PARTIAL BIOCHEMICAL CHARACTERIZATION OF THE ACTIVATED ESTERASE REQUIRED IN THE COMPLEMENT-DEPENDENT CHEMOTAXIS OF RABBIT POLYMORPHONUCLEAR LEUKOCYTES*

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Organophosphorus inactivators of serine esterases inhibit the chemotactic response of rabbit polymorphonuclear leukocytes to the complement-dependent chemotactic factor (1).[1] The organophosphorus inhibitors employed in demonstrating this inhibition were mainly several homologous series of phosphonate esters. Two general types of experimental procedures were used to elicit the inhibition of the chemotactic responsiveness of the leukocytes. In the first, inhibition was obtained on incubating leukocytes with a phosphonate ester in the absence of the chemotactic factor. The cells when washed free of inhibitor were shown to have lost their ability to respond to the chemotactic factor. This was termed "cell-dependent inhibition." In the second procedure, cells suspended in a phosphonate inhibitor and placed in the upper compartment of a chamber used to measure chemotactic response were acted upon by the chemotactic factor diffusing from the lower compartment. The inhibition obtained under these conditions was called "chemotactic factor–dependent inhibition." The concentration of inhibitor, kind of phosphonate, and duration of contact of cells and inhibitor were manipulated so that a high degree of chemotactic factor–dependent inhibition was obtainable in the complete absence of cell-dependent inhibition (1).

It was concluded that these two kinds of inhibition were due to the inactivation of two serine esterases. In the cell-dependent reaction, there is inhibition of what we term the "activated esterase." This esterase exists in or on the leukocyte in an already activated state and is, therefore, inhibitable by organophosphorus compounds in the absence of the chemotactic factor. The chemotactic factor–dependent inhibition is considered due to the inhibition of a second esterase which we term the "*activatable*

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[1] The complement-dependent chemotactic factor used in the preceding and present paper is the activated complex of the fifth, sixth, and seventh components of complement (C'5, C'6, C'7).

esterase." This enzyme is present in or on the cell in a precursor form which is not susceptible to inactivation by the organophosphorus inhibitor. The chemotactic factor activates this esterase; the esterase then is capable of being inhibited by the organophosphorus compound (1).

In the preceding study, the esterases were characterized only by their ability to be inhibited by several homologous series of *p*-nitrophenylethyl phosphonates. In order to establish the exact role of these enzymes in the chemotactic process it is both desirable and necessary to isolate the esterases from the leukocytes in pure form, and establish their precise biochemical function. For this purpose it is requisite that additional means of characterizing the esterases be available so that even though free of the cell they still can be identified as the specific esterases concerned with chemotaxis. It is the furnishing of such a further identifying characteristic for the *activated* esterase of chemotaxis which is the subject of this paper.

Organophosphorus inhibitors, like the phosphonate esters, inactivate serine esterases by irreversibly binding to the active site of the enzyme. As repeatedly shown, substrates or competitive inhibitors which combine reversibly with the active site of the serine esterase, when present at the same time as the organophophorus inhibitor, can protect the enzyme from inactivation by preventing access of the inhibitor to the enzymatic site (reviewed in reference 2).

With this in mind, a wide variety of simple carboxylic acid esters, amino acid esters, and phosphate esters were incubated with rabbit polymorphonuclear leukocytes in the presence of an inhibitory concentration of phosphonate ester. At the end of the incubation period the cells were washed and tested for their ability to respond to the chemotactic factor. As will be shown, acetate esters of a particular type, and only acetate esters, were found capable of protecting the rabbit leukocyte against this cell-dependent inhibition by phosphonates. Moreover, the conditions under which the protection could be demonstrated were those to be expected if the protecting acetate esters were competing with the phosphonate for the active site of the enzyme.

Materials and Methods

The method for collecting the rabbit polymorphonuclear leukocytes and carrying out the tests of chemotaxis were the same as described in the preceding paper (1). The *p*-nitrophenyl ethyl 5-chloropentyl phosphonate, the *p*-nitrophenyl ethyl hexyl phosphonate, *p*-nitrophenyl ethyl phenylpropyl phosphonate, and *p*-nitrophenyl ethyl phenylbutyl phosphonate were the same as used in the preceding study.[2] Ethyl formate, ethyl acetate, ethyl propionate, ethyl butyrate, butyl acetate, glyceryl triacetate (triacetin), acetylcholine bromide, and *p*-nitrophenyl acetate, were Eastman White Label chemicals, or of equivalent quality. The acetoxy acetic acid was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis. The tosyl-L-arginine methylester (TAMe), acetyl-L-tyrosine ethyl ester, and glycine ethyl ester were from Mann Research Laboratories, N.Y. The adenosine 5'triphosphate (ATP), and 1,6-fructose diphosphate were from Sigma Chemical Co., St. Louis, Mo. The 6-glucose phosphate was

[2] In what follows, the *p*-nitrophenyl ethyl phosphonates will be named only according to the nature of the R group, the presence of the *p*-nitrophenoxy and ethoxy groups being implied.

from the Schwarz Laboratories Inc., N.Y. These phosphates and the lithium salt of acetyl phosphate were generously provided by Dr. L. Corwin, Division of Biochemistry, Walter Reed Army Institute of Research.

Medium 199 was used to dissolve the carboxylic acid esters, and phosphonate esters and to suspend the polymorphonuclear leukocytes (1).

The tests of the protective action of the various esters against cell-dependent inhibition by the phosphonate were carried out as follows. In one tube 1 ml of 6×10^6 polymorphonuclear leukocytes was added to 1 ml of the appropriate concentration of the ester whose protective ability against inhibition by phosphonates was to be tested, and then 1 ml of an inhibitory concentration of phosphonate ester was added. In a second tube, was placed 1 ml of cells, 1 ml of the same concentration of ester, and 1 ml of medium 199. The third tube contained 1 ml of cells, 1 ml of phosphonate ester, and 1 ml of medium 199. All tubes were allowed to stand with intermittent shaking for 1 hr at room temperature and then centrifuged in the cold. The leukocytes were washed one time with medium 199, resuspended in 3 ml of medium 199 with 0.5% ovalbumin (1), and then tested for their ability to respond to the chemotactic factor as described (1).

In testing the ability of the phosphate, amino acid, and simple carboxylate esters to inhibit chemotaxis, 1 ml of 6×10^6 leukocytes was mixed with 1 ml of ester at three times the desired final concentration, and 1 ml of 0.5% ovalbumin, all in medium 199. This cell suspension was placed in the upper compartment of the chamber and the ability of the cells to respond to the chemotactic factor present in the lower compartment of the chamber was tested in the usual way (1).

The esterase activity of the intact leukocytes against the various carboxylic and phosphate esters was measured manometrically in the Warburg apparatus. 15-ml flasks were employed; the number of cells was 7.5×10^7 unless otherwise indicated, and the final ester concentration 0.02 M, bicarbonate concentration 0.024 M, all at a pH of 7.4 in a total volume of 3 ml of 0.15 M NaCl. The suspension was equilibrated with 5% CO_2 and 95% N_2 at 37°C.

The inhibition of the aliesterase activity of the polymorphonuclear leukocytes by phosphonate esters was determined by incubating for 1 hr at room temperature 1 volume of 1.3×10^6–2.3×10^6/ml leukocytes in 0.15 M saline, with 1 volume of three times the desired concentration of phosphonate in medium 199, and 1 volume of medium 199. At the end of the hour, 0.4 ml of the mixture was pipetted into a Warburg flask, containing 2.6 ml of the carboxylic acid ester, and bicarbonate in 0.15 M saline. The final concentration of carboxylic acid ester and bicarbonate was the same as described above.

RESULTS

The Chemical Nature of the Esters which Protect against Phosphonate Inhibition of Chemotaxis.—Table I shows the results of studies testing the ability of a variety of simple carboxylic acid esters, amino acid esters, and phosphonate esters to prevent the cell-dependent inhibition of the chemotactic ability of rabbit polymorphonuclear leukocytes by 5×10^{-4} M 5-chloropentyl phosphonate. As noted in the section on Materials and Methods, every ester tested for its protective ability was also tested for its effect on the chemotactic behavior of the leukocytes after incubation for 1 hr with the leukocytes in the absence of the phosphonate. None of the esters reported in Table I had any irreversible effect on the chemotactic responsiveness of the leukocytes. All experiments whose results are given in Table I were done at least twice. Glyceryl triacetate (triacetin) was used as the standard protecting agent in every experiment.

The high degree of specificity of the structure of those compounds able to

TABLE I

Specificity of the Prevention by Various Esters of the Cell-Dependent Inhibition of Chemotaxis Induced by 5-Chloropentyl Phosphonate

Experiment	Ester	5-Chloropentyl phosphonate	No. of cells responding	Inhibition
		5×10^{-4} M		%
A	—	−	282	—
	—	+	176	38
	Glyceryl triacetate (8.3×10^{-3} M)	+	285	0
	Glyceryl triacetate (3.3×10^{-3} M)	+	246	13
	Ethyl formate (8.3×10^{-3} M)	+	189	33
	Ethyl acetate (8.3×10^{-3} M)	+	302	0
	Ethyl propionate (8.3×10^{-3} M)	+	169	40
	Ethyl butyrate (8.3×10^{-3} M)	+	148	53
	Adenosine 5′triphosphate (3.3×10^{-3} M)	+	150	47
	Fructose 1,6-diphosphate (3.3×10^{-3} M)	+	150	47
	Glucose 6 phosphate (3.3×10^{-3} M)	+	147	48
B	—	−	210	—
	—	+	150	29
	Glyceryl triacetate (8.3×10^{-3} M)	+	233	0
	Butyl acetate (8.3×10^{-3} M)	+	225	0
C	—	−	177	—
	—	+	80	55
	Glyceryl triacetate (8.3×10^{-3} M)	+	175	1
	Tosyl-L-arginine methylester (1×10^{-2} M)	+	68	61
	Acetyl-L-tyrosine ethylester (5×10^{-3} M)	+	91	49
	Acetylcholine (1×10^{-2} M)	+	83	53
D	—	−	185	—
	—	+	96	48
	Glyceryl triacetate (8.3×10^{-3} M)	+	180	3
	Glycine ethylester (8.3×10^{-3} M)	+	98	47
	Acetoxyacetic acid (8.3×10^{-3} M)	+	147	21
	Sodium β-glycerophosphate (8.3×10^{-3} M)	+	66	65
E	—	−	211	—
	—	+	100	52
	Glyceryl triacetate (8.3×10^{-3} M)	+	226	0
	Glyceryl triacetate (3.3×10^{-3} M)	+	159	24
	Acetylphosphate (8.3×10^{-3} M)*	+	196	5
	Acetylphosphate (3.3×10^{-3} M)	+	119	42
	Acetoxyacetic acid (8.3×10^{-3} M)	+	194	7
	Acetoxyacetic acid (3.3×10^{-3} M)	+	160	23
	Sodium acetate (8.3×10^{-3} M)	+	102	51

* Somewhat less than 8.3×10^{-3} M because of slight insolubility of this concentration of lithium acetyl phosphate.

protect is evident from Table I. The only esters able to prevent 5-chloropentyl phosphonate from inhibiting the chemotactic responsiveness of rabbit leukocytes were the acetates, glyceryl triacetate, ethyl acetate, butyl acetate, acetoxy acetic acid (CH_3COOCH_2COOH), and acetyl phosphate. Acetylcholine, however, was not able to protect, nor was sodium acetate. Other simple esters such as ethyl formate, ethyl propionate, and ethyl butyrate were not protective. All three amino acid esters tested including ethyl glycine (ethyl α-amino acetate) were inactive, as were the phosphate esters exept acetyl phosphate.

Ability of Glyceryl Triacetate to Protect against Inhibition by Various Phosphonate Esters.—If the protection afforded the chemotactic capacity of the

TABLE II

The Ability of Glyceryl Triacetate to Protect Against Cell-Dependent Inhibition by Four Phosphonate Esters

Experiment	Phosphonate	Glyceryl triacetate	Cells responding to chemotactic factor	Inhibition
		M		%
A	—	—	280	
	Phenylpropyl (3.3×10^{-4} M)	—	139	50
	Phenylpropyl (3.3×10^{-4} M)	8.33×10^{-3}	238	15
	Hexyl (2.5×10^{-4} M)	—	167	41
	Hexyl (2.5×10^{-4} M)	8.33×10^{-3}	216	23
B	—	—	130	
	Phenylbutyl (2×10^{-5} M)	—	8	94
	Phenylbutyl (2×10^{-5} M)	8.33×10^{-3}	108	17
	5-chloropentyl (5×10^{-4} M)	—	55	48
	5-chloropentyl (5×10^{-4} M)	8.33×10^{-3}	122	6

polymorphonuclear leukocyte by the various acetate esters is due to their ability to compete with the phosphonate for the active site of the enzyme, then the protection should be demonstrable with phosphonates other than the 5-chloropentyl phosphonate. Table II shows that this is the case. Glyceryl triacetate (triacetin) is able to protect the chemotactic capacity of rabbit leukocytes not only against cell-dependent inhibition by 5-chloropentyl phosphonate, but also against inhibition by the hexyl, phenylpropyl, and phenylbutyl phosphonates (Table II). The hexyl and phenylbutyl phosphonates are the two most potent inhibitiors of the activated esterase so far known (see reference 1).

The Degree of Protection as a Function of the Concentration of Phosphonate Inhibitor and of Carboxylic Acid Ester.—If the protection afforded the leukocyte by the acetate esters is due to competition with the phosphonate for the active site of the enzyme, then the degree of protection should depend on the relative concentrations of inhibitor and protector. This was tested by deter-

mining the protection afforded by various concentrations of glyceryl triacetate at different levels of hexyl phosphonate. The results are shown in Table III. There it is seen that 8.3×10^{-3} M glyceryl triacetate was unable to protect against inhibition by 5.0×10^{-4} M hexyl phosphonate. The same concentration of glyceryl triacetate completely protected the chemotactic activity of the leukocyte against the 29% inhibition given by 2.0×10^{-4} M phosphonate. Reducing the concentration of glyceryl triacetate to 4.7×10^{-4} M in the presence of the same low concentration of phosphonate resulted in only 41% protection.[3]

TABLE III

The Effect of Variation of the Concentrations of Hexyl Phosphonate and Glyceryl Triacetate on the Degree of Protection

Concentration of hexyl phosphonate	Concentration of glyceryl triacetate	No. of cells responding to chemotactic factor	Inhibition
10^{-4} M	10^{-3} M		%
0	0	275	100
5	0	0	100
5	8.3	0	100
5	4.1	0	100
3.5	0	57	79
3.5	8.3	76	72
3.5	4.1	53	81
2.5	0	162	41
2.5	8.3	211	23
2.5	4.1	194	30
2.0	0	196	29
2.0	8.3	275	0
2.0	4.1	229	17

Results intermediate to these were obtained at intermediate concentrations of phosphonate.

The Correspondence between the Ability of Carboxylic Acid Esters to Protect against Cell-Dependent Inhibition by Phosphonates and Their Ability to Serve as Direct Inhibitors of Chemotaxis.—If a carboxylic acid ester can compete with the phosphonate for the active site of the enzyme, the carboxylic acid ester should also be able to compete for the same enzyme site with the natural substrate in the leukocyte. This implies that those carboxylic acid esters which

[3] Per cent protection is defined as the difference between the per cent inhibition due to the phosphonate alone and the per cent inhibition due to the phosphonate in the presence of the protective substance divided by the inhibition due to phosphonate alone, all multiplied by 100.

protect against cell-dependent inhibition of chemotaxis by phosphonates should themselves prevent chemotaxis.

The ability of various esters to prevent chemotaxis was tested as described in the section on Materials and Methods. Table IV shows the results obtained. Of the esters tested, glyceryl triacetate, ethyl acetate, and butyl acetate when mixed with the cell suspension in the upper compartment of the chamber inhibited chemotaxis whereas, acetylcholine, ethyl formate, and ethyl butyrate were inactive. The slight inhibition obtained with ethyl propionate was confirmed in a subsequent experiment. It is of interest, although not germane to the

TABLE IV
Prevention of Chemotaxis by Ester Substrates

Experiment	Ester	Concentration	No. of cells responding	Inhibition
		10^{-2} M		%
A	—		138	
	Glyceryl triacetate	8.3	63	54
	Acetyl-L-tyrosine ethylester	5.0	20	86
	Tosyl-L-arginine methylester	10	135	2
	Acetylcholine	10	128	7
B	—		188	
	Glyceryl triacetate	8.3	101	46
	Butyl acetate	8.3	100	47
	Ethyl acetate	8.3	130	31
	Ethyl formate	8.3	198	0
	Ethyl propionate	8.3	164	14
	Ethyl butyrate	8.3	199	0
	Adenosine 5'triphosphate (ATP)	3.0	90	52
	Glucose 6-phosphate	10	195	0
	Fructose 1,6-diphosphate	10	190	0

present paper, that acetyl-L-tyrosine ethyl ester and ATP were also effective inhibitors, even though amino acid esters such as tosyl-L-arginine methyl ester, and phosphate esters such as glucose 6-phosphate and fructose 1, 6-diphosphate, were not. The prevention of chemotaxis by acetyl-L-tyrosine ethyl ester and ATP is presumably due to mechanisms other than the inhibition of the activated esterase.

Inability to Relate the Activated Esterase of Chemotaxis to Known Aliesterase(s).—Rabbit leukocytes contain an aliesterase or esterases capable of splitting ethyl formate, ethyl acetate, ethyl propionate, and ethyl butyrate (3, 4). It was, therefore, of interest to see if the activated esterase of chemotaxis could be related to this known aliesterase(s).

Rabbit leukocytes were incubated for 1 hr at room temperature with con-

centrations of 5-chloropentyl phosphonate varying from 6.0×10^{-4} M to 3.8×10^{-9} M. The esterase activity remaining on the leukocyte was then determined using 0.02 M glyceryl triacetate, butyl acetate, or ethyl butyrate as the substrate. Table V shows that the concentration of phosphonate which gave 50% inhibition of esterase activity with each substrate was somewhat above 6.0×10^{-9}–3.8×10^{-9} M. This level of the 5-chloropentyl phosphonate is about 10,000 times less than the concentration, which under essentially the same conditions, gives 50% cell-dependent inhibition of chemotaxis (1). These results indicate that the activated esterase of chemotaxis is not the same as the aliesterase

TABLE V

Effect of 5-Chloropentyl Phosphonate on Aliesterase Activity of Rabbit Polymorphonuclear Leukocytes

Substrate*	No. of leukocytes per Warburg flask	Phosphonate concentration	Activity	Inhibition
		M	μl CO_2/hr	%
Glyceryl triacetate	6×10^7	0	210	
		6.7×10^{-8}	20	90
		1.5×10^{-8}	47	77
		3.8×10^{-9}	180	14
Butyl acetate	6×10^7	0	102	
		9.4×10^{-8}	8	92
		2.4×10^{-8}	36	65
		6.0×10^{-9}	90	12
Ethyl butyrate	4×10^7	0	321	
		9.4×10^{-8}	0	100
		2.4×10^{-8}	165	49
		6.0×10^{-9}	293	9

* In each case, the concentration of substrate was 0.02 M, $NaHCO_3$ 0.02 M, NaCl 0.15 M, pH 7.4 and total volume 3.0 ml.

responsible for splitting glyceryl triacetate, butyl acetate, and ethyl butyrate. Moreover, it confirms the hypothesis of Wong and Rossiter (4) that the same enzyme is responsible for splitting all of the latter three substrates.

DISCUSSION

The protection afforded by the carboxylic acid esters against inhibition of the activated esterase of chemotaxis by phosphonates is highly specific (Table I); it is not restricted to a single phosphonate (Table II); and the degree of protection depends upon the relative concentrations of protective ester and inhibitor (Table III). These findings are expected if the carboxylic acid ester competes with the phosphonate for the enzymatic site of the activated esterase. The finding that the protective esters are able to prevent chemotaxis (Table IV) is also in

accord with the hypothesis that these esters combine with the enzymatic site of the activated esterase.

The specificity in the structure of the protective esters is striking. The ester must be an acetate; ethyl acetate protects, but ethyl formate with one less carbon, or ethyl propionate with one more does not. Butyl acetate protects; its isomer, ethyl butyrate, is completely inactive. Acetyl phosphate protects, but the other three phosphates tested which lacked the acetyl group did not. Yet, not all acetates protect. Acetylcholine in which the acetate is esterified to a positively charged alcohol is completely inactive as a protecting agent. However, acetoxyacetic acid in which the acetic acid is esterfied to the negatively charged 2-hydroxyacetic acid, and acetyl phosphate, in which the acetic acid is bound to the negatively charged phosphate are protective. Glycine ethyl ester, in which the acetic acid contains an amino group on the α-carbon is not protective. Moreover, an ester linkage seems necessary; sodium acetate is not protective.

The protection tests only indicate, in a qualitative fashion, that the cell-bound–activated esterase is able to bind the protecting ester to the active site of the enzyme. They do not specify that the enzyme is capable of splitting the esters. Thus, it is impossible to state that the activated esterase is able to hydrolyze any of the esters which were found to be protective. Nevertheless, on the basis of the work described here, one can say that the activated esterase of chemotaxis is an enzyme capable of being competitively inhibited by, or of hydrolyzing, neutral acetate esters, and acetates bound to negatively charged groups, or of doing both. Acetylesterases (acetylases) are the presently known enzymes which best fit these specificity characteristics. The best known acetylesterase is citrus fruit acetylesterase (5–7), but similar enzymes have been reported in mammalian tissue as well (8). They have not been reported in mammalian leukocytes. On the basis of this work a search for such an enzyme in rabbit polymorphonuclear leukocytes is indicated.

The 10,000-fold difference in sensitivity to phosphonate inhibtion between the known aliesterase of rabbit leukocytes and the activated esterase of chemotaxis (Table V) indicates that the two esterases are different. Experiments not reported here suggest that the activated esterase is not part of the endogenous or exogenous glycolytic cycle of the leukocyte. In the presence and absence of added glucose, acid production as measured in the Warburg apparatus was unaffected by prior treatment of the leukocyte with concentrations of 5-chloropentyl, hexyl, or phenylbutyl phosphonate capable of giving 70–100% cell-dependent inhibition.

To the extent that an enzyme is defined in terms of the substrates upon which it acts, neither this work, nor that reported in the preceding paper, has identified the activated esterase. Nevertheless, the phosphonate inhibition profiles reported in the preceding study, and the specificity of the binding of acetates to

the active site of the enzyme described here should make such an identification possible. This awaits further work.

SUMMARY

It was shown in the preceding paper that incubation of the rabbit polymorphonuclear leukocytes with phosphonate esters leads to an irreversible inhibition of the ability of the leukocyte to respond to the chemotactic factor. This "cell-dependent inhibition" was attributed to the inactivation by the phosphonates of an esterase existing in or on the leukocyte in an already activated state. As shown in this paper, incubating the leukocyte with phosphonate in the presence of certain esters prevents this cell-dependent inhibition. The protection is specific; the ester must be an acetate. Ethyl formate, ethyl propionate, ethyl butyrate, glucose 6-phosphate, fructose 1,6-diphosphate, ATP, tosyl arginine methyl ester, or acetyl tyrosine ethyl ester do not protect. The protection is independent of the phosphonate used to inhibit, and the degree of protection depends on the relative concentrations of acetate and phosphonate. Those acetates which protect are also the esters which inhibit chemotaxis when added to the leukocyte in the upper part of the chemotaxis chamber. It is concluded that the activated esterase is an enzyme capable of specifically splitting, or binding acetates, or doing both. Presumably the esterase is some type of acetylesterase or acetylase. The known aliesterase present in the leukocyte is not the activated esterase. Inhibition of the activated esterase by phosphonates has no effect on endogenous or exogenous glycolysis.

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