvement of up to 25 per cent of PMNG in clumping was graded as 1 plus, up to 50 per cent as 2 plus, up to 75 per cent as 3 plus, and up to 100 per cent as a 4 plus reaction. It should be noted that mononuclear cells participated not at all in this reaction. Following 15 minutes of mixing, tubes of exudate containing pneumococcus were easily identified from control preparations because of the large aggregates of tightly agglutinated PMNG. Phagocytosis by this time was assumed to be better than 95 per cent but could not be accurately documented within the densely packed masses of cells. Further rotation for 30 minutes resulted in the formation of large wads of agglutinated cells, with very few individual PMNG found in the supernatant. An accurate assay of cellular viability of the leukocytic clumps could not be made, in view of their dense structure. From Wright stains of the dense cellular clumps, the PMNG on the periphery contained ingested bacteria, and a high degree of granularity was observed. In the interior of these clumps, however, a marked loss of cytoplasmic granules was noted, and from histologic sections it was determined that extensive vacuolation had occurred. These morphologic changes are not the subject of this report and will be discussed elsewhere from an electron micrographic point of view.[13]

Influence of Physical Factors on the Phagocytosis-Clumping Reaction. In view of the extraordinary rapidity of the clumping reaction that followed phagocytosis, it was of interest to determine the effect of rotational rate, temperature and pH on the reaction. As in the preceding section, exudate for each experiment was heparinized when harvested and before addition of pneumococcus.

By varying the rate of rotation, it was found that phagocytosis and clumping of PMNG was less extensive and developed more slowly when the mixing rate was 2 cycles per minute. Paired samples rotated at a rate of either 4 or 9 cycles per minute revealed more vigorous phagocytosis, and larger cellular aggregates were formed as the rate of mixing was increased. Mixing by hand at a rate of 9 inversions per minute produced less clumping of cells than was produced by the mechanical rotator at identical temperatures, and was thought to reflect differences of efficiency in the mixing procedure.

Temperature also influenced the reaction rate of both phagocytosis and the clumping reaction (Table I). Little or no phagocytosis or clumping was observed at 4° C. although by 60 minutes of mixing, microscopic evidence for both was found. Only minor differences in the

intensity of the 2 reactions were found at 25°, 37°, 40° and 50° C. although at the higher levels it was thought both developed more rapidly. Phagocytosis and loose clumping of cells was found at 54.5° C., but at this temperature viability diminished rapidly, and it was felt that both clumping and phagocytosis began immediately after preheated exudate

TABLE I

INFLUENCE OF TEMPERATURE UPON PHAGOCYTOSIS AND ADHESIVENESS OF RABBIT LEUKOCYTES OBTAINED FROM PERITONEAL EXUDATE AFTER MIXING WITH ROUGH STRAIN PNEUMOCOCCUS FOR 30 MINUTES

Temperature of exudate (° C.)	Phagocytosis by PMNG	Microscopic clumping by PMNG
4.0	Low	+
24.5	High	++++
37.0	High	++++
42.0	High	++++
54.5	Moderate	Loose aggregates

TABLE II

INFLUENCE OF HYDROGEN ION CONCENTRATION UPON PHAGOCYTOSIS AND ADHESIVENESS OF RABBIT LEUKOCYTES OBTAINED FROM PERITONEAL EXUDATE MIXED WITH ROUGH STRAIN PNEUMOCOCCUS FOR 30 MINUTES AT 37° C.

Condition of experiment	pH of buffered exudate and pneumococcus combination: Before rotational mixing	pH of buffered exudate and pneumococcus combination: After 30 min. mixing	Phagocytosis by PMNG	Microscopic clumping by PMNG
Control	7.80	8.00	high	++++
Added 1.0 N HCl*	4.80	6.00	low	++
"	5.80	6.40	moderate	++++
"	6.80	7.50	high	++++
Added 1.0 N NaOH	8.80	8.20	high	++++
Control	7.70	7.80	high	++++
Tris buffer † (5.2)	5.70	5.90	very low	++
" (6.2)	6.35	6.50	high	++++
" (7.2)	7.30	7.30	high	++++
" (8.2)	8.20	8.10	high	++++
" (8.6)	8.50	8.50	high	++++
Control	7.40	7.60	high	++++
Glycine buffer † (2.6)	4.45	4.65	none	o
" (3.6)	6.75	7.05	high	++++
" (8.6)	8.20	8.20	high	++++
" (9.6)	9.30	9.20	high	+++
" (10.6)	9.90	9.85	low	o

* Either 1.0 N HCl or NaOH was added to achieve desired initial pH of exudate.

† A volume of 10 ml. of either Tris or glycine buffer prepared to the pH indicated in parentheses was added to an equal volume of iced exudate before pneumococcus was introduced.

and pneumococcus were combined, but the high temperature inhibited the reactions in a short time.

A selected series of buffers were next added to exudate in order to determine the influence of pH on both reactions (Table II). Most exudates were found to have a pH ranging from 7.4 to 7.8 after addition of heparin. Despite drastic changes of pH accomplished with the buffers, phagocytosis and leukocytic clumping were vigorous over a broad range. Both were blocked at pH 4.45 although the cells appeared viable by trypan blue staining and a return of activity was noted in the pH range of 4.8 to 5.7. Nearer neutrality and above, the reactions were vigorous up to a high of pH 9.3. Above this level, denaturation of protein killed the cells.

Effect of Divalent Cations on Phagocytosis and Clumping. Both Brittingham [14] and Garvin [2] found that deionization of systems containing white cells substantially reduced the adhesiveness of PMNG to foreign surfaces as well as to each other. Accordingly, a 1 per cent solution of EDTA was used in a ratio of 1 in 9, and a chemical assay revealed no calcium or magnesium in the system. Trials with less concentrated preparations of EDTA were not as effective. Under these circumstances, little or no phagocytosis was found and clumping of PMNG was blocked completely even though cellular viability remained high. When calcium chloride was added to the deionized system in amounts sufficient to saturate the binding sites of EDTA and also restore concentrations to physiologic levels, both phagocytosis and cellular clumping were restored. Chemical measurement revealed low levels of magnesium ion as well as near physiologic concentrations of calcium. Substitution of equimolar amounts of magnesium chloride instead of calcium chloride to replace divalent cations to the system restored phagocytosis and clumping by white cells but did not exceed activity produced by calcium replacement. When magnesium was used as in this instance, calcium could not be detected in the system, yet cellular viability remained high.

A second approach to this problem involved deionization with a cation exchange resin. Immediately after the peritoneal cavity of the donor rabbit was opened, 10 ml. aliquots of exudate were mixed with 20 gm. of resin; heparin was not added. Gentle but thorough mixing by hand inversion every 5 seconds for 10 minutes at 25° C. assured complete extraction of cations with minimal trauma to cells. The beads of resin settled after 5 to 10 seconds, leaving a supernatant with only slightly fewer PMNG in suspension than were present before the extraction procedure. No calcium or magnesium ions could be detected in the supernatant following extraction (Table III). After addition of pneumococcus to exudate treated with cation exchange resin, phago-

TABLE III

INFLUENCE OF DIVALENT CATIONS UPON PHAGOCYTOSIS AND ADHESIVENESS OF RABBIT LEUKOCYTES OBTAINED FROM PERITONEAL EXUDATE MIXED WITH ROUGH STRAIN PNEUMOCOCCUS FOR 30 MINUTES AT 37° C.

	One per cent EDTA *						Cation exchange resin †						
	Experiment no.				Phagocytosis by PMNG	Microscopic clumping by PMNG	Experiment no.					Phagocytosis by PMNG	Microscopic clumping by PMNG
	1	2	3	4			31	32	33	34	30 π		
MAGNESIUM LEVELS													
Control exudate (Meq/l.)	0.48	0.24	0.48	0.52	high	++++	2.0	0.4	0.4	0.9	0.4	high	++++
Deionized exudate (Meq/l.)													
After deionization procedure	0	0	0	0	low	++++	0	0	0	0	0	low	0
After $CaCl_2$ added ‡	0.20	0.10	0.16	0	high	0	0	0	0	0	0	moderate	+++
After $MgCl_2$ added §	> 3.6	> 3.6	> 3.6	3.1	high	++++	2.6 π	1.2	0.9	1.4	1.4	high	++++
After $MgCl_2$ and $CaCl_2$ added ¶	N.D.	N.D.	N.D.	N.D.	high	++++	2.6 π	1.1	1.0	0.85	1.5	high	++++
CALCIUM LEVELS													
Control exudate (Meq/l.)	4.8	5.0	6.5	6.7	high	++++	4.35	3.8	3.5	4.3	4.5	high	++++
Deionized exudate (Meq/l.)													
After deionization procedure	0	0	0	0	low	0	0	0	0	0	0	low	0
After $CaCl_2$ added ‡	5.5	7.2	8.0	8.0	high	++++	8.0	4.8	4.9	4.5	6.0	moderate	+++
After $MgCl_2$ added §	0	0	0	0	high	++++	0 π	0	0	0	0	high	++++
After $MgCl_2$ and $CaCl_2$ added ¶	N.D.	N.D.	N.D.	N.D.	high	++++	7.6 π	4.9	4.8	5.2	6.0	high	++++

* Measurements of calcium and magnesium in these experiments were made by phosphate methods of Roe and Kahn [10a] and Denis [10b] respectively.

† Calcium levels were determined by the Ferro and Ham [11] method and magnesium by the technique of Neill and Neely.[12]

‡ 0.5 ml. 0.025 M $CaCl_2$ added to 4.5 ml. exudate.

§ 0.5 ml. 0.03 M $MgCl_2$ added to 4.5 ml. exudate.

¶ 0.5 ml. 0.025 M $CaCl_2$ and 0.1 ml. 0.03 M $MgCl_2$ added to 4.5 ml. exudate.

π 0.1 ml. 0.03 M $MgCl_2$ added to exudate deionized by cation exchange resin.

cytosis and clumping of PMNG were inhibited significantly but not as completely as achieved with EDTA. Restoration of calcium and magnesium to the system either singly or together revealed the two together to be most effective in bringing back both phagocytosis and leukocytic clumping. It was of interest to find that magnesium exceeded calcium when used alone in this regard.

Influence of Other Cations. In view of the requirement for either magnesium or calcium ions for PMNG obtained from rabbit peritoneal exudate to demonstrate both phagocytosis and adhesiveness, it was of interest to determine whether other cations were as essential. In the experiments summarized in this section, a series of monovalent, divalent, and trivalent cations were added separately to peritoneal exudate previously deionized by mixing with cation exchange resin. It was apparent that none of the monovalent ions were effective in restoring adhesiveness of PMNG even though phagocytosis of moderate intensity was present (Table IV). Unfortunately, several of the cations, particularly silver and copper, were of low solubility and were associated with toxic changes in both pneumococcus and leukocytes.

TABLE IV

INFLUENCE OF VARIOUS CATIONS UPON THE PHAGOCYTOSIS AND ADHESIVENESS OF RABBIT LEUKOCYTES OBTAINED FROM PERITONEAL EXUDATE MIXED WITH ROUGH STRAIN PNEUMOCOCCUS FOR 30 MINUTES AT 37° C.*

Source of cation †	Molarity of cation source	Final concentration of cation source	Cellular viability as per trypan blue	Phagocytosis by PMNG	Microscopic clumping by PMNG
AgCl	0.05	0.005	90%	moderate	+
CuCl	0.05	0.005	90	none ‡	o
HgCl	0.05	0.005	75	very low	+
NaCl	0.155	0.016	95	moderate	+
$CaCl_2$	0.025	0.0025	95	moderate to high	++ to +++
$CdCl_2$	0.025	0.0025	90	low to moderate	+ to ++
$FeCl_2$	0.025	0.0025	95	low	+
$MgCl_2$	0.03	0.003	95	high	+++ to ++++
$MnCl_2$	0.025	0.0025	90	high	++++
$NiCl_2$	0.025	0.0025	95	high	+++ to ++++
$PbCl_2$	0.025	0.0025	90	moderate	++ to +++
$AlCl_3$	0.016	0.0016	90	low	++
$FeCl_3$	0.016	0.0016	95	moderate	++++
$HAuCl_3$	0.005	0.0005	85	none	+
Deionized control			95	moderate	+

* Exudate deionized by mixing with cation exchange resin.

† Cations dissolved in distilled water and 0.5 ml. solution added to 4.5 ml. exudate before mixed with bacteria.

‡ Pneumococcus agglutinated.

Addition of divalent cations to exudate elicited a different response, but it was not uniform (Table IV). In general, adhesiveness of PMNG was closely associated with phagocytosis. Magnesium, manganese and nickel salts exhibited most activity, whereas divalent iron and cadmium salts were least active. It was somewhat surprising under the conditions of these experiments to find that calcium chloride was not as efficient as some other cation preparations.

Trivalent iron was more effective in restoring adhesiveness of PMNG than ferrous chloride, and once again, this effect was associated with vigorous phagocytic activity. Aluminum was less active than ferric chloride, and the other trivalent ion, gold, was least effective since no phagocytosis was seen.

These results seemed to indicate that the various cations exerted a rather nonspecific mass action effect on this aspect of cellular behavior, perhaps involving several enzymes rather than acting as a highly specific cofactor for a single system.

Influence of Complement. Since the results with divalent cations in the preceding section suggested that one or more enzymes figured in both phagocytosis and PMNG sticking, it became of interest to investigate the systems that might bear on the question. Of enzymes known to require divalent cations that in turn might figure in phagocytosis, complement was an obvious prime prospect. To cite a relevant example, Nelson and co-workers [6] defined rather clearly the essential role of complement in both the immune adherence reaction of bacteria and in the clumping together of platelets contained in citrated blood upon exposure to antigen-antibody complexes. Exclusion of complement prevented adhesion by these particles, whereas restoration of complement was attended by a return of clumping activity.[6] The next series of experiments were designed to determine if complement was required for phagocytosis and clumping of PMNG exposed to pneumococcus in our system.

We found the activity of complement in serum from rabbits and in peritoneal exudate to be too low for measurement by the conventional hemolytic assay method. Accordingly, fresh guinea pig serum was used to fortify the system under study. Heating at 56° C. for 20 minutes or longer inactivated the hemolytic activity of guinea pig serum and presumably the same components of complement in material obtained from rabbits.

In the first series of experiments, peritoneal exudates from 9 rabbits were examined. EDTA was added as before. The white cells were separated by centrifugation at 800 r.p.m. at 4° C., and the supernatants were heated for 20 to 60 minutes at 56° C. Fresh cells from a second

rabbit and the heated supernatant were then recombined to the original volume and then rough pneumococcus was added for rotational mixing at 37° C. Either calcium or magnesium chloride was added to the system in sufficient quantity to saturate the EDTA and restore physiologic levels of cation to the system. It was evident that even though phagocytosis by PMNG was suppressed, the heating procedure influenced clumping very little (Table V). Furthermore, restoration of

TABLE V

INFLUENCE OF COMPLEMENT ON THE PHAGOCYTIC AND ADHESIVE PROPERTIES OF RABBIT LEUKOCYTES OBTAINED FROM PERITONEAL EXUDATE MIXED WITH ROUGH STRAIN PNEUMOCOCCUS FOR 30 MINUTES AT 37° C.

EDTA-treated suspending medium	Cellular viability by trypan blue staining	Phagocytosis by PMNG	Microscopic clumping by PMNG
Control exudate	99%	very low	0
Control exudate plus $CaCl_2$ or $MgCl_2$	99	high	++++
Exudate heated 60 min., 56° C.*	99	low	++++
Exudate heated 60 min., 56° C., but 1.0 ml. fresh heated guinea pig serum added to 5 ml.*	99	low	++++
Exudate heated 60 min., 56° C., but 1.0 ml. fresh unheated guinea pig serum added to 5 ml.*	99	high	++++
Fresh unheated autologous rabbit serum †	99	high	++++
Fresh heated autologous rabbit serum †	99	moderate	++++

* 0.5 ml. 0.025 M $CaCl_2$ or 0.5 ml. 0.03 M $MgCl_2$ added to 4.5 ml. exudate supernatant.
† 2.0 ml. 0.025 M $CaCl_2$ added to 4.5 ml. serum to saturate the 0.25 ml. EDTA in 5 ml. serum.

both calcium and magnesium ions failed to restore the phagocytic activity. On the other hand, when fresh unheated guinea pig serum was added to the system, phagocytosis was enhanced appreciably. Incorporation of heated fresh guinea pig serum into the system, however, failed to restore the levels of phagocytosis to that of untreated exudate. This evidence suggested that complement was required for phagocytosis of rough pneumococcus but was not essential for cells of peritoneal exudate to adhere to each other. Unfortunately, such sharp separation of the two phenomena was not achieved when complement was inactivated by prior acidification of exudate. In this instance, phagocytosis was not predictably suppressed even though there was brisk clumping by leukocytes.

Influence of Metabolic Inhibitors. Fisher and Ginsberg [15] found that ingestion of influenza virus by leukocytes obtained from guinea pigs completely inhibited subsequent phagocytosis of yeast cells. This was shown to result from the blockade of intracellular glycolytic activity and could be duplicated by using receptor destroying enzyme (RDE) which blocked glucose phosphorylation. Along similar yet somewhat different lines, Philipson and Choppin [16] found that fixation of enteroviruses to erythrocytes depended upon intact viral terminal SH groups and that prior treatment with para-chloromercuric benzoic acid sodium salt (PCMB) blocked attachment to red cells by these viruses. Garvin [2] also reported that iodoacetamide prevented leukocytic sticking to glass beads when used in a concentration of 4×10^{-3} M. In the light of evidence of this sort, the influence of a series of metabolic blockers on leukocytic phagocytosis and adhesiveness was investigated.

The results of these experiments have been summarized in Table VI.

TABLE VI

INFLUENCE OF METABOLIC INHIBITORS UPON PHAGOCYTOSIS AND ADHESIVENESS OF RABBIT LEUKOCYTES OBTAINED FROM PERITONEAL EXUDATE MIXED WITH ROUGH STRAIN PNEUMOCOCCUS FOR 30 MINUTES AT 37° C.

Inhibitor	Final concentration used (Moles)	Viability by trypan blue staining (%)	Phagocytosis by PMNG	Microscopic clumping by PMNG
Iodoacetamide	4×10^{-3}	0–10	0	0
	4×10^{-4}	50–80	high	++
	4×10^{-5}	50–95	high	++ to +++
	4×10^{-6}	90–95	high	++++
	4×10^{-7}	99	high	++++
Dinitrophenol	0.4×10^{-1}	0–50	0	0
	0.4×10^{-2}	50–85	low to moderate	++ to +++
	0.4×10^{-3}	85–90	high	++++
Sodium cyanide	4×10^{-1}	0	0	0
	4×10^{-2}	50	0	0
	4×10^{-3}	85–90	moderate	++++
Parachloromercuric benzoic acid (PCMB)	1×10^{-2}	0–10	0	0
	1×10^{-3}	40	0	0
	1×10^{-4}	90	high	++++
PCMB plus glutathione 1×10^{-2} M	1×10^{-2}	0	0	0
	1×10^{-3}	80	0	0
	1×10^{-4}	95	high	++++
Glutathione	1×10^{-2}	95	high	++++
Sodium fluoride	2×10^{-1}	25	0	0
	2×10^{-2}	50	low	0
	2×10^{-3}	75	high	++++
	2×10^{-4}	99	high	++++
	to 2×10^{-9}	99	high	++++

The first 4 compounds tested were added directly to the heparinized leukocyte-pneumococcus combination before rotational mixing was begun. An iodoacetamide concentration of 4×10^{-3} M killed most PMNG as determined by trypan blue supravital stains and prevented both phagocytosis and clumping. Concentrations of 4×10^{-4} and 4×10^{-5} varied in lethality and interfered with the reactions, whereas at 4×10^{-6} M, both phenomena were recorded and the white cells appeared viable and otherwise unaltered. When dinitrophenol (DNP) was used, the results were quite similar to those obtained with iodoacetamide except that the concentration lethal for PMNG was 0.4×10^{-1} M. Only after leukocytes were killed by exposure to DNP were both phagocytosis and clumping prevented. Approximately the same results were recorded after cells from exudate were exposed to sodium cyanide, and once again only concentrations lethal to PMNG prevented phagocytosis and clumping.

The next two compounds (Table VI) were added to heparinized exudate and mixed by rotation for 30 minutes at 37° C. before the pneumococcus suspension was added. Para-chloromercuric benzoic acid sodium salt (PCMB) killed all PMNG exposed to 1×10^{-2} M and about 50 per cent of cells subjected to 1×10^{-3} M. In more dilute solutions of PCMB all leukocytes were viable and phagocytosis and clumping were unimpaired. It should be noted that addition of glutathione to the preparations failed to reduce the lethal effect of PCMB or otherwise alter the behavior of the exudate cells. Sodium fluoride in less than lethal concentrations, below 2×10^{-3} M, did not impede either phagocytosis or clumping by PMNG.

Influence of Enzyme Inhibitors. Previous investigators have suggested that the earliest events of the inflammatory reaction involved unspecified proteolytic reactions.[17,18] Proof that this might be true was derived in one instance from *in vivo* studies with agents known to block proteolytic enzyme activity *in vitro*.[19] It thus seemed appropriate to study similar reagents in our experimental model. Two well known proteolytic enzyme inhibitors were investigated (Table VII). The first, soybean trypsin inhibitor (SBTI), was completely without biologic effect even though tested over a wide range of concentrations. The potency of the SBTI preparations was demonstrated *in vitro* by blocking the activation of plasmin by streptokinase. A second agent, epsilon aminocaproic acid (EACA) proved quite different because both phagocytosis and clumping were inhibited by concentrations varying from 0.3 M to 0.5 M, yet the viability of PMNG remained high. Both phenomena were recorded with increasing vigor at lower concentrations and little or no effect of EACA could be detected at 0.05 M and 0.01 M.

This result with EACA led to study of other agents known to have potential proteolytic blocking activity by their capacity to act either as competitive inhibitors or as substrate analogues in the reaction. One of these substances, p-tosyl-L-arginine methylester (TAME), has been reported to inhibit experimental inflammation but in our hands no evi-

TABLE VII

INFLUENCE OF ENZYME INHIBITORS UPON PHAGOCYTOSIS AND ADHESIVENESS OF RABBIT LEUKOCYTES OBTAINED FROM PERITONEAL EXUDATE MIXED WITH ROUGH STRAIN PNEUMOCOCCUS FOR 30 MINUTES AT 37° C.

Inhibitor *	Final concentration used (Moles/ml.)	Viability by trypan blue staining (%)	Phagocytosis PMNG	Microscopic Clumping by PMNG
Crystalline soybean trypsin inhibitor	0.0625 mg./ml. through	95	high	++++
	2.0 mg./ml.	95	high	++++
Epsilon aminocaproic acid	0.01	95	high	++++
	0.05	95	high	+++ to ++++
	0.1	95	low to moderate	++ to +++
	0.2	95	low to moderate	+ to ++
	0.3	95	o to low	o to +
	0.4	95	o to low	o to +
	0.5	95	o to low	o to +
P-tosyl-L arginine methylester	0.01	90	high	++++
	0.001	90	high	++++
L-cysteine	0.05	90	high	++++
	0.005	95	high	++++
	0.0005	95	high	++++
L-lysine-ethyl-ester-dihydrochloride†	0.05	90	low	+++
	0.005	90	high	++++
	0.0005	90	high	++++
N-acetyl-L-tryptophan	0.02	75	moderate	(? denatured)
	0.002	95	high	++++
N-acetyl-DL-tryptophan	0.02	95	high	++++
	0.002	95	high	++++
N-acetyl-L-tyrosine-ethyl ester	0.008	95	high	++++
	0.0008	95	high	++++
L-canavanine sulfate	0.0075	95	high	++++
	0.00075	95	high	++++

* Inhibitor: 0.5 ml. added to 4.5 ml. exudate.
† Final pH was below 7.

dence for interference with either phagocytosis or clumping by PMNG was found [20] (Table VII). It was also of interest that cellular viability remained high in all concentrations studied.

Influence of Proteolytic Enzymes. Because of the rather disappointing results obtained with most of the enzyme inhibitors, the experimental approach was reversed so as to determine the effect of several activated enzymes on these phenomena. This possibility seemed especially worth while with respect to trypsin since this agent has been used to dislodge and disperse sheets of tissue culture cells stuck to the glass surface of growth flasks. On the other hand, previous studies *in vivo* with two other enzymes, hyaluronidase and varidase (SK-SD), had proved disappointing since the sticking of leukocytes to damaged vascular endothelium was not found to be altered.[1,4] In the earlier studies, trypsin proved too traumatic for definitive microcirculatory studies and consequently was not subjected to the same scrutiny. The results of these studies are recorded in Table VIII.

In each instance, enzyme was added to heparinized exudate as dry powder and twofold dilutions were made. Neither hyaluronidase nor varidase in high concentrations altered either of the two phenomena. Trypsin, however, did change the aggregation of PMNG in two different patterns, depending upon the concentrations utilized. In the lower dilutions, beginning at 2.5 units per ml., trypsin induced substantial microscopic aggregation of PMNG before the bacterium was added to the mixture. At levels above 781 units per ml., this effect disappeared. After mixing with pneumococcus, these pre-clumped leukocytes phagocytosed briskly, became even more intensely aggregated and generally exhibited no ill effects from pretreatment with trypsin. Higher concentrations of trypsin, beginning at 6,250 units per ml., prevented even the slightest tendency for aggregation by cells before the addition of bacteria and also prevented in part phagocytosis and clumping after exposure to pneumococcus. Needless to say, trypan blue-stained smears indicated that most leukocytes were viable during the time of study. These results presumably reflected action by the proteolytic enzyme since addition of SBTI blocked all response to trypsin.

In Vivo Experiments

The Influence of Proteolytic Enzyme Inhibitors on the Sticking of Leukocytes to Injured Endothelium Within the Ear Chambers of Rabbits. After it was found that EACA prevented the clumping of PMNG exposed to pneumococcus *in vitro,* it was imperative that this agent be investigated in the microcirculation of the rabbit ear chamber. From studies reported by Lee [20] and Zweifach, Nagler and Troll,[19] as well as from extensive data derived from use in human beings, it was apparent that a wide margin of safety attended the intravenous administration of EACA. Our experiments were, therefore, directed only toward an

examination of the sticking reaction of leukocytes induced by heat injury to the ear chambers of rabbits.

In a series of 6 animals, sufficient EACA suitable for parenteral use was given by continuous infusion via the opposite ear to maintain blood levels of approximately 0.3 M or more for 6 to 7 hours. In this way it was possible to obtain satisfactory pre-injury observations for an hour or so in order to determine that EACA did not alter the circulation significantly and that the numbers of both circulating leukocytes and platelets remained stable. Following burn injury according to methods

TABLE VIII

INFLUENCE OF PROTEOLYTIC ENZYMES UPON THE PHAGOCYTOSIS AND ADHESIVENESS OF RABBIT LEUKOCYTES OBTAINED FROM PERITONEAL EXUDATE MIXED WITH ROUGH STRAIN PNEUMOCOCCUS FOR 30 MINUTES AT 37° C.

Enzyme used	Final concentration of enzyme	Viability by trypan blue staining (%)	Phagocytosis by PMNG	Microscopic clumping by PMNG
Bovine testicular hyaluronidase	0.117 TRU/ml. through			
	15.0 TRU/ml.	95	high	++++
Varidase	0.078 units SK/ml. through			
	10.0 units SK/ml.	95	high	++++
Trypsin	0.025 units/ml.	95	high	++++
	0.25 units/ml.	95	high	++++
	2.5 * units/ml.	95	high	++++
	25 * units/ml.	95	high	++++
	250 † units/ml.	95	high	++++
Trypsin	97 units/ml.†	95	high	++++
	195 units/ml.†	95	high	++++
	390 units/ml.†	95	high	++++
	781 units/ml.†	95	high	++++
	1562 units/ml.	95	high	++++
	3125 units/ml.	95	high	+++
	6250 units/ml.‡	95	high	++
	12500 units/ml.‡	95	high	++
Trypsin	195 † units/ml.	95	high	++++
	390 † units/ml.	95	high	++++
	781 * units/ml.	95	high	+++ to ++++
	1562 units/ml.	95	high	+ to +++
	3125 units/ml.	95	moderate	+ to ++
	6250 ‡ units/ml.	95	low	+
	12500 ‡ units/ml.	95	high	+
	25000 ‡ units/ml.	95	high	+

* Clumping of 2+ intensity developed after exposed to trypsin and before organisms added.

† Clumping of 3+ intensity developed after exposed to trypsin and before organisms added.

‡ Absolutely no leukocytic clumping was found before organisms added.

described earlier, the margination and sticking of leukocytes developed within 5 to 15 minutes and progressed in the same way as seen in untreated animals.[21] There was no impairment of leukocyte diapedesis or other exudative phenomena so that the inflammatory reaction was unchanged. This was so when as much as 6.0 gm. EACA was given each 2 hours in order to maintain concentration within the circulation of at least 0.5 M. As an aside, it was interesting to find a complete lack of influence of EACA upon the few thrombotic events noted with this preparation. Animals that received as much as 15.0 gm. EACA over 5 to 7 hours showed no immediate or delayed adverse reaction to the material.

In keeping with the above studies and also with observations recorded in the literature,[18] another proteolytic enzyme inhibitor, SBTI, was tested in the ear chamber preparation. Only two animals were examined with this agent since appreciable quantities of the crystalline enzyme inhibitor were prohibitively expensive. The first animal was given 0.6 gm. crystalline SBTI intravenously 1 hour before the ear chamber was damaged by heat. No reaction attended administration of the material and leukocytic sticking developed briskly after the trauma. In the second rabbit, 1.654 gm. crystalline SBTI was administered in the same manner and again, there was no discernible effect on the behavior of white cells after the ear chamber was damaged by heat.

Discussion

It was apparent that the clumping of PMNG, after the process of phagocytosis, depended upon an as yet unknown alteration of the cell membrane that appeared upon ingestion of particulate matter. A clear separation of phagocytosis from the aggregation of cells was achieved only with difficulty. This fact suggested that although the surface alterations involved in the two phenomena might be closely related, they were not necessarily identical. From a teleologic point of view, the close relationships of these events, i.e., phagocytosis and leukocytic aggregation, was not unexpected since the adherence of white cells to a stationary surface is almost always required before phagocytosis develops *in vivo*.[22] Even though it is tempting to extrapolate our results obtained *in vitro* to the endothelial sticking of leukocytes during inflammation, it must be recalled that the cells under study were obtained from peritoneal exudate and not from the bloodstream. This could mean that they had already undergone an irreversible physicochemical change that made their behavior unlike that of their intravascular counterparts. Such a possibility could not be discounted even though the PMNG performed vigorously as phagocytes. Finally, when the

in vitro findings were tested in rabbits equipped with plastic ear chambers, EACA failed either to inhibit the intravascular leukocytic sticking reaction or to modify inflammation induced by heat injury. Even so, information derived from study of exudate cells should assist in future experiments employing leukocytes derived from the circulating blood.

It was not surprising to find that certain divalent cations, particularly magnesium and calcium, were required for both reactions since enzymes require such ions as cofactors. Similar results were obtained with white cells from the venous blood of man by Garvin [2] and also with platelets from man by Zucker and Borrelli.[23] The notable lack of specificity for given ions, however, suggested that polymerization of fibrinogen to fibrin was not the principal mechanism concerned since this reaction is singularly dependent upon ionized calcium or strontium.[23] On the other hand, from results obtained with EACA, other enzymes could be implicated and a proteolytic system seemed most likely. A broad, rather nonspecific blockade of proteolytic activity would be likely, with the high concentrations of EACA required, but we have not been able so far to identify specific systems that might be involved.[24]

Our failure to confirm the results of Garvin,[2] wherein iodoacetamide in concentrations of 4×10^{-3} or more prevented adhesion of leukocytes from venous blood to glass beads, may have been due to differences in technique as well as in experimental species. In our hands, this concentration of iodoacetamide was lethal to PMNG from the exudate of rabbits and thereby precluded both phagocytosis and clumping. Sublethal amounts of iodoacetamide in no way modified the reaction. Similarly, negative results were also obtained with other agents previously reported to block adhesion of viral particles to cells. In particular, unlike the findings of Philipson and Choppin [16] with attachment of enteroviruses to erythrocytes, PCMB failed to alter either the ingestion of bacteria or the aggregation of cells unless lethal concentrations were used. Along somewhat different lines, lack of an effect by sublethal concentrations of dinitrophenol seemed to indicate that a glycolytic mechanism was not involved in the reaction. In this same general area, Fisher and Ginsberg [15] found that prior ingestion of influenza virus by phagocytes or treatment with receptor-destroying enzyme inactivated glucose phosphorylation and thereby prevented phagocytosis of bacteria. We have not as yet used this approach but plan to do so in the near future.

Most of our data tended to eliminate complement as a cofactor in this system. As a consequence, the combination of antigen and antibody to produce the immune adherence phenomenon described by Nelson and Nelson [5] did not appear to figure in the phagocytosis and clumping in our system. It must be remembered, however, that in view of the

minute quantities of reactants required for immunologic reactions and also because some of our data with complement were not entirely confirmatory, this mechanism should not be eliminated from consideration.

Finally, in keeping with the well-known effect of trypsin for dispersing sheets of tissue culture cells, it was relevant that high concentrations of this enzyme prevented clumping of leukocytes and reduced but did not eliminate the phagocytic process. The mechanism for this effect is not known although it must relate to surface structural features that the enzyme alters. Such a possibility seemed quite likely since low concentrations of trypsin had the opposite effect and enhanced both reactions.

From this point it may be permissible to postulate that injury leading to inflammation results in an enhanced level of proteolytic enzyme activity both within tissues and perhaps intravascularly. Much evidence can be cited to support this fact.[18] There may then follow an alteration of surface characteristics of both endothelium and circulating white cells that leads to increased adhesiveness and eventually full-fledged sticking. The same or similar substances might also increase platelet adhesiveness and simultaneously activate the conversion of fibrinogen to fibrin. Such a hypothesis would explain our *in vitro* results, but, so far, it has not been possible to obtain confirmation in living animals. The multiplicity of systems involved, receptive to different inhibitors, may explain some of our difficulty to date.

Although the effect of phagocytosis in bringing about clumping of leukocytes was not an original observation,[8] it was not widely known. Undoubtedly, this may be one of the mechanisms whereby leukocytes disappear from the bloodstream to give a transient, peripheral neutropenia during bacteremia or following administration of endotoxin. Presumably, phagocytosis of bacteria or macromolecules alters the cell membrane, leads to massive clumping of both PMNG and platelets, and these in turn become sequestered in the lungs and elsewhere at the height of the reaction. There may subsequently ensue intracellular degranulation, cytolysis, and in some instances release of endogenous pyrogen.

Summary

An *in vitro* system was devised in order to measure directly the adhesiveness of leukocytes involved in the acute inflammatory reaction. It was found that white cells obtained from the peritoneal exudate of rabbits clumped together rapidly upon ingestion of rough pneumococcus, and separation of the two phenomena proved difficult. The requirement for cations in the system suggested that an enzymatic mechanism was involved, but the lack of specificity for calcium seemed to elimi-

nate the clotting mechanism from consideration. Of the many substances tested, a proteolytic enzyme antagonist, epsilon aminocaproic acid (EACA), proved consistently effective in blocking the reactions. Low levels of trypsin activity accentuated both events, whereas high concentrations of trypsin inhibited them.

From these results it was postulated that injury activates dormant proteolytic systems as yet unidentified within tissue; these alter the surface characteristics of cells and lead to their increased stickiness. An attempt to test this thesis in the rabbit ear chamber preparation damaged by heat proved disappointing since neither EACA nor soybean trypsin inhibitor modified the inflammatory reaction.

References

1. Allison, F., Jr., and Lancaster, M. G. Studies on the pathogenesis of acute inflammation. II. The relationship of fibrinogen and fibrin to the leucocytic sticking reaction in ear chambers of rabbits injured by heat. *J. Exper. Med.*, 1960, **111**, 45–64.
2. Garvin, J. E. Factors affecting the adhesiveness of human leucocytes and platelets *in vitro*. *J. Exper. Med.*, 1961, **114**, 51–73.
3. Allison, F., Jr., and Lancaster, M. G. Studies on the pathogenesis of acute inflammation. III. The failure of anticoagulants to prevent the leucocytic sticking reaction and the formation of small thrombi in rabbit ear chambers damaged by heat. *J. Exper. Med.*, 1961, **114**, 535–554.
4. Allison, F., Jr.; Lancaster, M. G., and Baxter, M. D., Jr. Studies on the pathogenesis of acute inflammation. IV. The failure of papain and hyaluronidase to modify leukocytic sticking within rabbit ear chambers injured by heat. *Am. J. Path.*, 1962, **41**, 163–182.
5. Nelson, D. S., and Nelson, R. A., Jr. On the mechanism of immune adherence. I. Differentiation from acid-adhesion of bacteria to erythrocytes. *Yale J. Biol. & Med.*, 1959, **31**, 185–200.
6. Siqueira, M., and Nelson, R. A., Jr. Platelet agglutination by immune complexes and its possible role in hypersensitivity. *J. Immunol.*, 1961, **86**, 516–525.
7. Hirsch, J. G. Phagocytin: a bactericidal substance from polymorphonuclear leucocytes. *J. Exper. Med.*, 1956, **103**, 589–611.
8. Wood, W. B., Jr. Personal communication.
9. Gomori, G. Preparation of Buffers for Use in Enzyme Studies. In: Methods in Enzymology. Colowick, S. P., and Kaplan, N. O. (eds.). Academic Press, Inc., New York, Vol. 1, 1955, p. 138.
10. Levinson, S. A., and MacFate, R. P. Clinical Laboratory Diagnosis. Lea & Febiger, Philadelphia, 1956, ed. 5, (a) p. 300; (b) p. 372.
11. Ferro, P. V., and Ham, A. B. A simple spectrophotometric method for the determination of calcium. *Am. J. Clin. Path.*, 1957, **28**, 208–217.
12. Varley, H. In: Practical Clinical Biochemistry. Interscience Publishers, Inc., New York, 1958, ed. 2, p. 377.
13. Lockwood, W. R., and Allison, F., Jr. Unpublished data.
14. Brittingham, T. E. Observations on mechanism and prevention of non-

specific agglutination of leukocytes. *Proc. Soc. Exper. Biol. & Med.*, 1958, **99**, 252–257.

15. FISHER, T. N., and GINSBERG, H. S. The reaction of influenza viruses with guinea pig polymorphonuclear leucocytes. II. The reduction of white blood cell glycolysis by influenza viruses and receptor-destroying-enzyme (RDE). *Virology*, 1956, **2**, 637–655.
16. PHILIPSON, L., and CHOPPIN, P. W. On the role of virus sulfhydryl groups in the attachment of enterovirsuses to erythrocytes. *J. Exper. Med.*, 1960, **112**, 455–478.
17. UNGAR, G. The Fribrinolytic System and Inflammation. In: The Mechanism of Inflammation. An International Symposium. JASMIN, G., and ROBERT, A. (eds.). Acta, Inc., Medical Publishers, Montreal, 1953, pp. 151–156.
18. MILES, A. A., and WILHELM, D. L. Enzyme-like globulins from serum reproducing the vascular phenomena of inflammation. I. An activable permeability factor and its inhibitor in guinea-pig serum. *Brit. J. Exper. Path.*, 1955, **36**, 71–81.
19. ZWEIFACH, B. W.; NAGLER, A. L., and TROLL, W. Some effects of proteolytic inhibitors on tissue injury and systemic anaphylaxis. *J. Exper. Med.*, 1961, **113**, 437–450.
20. LEE, L. Reticuloendothelial clearance of circulating fibrin in the pathogenesis of the generalized Shwartzman reaction. *J. Exper. Med.*, 1962, **115**, 1065–1082.
21. ALLISON, F., JR.; SMITH, M. R., and WOOD, W. B., JR. Studies on the pathogenesis of acute inflammation. I. The inflammatory reaction to thermal injury as observed in the rabbit ear chamber. *J. Exper. Med.*, 1955, **102**, 655–668.
22. WOOD, W. B., Jr. Studies on the cellular immunology of acute bacterial injections. *The Harvey Lectures*, 1951–1952, **47**, 72–98.
23. ZUCKER, M. B., and BORRELLI, J. Some effects of divalent cations on the clotting mechanism and the platelets of EDTA blood. *J. Appl. Physiol.*, 1958, **12**, 453–460.
24. ALKJAERSIG, N.; FLETCHER, A. P., and SHERRY, S. ϵ-Aminocaproic acid: an inhibitor of plasminogen activation. *J. Biol. Chem.*, 1959, **234**, 832–837.