THE DEACTIVATION OF RABBIT NEUTROPHILS BY CHEMOTACTIC FACTOR AND THE NATURE OF THE ACTIVATABLE ESTERASE*

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A factor generated during the sequential interaction of the first seven of the nine components of complement (C′) is chemotactic for polymorphonuclear leukocytes (1–3). This factor, referred to as $C'(5, 6, 7)_a$, has been identified as the activated trimolecular complex consisting of the fifth (C′5), sixth (C′6), and seventh (C′7) components of complement. $C'(5, 6, 7)_a$ mediates the selective, unidirectional migration of polymorphonuclear leukocytes in vitro, and it may also be a mediator in immunologically induced acute inflammatory reactions involving vascular structures of the skin (4) and kidneys (5). In addition, a complement-associated chemotactic factor can be demonstrated for brief periods of time in the plasma of rabbits following injection of zymosan (2).

Organophosphorus inhibitors such as diisopropylphosphofluoridate or *p*-nitrophenyl ethyl phosphonates are specific, irreversible inhibitors of serine esterases. They inhibit the $C'(5, 6, 7)_a$-induced chemotaxis of rabbit polymorphonuclear leukocytes by two different mechanisms (6, 7). In the first, the leukocytes and organophosphorus inhibitor are incubated together for an appropriate period of time. After being washed free of inhibitor, the leukocytes are found to have lost their chemotactic responsiveness. This "cell-dependent inhibition" is believed to be caused by the irreversible inactivation by the organophosphorus inhibitor of an esterase existing in or on the cell in an already activated state (6). This so-called "activated esterase" has a distinct affinity for acetate esters, suggesting that it might be similar in its activity to an acetylase or acetyl esterase (7).

The second method of inhibition is to incubate the organophosphorus compound with neutrophils during the chemotactic assay. Under the proper experimental conditions, the resulting inhibition is due neither to an inactivation of the activated esterase nor to a direct action on the chemotactic factor. Rather, this so called "chemotactic factor–dependent inhibition" is the result of inhibition of a second esterase, the "activatable esterase." This latter esterase is present in or on the cell in a phosphonate-insusceptible form which is enzymatically inert. Contact of the leukocyte with the chemotactic factor leads to a conversion of the enzyme to an active serine esterase that is susceptible to the inhibitory action of the organophosphorus compound (6).

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The serine esterases, although a restricted class of enzymes, still comprise very many different kinds of esterases. In order to establish the biochemical role of the activatable esterase in chemotaxis, it will be necessary to isolate this enzyme in pure form. For this purpose, it is necessary that additional means of characterizing the enzyme be available. The furnishing of such possible further identifying characteristics for the activatable esterase is in part the subject of this paper.

Previous observations by one of us (P.A.W.) have indicated that incubation for appropriate periods of time of polymorphonuclear leukocytes with preparations containing $C'(5, 6, 7)_a$ resulted in an irreversible loss in the ability of the leukocytes to give a chemotactic response to $C'(5, 6, 7)_a$. We have termed the specific, irreversible loss of responsiveness induced in this manner "deactivation." The first part of this paper is devoted to showing that deactivation arises from the action of $C'(5, 6, 7)_a$ on the leukocyte. The second portion will demonstrate that the phosphonate esters prevent deactivation in a manner which indicates that the activatable esterase is involved in deactivation. Lastly it will be shown that simple aromatic amino acid derivatives specifically prevent deactivation and also inhibit chemotaxis, suggesting that the activatable esterase is a serine esterase with a particular affinity for compounds containing aromatic amino acids.

Materials and Methods[1]

Chemotaxis.—The ability of polymorphonuclear leukocytes (neutrophils) to migrate toward a concentration gradient of $C'(5,6,7)_a$ was measured by the use of chambers with micropore filters. Rabbit neutrophils were obtained from a glycogen-induced peritoneal exudate and suspended in 0.5% ovalbumin. Details of these techniques are described elsewhere (2, 6).

Activated Serum.—The complement-associated chemotactic factor was generated in fresh rabbit serum by the addition of immune complexes, followed by incubation at 37°C for 1 hr and removal of the precipitate by centrifugation (2). Rabbit IgG, obtained from high-titer antisera to bovine serum albumin (BSA), was purified by ion-exchange chromatography (4). In order to activate serum, and thus generate the chemotactic factor, 100 μg antibody N, with antigen at equivalence, was mixed with each 0.1 ml of fresh serum. The chemotactic factor was also generated by addition of zymosan to fresh rabbit serum (2). Unless otherwise indicated, "activated serum" refers to serum treated with an immune complex.

Isolation of $C'(5, 6, 7)_a$.—The partially purified nonactivated chemotactic factor, $C'(5, 6, 7)$, was obtained by eluting rabbit euglobulin from triethylaminoethylcellulose (TEAE-cellulose). The $C'(5, 6, 7)$ was then activated by incubation with sensitized erythrocyte stromata containing the first four reacting components of complement. These procedures have been described in detail in earlier reports (2, 3). $C'(5, 6, 7)$, not interacted with the first four complement components and therefore not chemotactically active, is referred to as the "nonactivated TEAE fraction." The material which has been reacted so as to make it chemotactically active [rich in $C'(5, 6, 7)_a$] is termed the "activated TEAE fraction."

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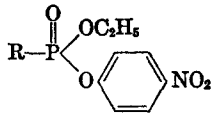
[1] The "Principles of Laboratory Animal Care" as promulgated by the National Society for Medical Research were observed during this study. Animal facilities were provided by the Animal Care Branch of the Armed Forces Institute of Pathology.

Phosphonate Esters.—The *p*-nitrophenyl ethyl phosphonates with the structure



in which R is either a phenylalkyl, alkyl, or chloroalkyl group, were the same as used previously (6). In what follows, the phosphonates will be named only according to the R group, the presence of the *p*-nitrophenoxy and ethoxy groups being implied. The phosphonates were dissolved in acetone and then appropriately diluted to a final concentration of 0.5% acetone. This concentration of acetone had previously been determined not to interfere with the chemotactic behavior of the leukocyte (6).

Other Esters.—The simple carboxylic acid esters, phosphate esters, and amino acid esters employed here were the same as used previously (7). They were dissolved in medium 199. In addition to these esters, additional amino acid esters were also employed as well as *N*-acetyl amino acids. These were all obtained from Mann Research Laboratories (N. Y.) and used in the same way as the others.

Deactivation of Leukocytes.—In most "deactivation" experiments leukocytes were incubated at room temperature for 30 min with a 30% (v/v) concentration of chemotactic factor. The final leukocyte suspension was 2×10^6 cells/ml. The source of the chemotactic factor, $C'(5, 6, 7)_a$, was either activated homologous serum or the activated TEAE fraction. After incubation at room temperature for 30 min, the leukocytes were then diluted 10-fold in medium 199, centrifuged, and resuspended in 10% fresh rabbit serum for testing of their chemotactic responsiveness in chambers. In some experiments, the concentration of chemotactic factor and duration of incubation were varied as indicated.

The inhibition of the cell responsiveness was calculated as one, minus the ratio of cell counts of responding cells that had been treated with deactivating factor to the number of responding cells that were not pretreated in this manner, all multiplied by 100.

Protection against Deactivation.—In all deactivation experiments in which phosphonate esters or amino acid, carboxylic acid, or phosphate esters were used, the source of chemotactic factor was the *activated TEAE fraction* (see above), in order to obviate the problem of the hydrolysis of the phosphonate compounds or esters by enzymes present in rabbit serum (8). In addition, the final suspending medium for the polymorphonuclear leukocytes was 0.5% ovalbumin (6). The source of chemotactic factor (present in the lower compartment of chambers) was rabbit serum activated by incubation with immune complexes. In those experiments in which the protective effects of the amino acid esters against deactivation were studied, one volume of cells (6×10^6/ml) was added to one volume of ester and one volume of chemotactic factor. The final concentration of the ester is expressed in the tables. For controls, cell suspensions were incubated with the esters alone and with chemotactic factor alone. Reaction volumes were always kept constant. When the phosphonate esters were tested for their ability to prevent deactivation of polymorphonuclear leukocytes by the chemotactic factor, they were used in concentrations sufficiently low so that no cell-dependent inhibition (see introduction) was evident. The ability of certain compounds to protect neutrophils from deactivation caused by contact with the chemotactic factor was calculated as the ratio of the inhibition of chemotactic responsiveness (deactivation) in the absence of phosphonates or amino acid esters, minus the inhibition of cell responsiveness in the presence of the protective agent, to the inhibition of chemotactic responsiveness in the absence of the protecting substance. This ratio, multiplied by 100, was expressed as "per cent protection." For example, if cells treated with chemotactic factor and then assayed in chemotactic chambers had 40% of the chemotactic response of untreated cells (that is, they were inhibited 60%), whereas cells

treated with chemotactic factor in the presence of phosphonate gave a 70% response compared with normal untreated cells, the degree of protection from deactivation effected by the phosphonate would be 100[(60-30)/60] or 50% protection.

RESULTS

Deactivation of Polymorphonuclear Leukocytes by Activated Serum.—When polymorphonuclear leukocytes were incubated for 30 min at room temperature with activated rabbit serum, the cells were no longer able to respond chemo-

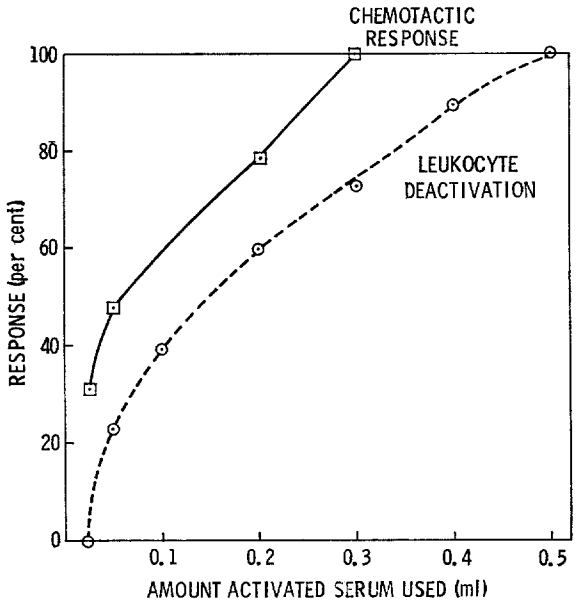


FIG. 1. Comparison of leukocyte-deactivating capacity and chemotactic activity of rabbit serum treated with immune complexes.

tactically, that is, they were "deactivated." The degree of deactivation was found to be a function of the concentration of activated serum: The larger the amount of serum employed, the greater was the deactivation (Fig. 1). Contact of cells for 30 min with 40% serum caused a 90% inactivation of polymorphonuclear leukocytes.

The treated serum was also tested for its chemotactic activity using untreated cells from the same batch. The chemotactic activity of the activated serum was directly proportional to its concentration (Fig. 1). The curves for change of chemotactic activity and leukocyte-deactivating activity with change of concentration of the same treated serum were closely parallel.

Deactivation of Polymorphonuclear Leukocytes by Serum as a Function of Time.—The ability of the same activated serum used in the above experi-

ment to cause deactivation of polymorphonuclear leukocytes was studied as a function of the duration of contact with cells. Deactivation of leukocytes was directly proportional to the duration of contact between cells and treated serum (Fig. 2). The curve of deactivation was linear during the first 30 min and then slowly leveled off. By 60 min all leukocytes had lost their chemotactic responsiveness. In this experiment, control cells were incubated for similar intervals with untreated rabbit serum; these cells did not lose their ability to respond to the chemotactic factor.

Ability of Partly Purified Chemotactic Factor to Deactivate Leukocytes.—Since it was considered likely that deactivation of polymorphonuclear leukocytes by

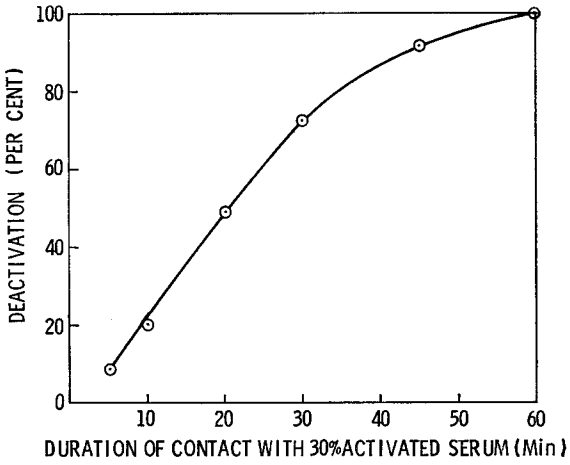


FIG. 2. Leukocyte-deactivating capacity of rabbit serum treated with immune complexes as a function of duration of incubation.

activated serum was due to the presence of the chemotactic factor, partially purified C′(5, 6, 7)$_a$ was used in experiments similar to those described above. The activated TEAE fraction, rich in the chemotactic factor, deactivated polymorphonuclear leukocytes to an extent proportional to the concentration of chemotactic factor, and the curve of deactivation paralleled the chemotactic activity (Fig. 3). In contrast, a fraction of nonactivated rabbit euglobulin, rich in C′(5, 6, 7), which was not chemotactically active, was incapable of deactivating leukocytes.

The degree of deactivation of leukocytes by the activated TEAE fraction also increased with duration of contact with the leukocytes (Fig. 4). The similarity in deactivating capacity and chemotactic activity of treated serum and the partly purified C′(5, 6, 7)$_a$, and the fact that the substances responsible for these two activities eluted from TEAE-cellulose under the same conditions

are strong evidence that these activities are in fact biologic properties of the same substance.

Failure to Generate Deactivating Factor in C'6-Deficient Serum.—The complement-associated chemotactic factor requires C'6 for its generation (2). The

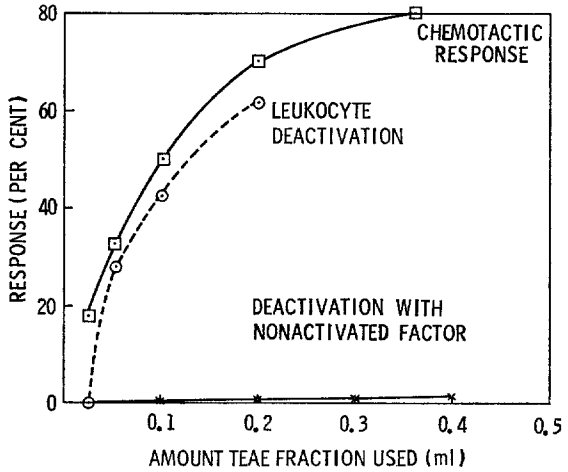


FIG. 3. Comparison of leukocyte-deactivating capacity and chemotactic activity with preparation rich in rabbit chemotactic factor. Nonactivated factor consists of C'(5,6,7) complex that has not reacted with prior complement components.

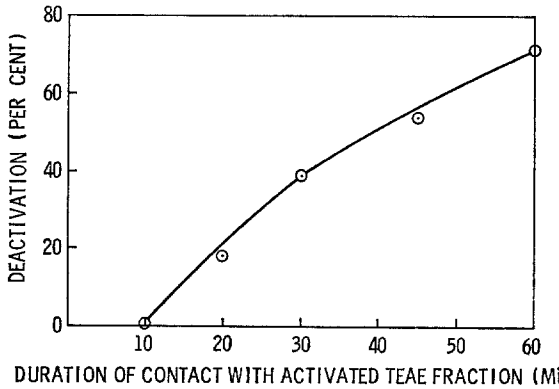


FIG. 4. Leukocyte-deactivating capacity of preparation rich in $C'(5,6,7)_a$, as a function of duration of contact with cells.

addition of zymosan or immune complexes to rabbit serum genetically deficient in C'6[2] failed to result in appearance of the chemotactic factor (2). This knowledge was applied in attempts to generate the leukocyte-deactivating factor in

[2] C'6-deficient rabbit serum was kindly supplied by Dr. Klaus Rother, Department of Pathology, New York University School of Medicine.

C'6-sufficient (normal) and C'6-deficient rabbit serum. The defective serum was found to be very poor in its ability to generate the deactivating activity (Table I), indicating that C'6 is required for the generation of the deactivating factor as well as the chemotactic factor.

Physical-Chemical Characteristics of Leukocyte-Deactivating Factor.—In order to obtain additional information on the relation of the deactivating factor to the chemotactic factor, serum was activated by incubation with immune complexes and then subjected to heating and dialysis. The effects of such treatment on chemotactic and deactivating abilities were then compared. Heating serum at 56°C for 1 hr caused only a 9% reduction in chemotactic activity and

TABLE I

Experiments A and B: Requirement for C'6 in the Generation of Leukocyte-Deactivating Factor

Experiment	Treatment of cells*	Leukocyte-deactivating capacity‡
A	Normal serum	0
	Normal serum activated with immune precipitate§	95
	C'6-deficient serum activated with immune precipitate	31
B	Normal serum	0
	Normal serum treated with zymosan‖	84
	C'6-deficient serum treated with zymosan	11

* 0.3 ml serum plus 1.5 × 10^6 PMN'S in a total volume of 1.0 ml incubated at room temperature for 20 min.

‡ Expressed as per cent reduction of chemotactic responsiveness of cells.

§ 0.1 ml serum plus 100 μgN antibody with antigen added at equivalence, in a final volume of 1.0 ml.

‖ 0.1 ml serum plus 10 mg zymosan in a final volume of 1.0 ml.

no loss of deactivating capacity (Table II). Similarly, dialysis had little effect on these two activities. These data on stability of the chemotactic factor and the leukocyte-deactivating factor, taken with the requirement for C'6 in the generation of both factors and the fact that both substances behave similarly in ion-exchange chromatography, indicate that the two factors are probably identical.

Ability of Phosphonate Esters to Protect against Deactivation.—The evidence just described makes it very likely that the action of the chemotactic factor on the leukocyte is responsible for deactivation. Since the phosphonate esters prevent the chemotactic action of $C'(5, 6, 7)_a$ on leukocytes, we tested the phosphonates for their ability to prevent deactivation by the same factor. The results of such experiments, performed as described in the section on Materials and Methods, are seen in Table III and Fig. 5. In all experiments, the concen-

TABLE II

Similarity in Properties of the Chemotactic Factor and Leukocyte-Deactivating Factor

Serum	Treatment of serum	Effect of treatment of serum on chemotactic activity (% activity)‡	Leukocyte-deactivating capacity of serum§
			(%)
Normal	None	0	4
Activated*	None	100	73
Activated	56°C, 1 hr	91	77
Activated	Dialyzed in medium 199, 4 hr	100	80

* Activated by incubation with immune complexes. See details in Materials and Methods

‡ 10% treated serum used in testing for chemotactic activity.

§ Expressed as per cent reduction of chemotactic responsiveness of cells. PMN's, 2×10^6, were incubated with 0.30 ml serum in final volume of 1.0 ml at room temperature for 30 min, then washed and resuspended in 10% normal serum for testing.

TABLE III

Ability of Phosphonate Esters to Protect Polymorphonuclear Leukocytes from Deactivation by Chemotactic Factor

Treatment of cells		Chemotaxis		
Phosphonates used (chloroalkyl series*)	Chemotactic factor	No. of cells responding	Inhibition	Protection
			%	%
3-chloropropyl	−	101	6	
3-chloropropyl	+	85	21	58
4-chlorobutyl	−	114	0	
4-chlorobutyl	+	119	0	100
5-chloropentyl	−	108	0	
5-chloropentyl	+	115	0	100
6-chlorohexyl	−	105	2	
6-chlorohexyl	+	65	40	20
Controls				
None	−	108	—	
None	+	54	50	

* Final concentration at 1×10^{-4} M. Leukocytes were incubated with or without chemotactic factor in the presence or absence of phosphonate esters for 30 min at room temperature. They were then washed, resuspended in 10% normal rabbit serum, and tested for chemotactic responsiveness.

tration of phosphonates was kept below that necessary to give cell-dependent inhibition.

Table III shows the results obtained with one of the three homologous series of phosphonates tested, the chloroalkyl phosphonates. These results are illustrative of those generally obtained. Incubation of the leukocytes with

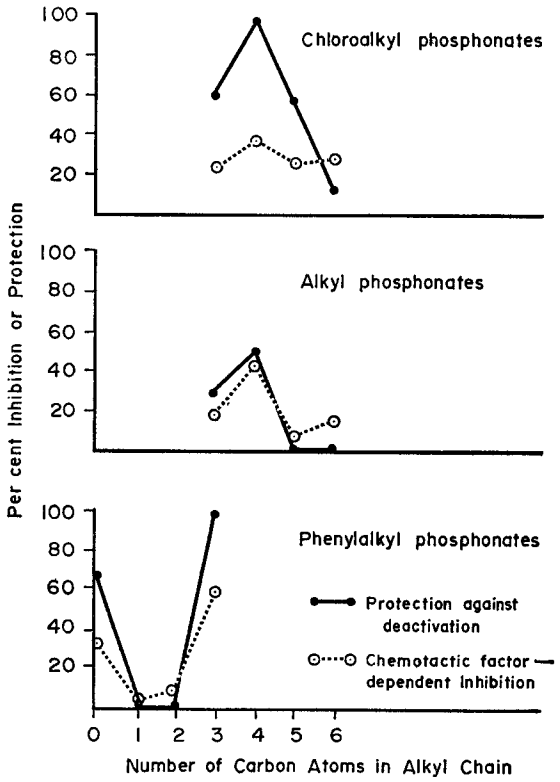


FIG. 5. Comparison of inhibition in chemotactic responsiveness of leukocytes (chemotactic factor–dependent inhibition) and protection against deactivation caused by cell contact with chemotactic factor, for three different classes of phosphonates.

the chemotactic factor alone led to a 50% decrease of chemotactic responsiveness; that is, they were 50% deactivated. Incubation of the leukocytes with the phosphonates alone was without significant effect in every instance. When the leukocytes were incubated with the chemotactic factor in the presence of the chlorobutyl, chloropentyl, or chlorohexyl phosphonate, however, there was 0, 0, and 40% inhibition of chemotaxis, respectively. In other words, depending on the phosphonate used, 20–100% protection against deactivation was achieved.

In Fig. 5, the per cent protection from deactivation given by each

TABLE IV

Ability of Various Esters and Acetyl Amino Acids to Protect Leukocytes from Deactivation by C′(5, 6, 7)α

Experiment	Esters (M concentration)	Chemotactic factor	No. of cells responding	Inhibition	Protection
				%	%
A	Triacetin (8×10^{-3})	−	135	0	
	" (8×10^{-3})	+	28	79	0
	Ethyl acetate (8×10^{-3})	−	159	0	
	" " (8×10^{-3})	+	32	76	4
	Butyl acetate (8×10^{-3})	−	137	0	
	" " (8×10^{-3})	+	38	71	0
	Acetyl phosphate (5×10^{-3})	−	134	0	
	" " (5×10^{-3})	+	57	57	23
	Acetylcholine (1×10^{-2})	−	139	0	
	" (1×10^{-2})	+	31	76	0
	TAMe* (5×10^{-3})	−	136	0	
	" (5×10^{-3})	+	43	68	8
	Acetyl-L-tyrosine ethyl ester (5×10^{-3})	−	113	14	
	" " " " (5×10^{-3})	+	88	33	56
	None	−	132		
	"	+	35	74	
B	Acetyl-L-tyrosine ethyl ester (1×10^{-3})	−	132	0	
	" " " " (1×10^{-3})	+	89	34	50
	Acetyl-L-phenylalanine ethyl ester (1×10^{-3})	−	128	4	
	Acetyl-L-phenylalanine ethyl ester (1×10^{-3})	+	98	27	61
	Acetyl-L-tryptophan ethyl ester (1×10^{-3})	−	148	0	
	Acetyl-L-tryptophan ethyl ester (1×10^{-3})	+	118	12	84
	Acetyl-L-tyrosine (1×10^{-3})	−	128	4	
	" " (1×10^{-3})	+	110	18	74
	Acetyl-L-tryptophan (1×10^{-3})	−	140	0	
	" " (1×10^{-3})	+	119	11	86
	Acetyl-DL-phenylalanine (1×10^{-3})	−	132	0	
	" " (1×10^{-3})	+	55	59	14

* Tosyl-L-arginine methyl ester.

TABLE IV—*Concluded*

Experiment	Esters (M concentration)	Chemotactic factor	No. of cells responding	Inhibition	Protection
				%	%
	Benzoyl-L-arginine methyl ester (1×10^{-3})	−	136	0	
	Benzoyl-L-arginine methyl ester (1×10^{-3})	+	49	63	9
	Acetyl-L-glycine (1×10^{-3})	−	118	11	
	" " (1×10^{-3})	+	49	63	9
	Acetyl-L-leucine (1×10^{-3})	−	104	22	
	" " (1×10^{-3})	+	41	70	0
	Acetyl-allo-L-isoleucine (1×10^{-3})	−	133	0	
	" " " (1×10^{-3})	+	39	71	0
	None	−	134	—	
	"	+	42	69	

phosphonate is plotted against the number of carbon atoms in the alkyl chain of the chloroalkyl phosphonates, the alkyl phosphonates, and the phenylalkyl phosphonates. The "protection profiles" were all obtained at a 1.0×10^{-4}M concentration of the phosphonates. The ability of the different members of a given homologous series of phosphonates to give protection against deactivation was compared only in experiments run at the same time with the same batch of cells and partially purified chemotactic factor. In the same figure the inhibition profiles for chemotactic-dependent inhibition given by the same phosphonates are also plotted. These are taken from Fig. 6 of reference 6.

It is evident from Fig. 5 that the protection profiles given by the chloroalkyl phosphonates, the alkyl phosphonates, and the phenylalkyl phosphonates are essentially the same as the "inhibition profiles" obtained when the identical three series of phosphonates were tested for their ability to give chemotactic factor–dependent inhibition. This implies that the "activatable esterase" is involved not only in chemotaxis but also in deactivation.

Ability of Aromatic Amino Acid Derivatives to Protect Polymorphonuclear Leukocytes from Deactivation.—If, as just demonstrated, phosphonate esters prevent deactivation by blocking the action of the activatable esterase, substrates or competitive inhibitors of this esterase might also protect against deactivation by reversibly interfering with the action of the enzyme. Therefore, carboxylic acid esters, amino acid esters, and phosphate esters were tested for their ability to prevent deactivation.

The various esters were incubated with the leukocytes and chemotactic factor as described in the section on Materials and Methods. The results are seen in Table IV. The simple acetate esters tested, namely triacetin, ethyl acetate, acetyl phosphate, etc., had previously been shown to prevent cell-dependent inhibition by the phosphonates and also to inhibit chemotaxis by interacting with the activated esterase (7). They were unable to protect completely against deactivation induced by the chemotactic factor (Table IV, Experiment A). (The 23% protection obtained with acetyl phosphate was not obtained in other experiments.) In contrast, aromatic amino acid esters such as acetyl-L-tyrosine ethyl ester and acetylated aromatic amino acids were distinctly effective in protecting against deactivation (Table IV, Experiment B). Acetyl-L-tyrosine ethyl ester had previously been shown to be completely ineffective in protecting against cell-dependent inhibition (7). The protection against deactivation given by the aromatic amino acid derivatives is specific; other nonaromatic amino acid esters, namely, tosyl-L-arginine methyl ester (TAMe) (Table IV, Experiment A), benzoyl-L-arginine methyl ester, and nonaromatic acetylated amino acids such as acetyl-L-glycine, acetyl-L-leucine, or acetyl-allo-L-isoleucine (Table IV, Experiment B), were not active.

Direct Inhibition of Chemotaxis by Various Amino Acid Derivatives.—If the aromatic amino acid derivatives prevent deactivation by interfering with the action of the activatable esterase and if, as already shown, this enzyme is required in chemotaxis, then one would expect that the same aromatic amino acid derivatives would also prevent chemotaxis. To test this inference, the various amino acid derivatives were examined for their effect on chemotaxis when added directly to the upper compartment of the chamber containing the leukocytes. Ethyl acetate, acetoxyacetic acid, and acetyl phosphate, which interfere with the "activated esterase" but do not inhibit the activatable esterase (7), were also tested. The results of the experiments, employing a final concentration of the amino acid compound of 2×10^{-3}M, are given in Table V. Acetyl-L-tyrosine ethyl ester and acetyl-L-tryptophan ethyl ester were highly inhibitory, as were acetyl-L-phenylalanine ethyl ester and acetyl-DL-phenylalanine (Table V, Experiment A). On the other hand, acetyl isoleucine and acetyl glycine were not inhibitory (Table V, Experiment A), while glycine ethyl ester, tosyl-L-arginine methyl ester, and benzoyl arginine methyl ester gave 12% or less inhibition (Table V, Experiment B). In other experiments these latter compounds did give higher inhibition than seen here. In general, however, the nonaromatic derivatives were distinctly less inhibitory than the aromatic ones. Ethyl acetate, acetoxyacetic acid, and acetyl phosphate inhibited, as was expected, due to their ability to interfere with the activated esterase (7). Thus, three groups of compounds have so far been found to more or less specifically prevent chemotaxis: simple acetates, which inhibit the activated esterase (7); aromatic amino acid derivatives, which presumably inhibit the activatable esterase; and ATP,

TABLE V

Experiments A and B: Direct Inhibition of Chemotaxis by Aromatic Amino Acid Compounds

Experiment	Material added to cell suspension	No. of cells responding*	Inhibition
			%
A‡	Acetyl-L-tyrosine ethyl ester	13	87
		8	92
	Acetyl-L-tryptophan ethyl ester	32	68
		19	81
	Acetyl-L-phenylalanine ethyl ester	57	43
		59	41
	Acetyl-DL-phenylalanine	38	62
		46	54
	Acetyl isoleucine	108	0
		96	0
	Acetyl glycine	100	0
	None (positive control)	98	—
	None (negative control)	0	—
B	Tosyl-L-arginine methyl ester	176	12
	Benzoyl-L-arginine methyl ester	195	2
	Glycine ethyl ester	224	0
	Acetyl-L-leucine	180	10
	Acetyl-L-glycine	100	50
	Ethyl acetate	55	72
	Acetoxyacetic acid	95	72
	Acetyl phosphate	105	49
	None (positive control)	200	—
	None (negative control)	0	—

* All chambers except the negative control contained 10% activated rabbit serum in the bottom compartment.

‡ Compounds were added at a concentration of 2×10^{-3} M. In Experiment A studies were done in duplicate.

which inhibits neither and whose mechanism of inhibition of chemotaxis is still unknown (7).

DISCUSSION

Sufficiently prolonged contact of the polymorphonuclear leukocytes with preparations containing the chemotactic factor, $C'(5, 6, 7)_a$, leads to a complete and irreversible loss in the chemotactic responsiveness of neutrophils to

$C'(5, 6, 7)_a$. The deactivating factor in serum or partially purified preparations and the chemotactic factor are similar in that they require C'6 for generation (Table I), both elute similarly in ion-exchange chromatography, and there is a relationship between deactivation and chemotaxis as revealed by the effects of phosphonate esters and aromatic amino acid derivatives. The phosphonate esters prevent deactivation and also prevent chemotaxis. Moreover, the profiles of protection against deactivation given by the three series of phosphonate esters were essentially the same as those found in the chemotactic factor–dependent inhibition (Fig. 5). This indicates that the same enzyme, the "activatable esterase," is involved in both processes, making it most likely that, in fact, $C'(5, 6, 7)_a$ is responsible for deactivation as well as chemotaxis.

There are several possible mechanisms to explain deactivation of neutrophils by the complement-associated chemotactic factor. One possibility is a more or less irreversible saturation of the hypothetical cell receptors by the chemotactic factor. Although phosphonate esters and aromatic amino acid derivatives prevent deactivation, it is improbable that they do so by interfering with the combination of the chemotactic factor and the putative receptors. Another possibility is that the activation of the proesterase results in an active enzyme that is exceedingly labile, either intrinsically or because of a natural inhibitor. This explanation is analogous to that offered by Mongar and Schild (9) for the antigen-induced desensitization of acute allergic responses. It is also possible that activation of the proesterase is required for the oriented movement of the cell induced by the chemotactic factor and that activation of all the proesterase prevents such oriented response to chemotactic factor. Finally, it is also possible that deactivation is nothing more than the utilization of a substrate or cofactor that is required for the chemotactic response of the neutrophil and that is present in very limited concentration on the cell. The data in this paper do not permit a selection of any one of these possibilities.

The manner in which the phosphonates prevent deactivation not only indicates that the activatable esterase is involved, but also throws light on the manner in which this esterase is activated. The phosphonates "protect" against, that is, prevent deactivation by irreversibly inhibiting the activatable esterase following its activation. Such protected cells are still able to give a chemotactic response to $C'(5, 6, 7)_a$, however, implying that there is still activatable esterase available within or on the cell in precursor form. Thus, in protecting against deactivation, the phosphonates prevent further activation of the activatable esterase. This implies that the enzymatic activity of the activatable esterase is required for the further activation of this enzyme. In other words, the activation of this esterase is, at least in part, an autocatalytic process. This is precisely analogous to the activation of C'1 to C'1a by an antigen-antibody complex, also a process at least partly autocatalytic (10).

In testing their ability to protect against deactivation, it was necessary that

the phosphonate esters be used at a concentration low enough not to affect the "activated esterase." It was therefore impossible to tell from these experiments whether the activated esterase was involved in deactivation. Acetate esters previously shown to prevent the action of the activated esterase did not prevent deactivation, however, indicating that deactivation can occur without the action of this second enzyme. This means that deactivation involves only part of the biochemical mechanism of chemotaxis. A further implication is that in chemotaxis the activated esterase either does not act in the same biochemical sequence as the activatable esterase, or if it does, its action is later than that of this enzyme.

The only other substances besides the phosphonates found to prevent deactivation were aromatic amino acid derivatives (Table IV). Nonaromatic amino acid derivatives of basic amino acids such as tosyl-L-arginine methyl ester or benzoyl-L-arginine methyl ester, or acetylated nonaromatic amino acids such as acetyl-L-glycine or acetyl-L-leucine, were more or less inactive in this regard.

The same aromatic amino acids that protect against deactivation also inhibit chemotaxis when added, with the leukocytes, to the upper compartment of the chamber (Table V).

One possible explanation for the ability of aromatic amino acid derivatives to protect against deactivation and to inhibit chemotaxis is that an enzyme that has a particular affinity for aromatic amino acids is required in both processes. As shown, the activatable esterase is involved in both chemotaxis and deactivation. It is therefore plausible to suggest that the activatable esterase is an enzyme with a particular affinity for aromatic amino acid esters and aromatic amino acid derivatives. Although plausibility is no guarantee of truth, it can serve as a guide for future work.

SUMMARY

As shown previously, immune complexes engender in rabbit serum a factor capable of inducing chemotaxis of rabbit polymorphonuclear leukocytes. This chemotactic factor consists of a complex of the fifth, sixth, and seventh components of complement. As demonstrated here, the polymorphonuclear leukocytes incubated with such treated rabbit serum lose their ability to respond chemotactically to the chemotactic factor. They are "deactivated."

The process of "deactivation" is a function of the duration of contact of the cells with, and the concentration of, the treated serum. There is a parallelism between the time course of deactivation and of chemotaxis, as well as the dose-response curves for the two processes. Chemotactic factor purified by isoelectric precipitation and ion-exchange chromatography produces deactivation in the same manner as the treated serum. The deactivating activity requires, as does the chemotactic factor, the sixth component of complement; like the chemotactic factor, it is heat-stable and nondialyzable. Deactivation is prevented

by the same phosphonate esters shown previously to prevent chemotaxis by the complement-associated chemotactic factor. The profiles of the phosphonates in protecting against deactivation are the same as the profiles for the chemotactic factor–dependent inhibition of chemotaxis. Aromatic amino acid derivatives prevent both chemotaxis and deactivation. We conclude from this evidence that the chemotactic factor is able to deactivate or induce chemotaxis depending upon experimental conditions.

The fact that the profiles given by the phosphonates for protection against chemotactic factor–dependent deactivation and for chemotactic factor–dependent inhibition of chemotaxis are the same indicates that the "activatable esterase" is involved in both processes. Acetate esters such as ethyl acetate and others shown previously to prevent chemotaxis by inhibiting the "activated esterase" do not prevent deactivation. This indicates that deactivation can occur without participation of the latter enzyme, implying that deactivation involves only a part of the biochemical mechanism of chemotaxis.

The protection against deactivation afforded by aromatic amino acid derivatives is specific, insofar as nonaromatic amino compounds and simple acetate esters have no effect. In addition, as stated, the aromatic amino acid derivatives inhibit deactivation and chemotaxis by the chemotactic factor. This latter finding, together with the demonstration of the involvement of the activatable esterase in both deactivation and chemotaxis, suggests that the activatable esterase of the rabbit polymorphonuclear leukocyte is a serine esterase with a special affinity for aromatic amino acid derivatives.

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