MECHANISMS OF THE INHIBITION OF CHEMOTAXIS BY PHOSPHONATE ESTERS*

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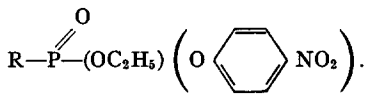
Fresh normal serum treated with immune complexes is chemotactic for polymorphonuclear leukocytes (PMN's) from a variety of species (1, 2). The factor in treated serum causing specific directional migration of PMN's has been identified as a molecular complex consisting of the fifth (C'5), sixth (C'6), and probably seventh (C'7) components of complement (2, 3).

The manner by which the chemotactic factor causes migration of leukocytes has proved difficult to analyze and is virtually unknown. The major difficulty in such analysis stems from the obvious requirement for living, intact cells. Previous work on the antigen-antibody–induced release of histamine from tissue slices and cell suspensions has clearly demonstrated that organophosphorous inhibitors such as diisopropylphosphofluoridate (DFP) and phosphonate esters are particularly suitable for the study of living cell systems (4). These compounds inactivate a specific group of enzymes, the serine esterases, in a characteristically irreversible manner (5). The *p*-nitrophenylethyl phosphonate esters have the general structure



Their inhibitory activity varies with changes in structure of the R group in a manner which is characteristic for such well defined esterases as trypsin, chymotrypsin, and acetylcholine esterase (5). There is also a characteristic variation in the relation of the structure of the phosphonate and its ability to inhibit the hemolytic activity of the first component of complement, C'1a (4), and the antigen-induced release of histamine from sensitized slices of guinea pig lung (4), and from rat peritoneal mast cells sensitized with rat homocytotropic antibody (6), or with rabbit anti-rat γ-globulin (7).

Because of the well defined nature of the inhibitory activity of these phos-

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phonates and the relation of their structure to the specificity of inhibition, they have been tested for their effects on the chemotaxis of PMN's by the complement-dependent chemotactic factor. Inhibition of chemotaxis was found. The characteristics of such inhibition have led to the view that an esterase exists in or on the cell in a phosphonate-insensitive, precursor form which is transformed directly or indirectly by the chemotactic factor into a phosphonate-sensitive, active enzyme. The esterase activated in this manner is required for the chemotactic response of the cell. In addition, it would appear that a second esterase exists in or on the cell in an already activated form, i.e. susceptible to inhibition by certain phosphonate esters without the intervention of the chemotactic factor, and that the activity of this second "activated" esterase is also required for the chemotactic response of the cell.

Materials and Methods

Animals.—Housing and care of animals used as leukocyte and serum donors were provided by the Animal Care Branch of the Armed Forces Institute of Pathology. The "Principles of Laboratory Animal Care" as promulgated by the National Society for Medical Research were observed in these studies.

Chemotaxis.—The system employing specially constructed stainless steel chambers with micropore filters (pore size 650 mμ) was used as previously described (2). Chemotaxis of rabbit polymorphonuclear leukocytes (PMN's) which were obtained from a glycogen-induced peritoneal exudate was determined by counts of cell numbers after migration through micropore filters. For most studies the period of chamber incubation, and thus cell migration, was 60 min.

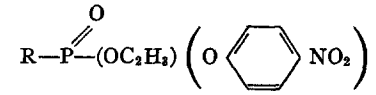
In previous experiments cell suspensions of rabbit PMN's for chemotactic studies were made up in 10% fresh rabbit serum (2). In the present study, it was found that cells were reactive when suspended in 0.5% ovalbumin (Nutritional Biochemical Corp., Cleveland, Ohio) in medium 199 at a pH of 7.4. All experiments were conducted with cells suspended in this albumin solution in order to obviate the problem of hydrolysis of phosphonate esters by an enzyme present in rabbit serum (8).

The sources of the chemotactic factor used in the studies included: (*a*) the unpurified form present in 10% rabbit serum which had been treated with an immune precipitate (2); (*b*) a semipurified form obtained by preparation of the active euglobulin fraction from fresh rabbit serum treated with immune complexes and fractionation of the euglobulin on triethylaminoethylcellulose (TEAE-cellulose); and (*c*) the highly purified form of the chemotactic factor obtained by fractionating the active TEAE-cellulose fraction in Pevikon block preparative electrophoresis. The details of the procedure for obtaining the semipurified and highly purified preparations of the chemotactic factor have been described previously (2).

The kind of preparation of the chemotactic factor used in the given experiment is indicated in the description of that experiment. As will be shown, the state of purity of the chemotactic factor does not influence the inhibitory effects of the phosphonates.

Density Gradient Ultracentrifugation.—In density gradient experiments, 0.2 ml of highly purified chemotactic factor was added to sucrose density gradient solutions (10–40% sucrose at pH 7.3 or 8.6) and centrifuged at 35,000 rpm in a swinging bucket rotor for 20 hr as previously described (2). Various fractions were analyzed for the sixth component of complement (C'6) and for chemotactic activity (2).

Phosphonate Inhibitors.—The p-nitrophenylethyl phosphonates of the general structure



where R represents the alkyl, phenylalkyl, ω-aminoalkyl, or ω-chloroalkyl chain were used.[1] They have been described previously (5). The phosphonate esters were dissolved in acetone diluted 1:10 or 1:100 (in medium 199 or appropriate phosphate buffer). The inhibitor under study was then appropriately diluted to a final acetone concentration of 0.5%. Diisopropylphosphofluoridate (DFP), kindly supplied by Dr. Bernard Jandorf, Army Chemical Corp., Edgewood, Md., was used in some studies. At the concentrations used DFP was readily soluble in medium 199 but acetone was added, for the purpose of control, in a final concentration of 0.5%.

Procedure for Demonstrating Inhibition.—Unless otherwise noted, two types of experimental procedures were used to determine the effects of the phosphonates on chemotaxis. The first procedure was designed to reveal the inhibitory effect of the phosphonate in the absence of the chemotactic factor. The cells, at a concentration of 2×10^6/ml, were preincubated at room temperature with an equal volume of phosphonate ester in medium 199. All concentrations of phosphonate esters were expressed as final concentrations after dilution with the cell suspensions. The cell suspensions contained 0.5% acetone. After an appropriate time of incubation, the cells were washed once with medium 199 to remove the inhibitor, centrifuged, and then resuspended in 0.5% ovalbumin. Control cells incubated with 0.5% acetone in medium 199 for the same length of time were carried through the same procedure. The response to the chemotactic factor of the cells pretreated with inhibitor compared to the response of the control cells was determined. The depressing effect of the phosphonate esters on cells in the absence of the chemotactic factor was termed *cell-dependent inhibition.*

The second type of procedure was designed to reveal any effect of the phosphonates on chemotaxis which was dependent upon the intervention of the chemotactic factor. The cells were incubated in medium 199 for 60 min in the absence of phosphonate inhibitor. They were washed once, and centrifuged precisely as described in the procedure to measure cell-dependent inhibition, but then resuspended in the appropriate phosphonate ester. The cells suspended in inhibitor were immediately placed in chambers and the chemotactic response measured after a 60 min period. The chemotactic responsiveness was also determined for control cells treated in exactly the same way except that they were resuspended in 0.5% acetone in medium 199 instead of inhibitor. The reduction in response to the chemotactic factor of the cells suspended in inhibitor compared to the control cells was termed the *chemotactic factor–dependent inhibition.*

Usually cell-dependent and chemotactic factor–dependent inhibition were determined in the same experiment under conditions where the same batch of cells was in contact with the same concentration of the inhibitor for the same length of time. If, under a given set of experimental conditions, cell-dependent inhibition was demonstrable, the chemotactic factor–dependent inhibition obtained was due either wholly or in part to the effect of the phosphonate on the cell alone. However, as will be described, under certain circumstances, no cell-dependent inhibition was obtained. One could then speak of a "true" or "pure" chemotactic factor–dependent inhibition.

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

Trypan Blue Studies.—In order to determine if generalized damage to the polymorphonuclear leukocytes was produced by contact with phosphonate esters, the following experiment was carried out. Leukocytes were incubated with three phosphonate esters at concentrations sufficient to cause significant cell-dependent inhibition of chemotaxis. The details of this procedure were exactly the same as described above for the study of cell-dependent chemotaxis. After washing, the leukocytes were tested for their ability to respond to the chemotactic factor. At the same time, a drop of the cell suspension was mixed with an equal volume of 0.4% trypan blue dye (Matheson Scientific, Inc., Cincinnati, Ohio) in 80% rabbit serum. After incubation at room temperature for 15 min, at least 100 cells were examined by light microscopy and the per cent of stained cells determined.

RESULTS

Time Course of the Chemotactic Response.—In order to study the possible inhibitory effects of phosphonate esters on the chemotactic response, it became necessary to define the time course of the response of the cells to the chemotactic factor in the absence of the inhibitor. In each of three experiments performed on different days, chemotaxis was measured after intervals of chamber incubation of 15, 30, 60, and 90 min. The source of the chemotactic factor was rabbit serum which had been incubated with immune complexes. The number of cells migrating through the filter at each interval was compared with the value obtained at 90 min, which was taken as the 100% value (Fig. 1). Within 15 min 25–34% of cells responded, and this figure more than doubled by the end of 30 min. By 1 hr, the response was virtually completed, and only a few additional cells were responding at 90 min. In most of the following experiments, the 60 min chemotactic response was selected in assessing inhibition by phosphonate esters.

Kinetics of Inhibition of the Chemotactic Response.—12 of the phosphonate esters were used in defining the time course of inhibition of chemotaxis utilizing activated rabbit serum as the source of the chemotactic factor. Representative results obtained with four phosphonates in both cell-dependent and chemotactic factor–dependent inhibition are given in Fig. 2. The curves of inhibition are expected to be a function of the concentration of the inhibitor; nevertheless any one inhibitor was studied at only one concentration. With the propyl phosphonate and 5-aminopentyl phosphonate at the concentrations used it is evident that progressive chemotactic factor–dependent inhibition was obtained in essentially the complete absence of any cell-dependent inhibition (Fig. 2, A and D). With 5×10^{-4} M phenyl phosphonate, there was distinct cell-dependent inhibition, but the chemotactic factor–dependent inhibition was clearly greater at every interval tested (Fig. 2, C). On the other hand, with 5-chloropentyl phosphonate at a concentration of 5.0×10^{-4} M, the time course of the two modes of inhibition was the same (Fig. 2, B). It is doubtful that the apparent lag in the course of inhibition over the first 15–30 min with 5-chloropentyl phosphonate and the propyl phosphonate is real. Repetition of the same experiment with the 5-chloropentyl phosphonate did not reveal any such lag. The same sort of lag was seen in a single experiment performed with

the phenyl phosphonate and pentyl phosphonate, but on repetition of the experiment, no such lag was found.

As a result of these studies, in all subsequent experiments cells were uni-

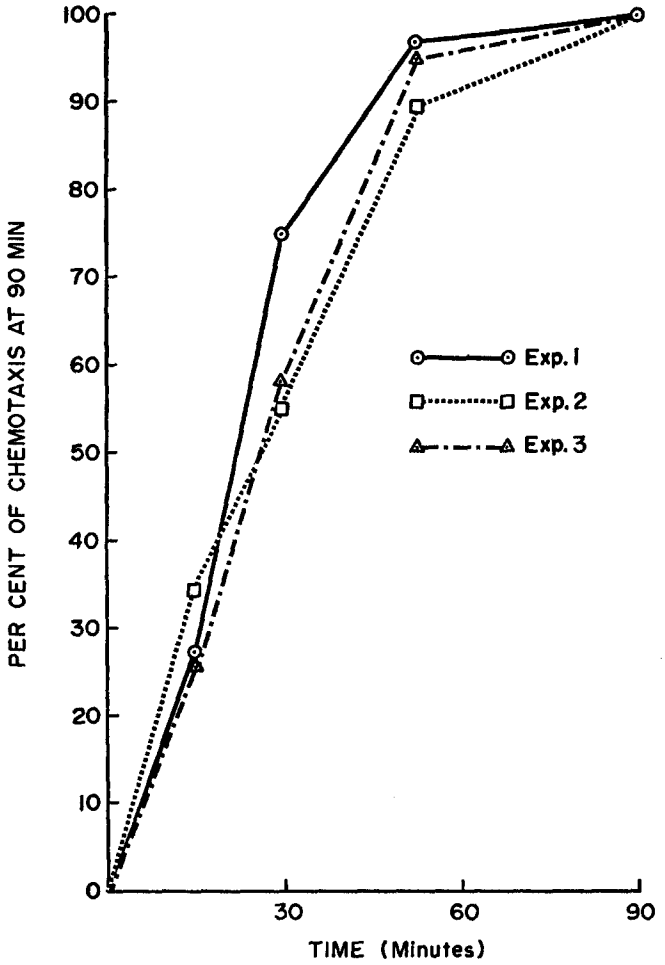

FIG. 1. The time course of chemotactic activity. The 90 min value was taken as the 100% value. Each experiment was performed on a different day.

formly placed in contact with each inhibitor for a 60 min period, whether for the study of cell-dependent or chemotactic factor–dependent inhibition.

Concentration Dependence of Phosphonate Inhibitors.—The effect of the concentration of phosphonate was determined for both cell-dependent and chemotactic factor–dependent inhibition. All experiments were duplicated at least once. The source of chemotactic factor included the partly purified prepara-

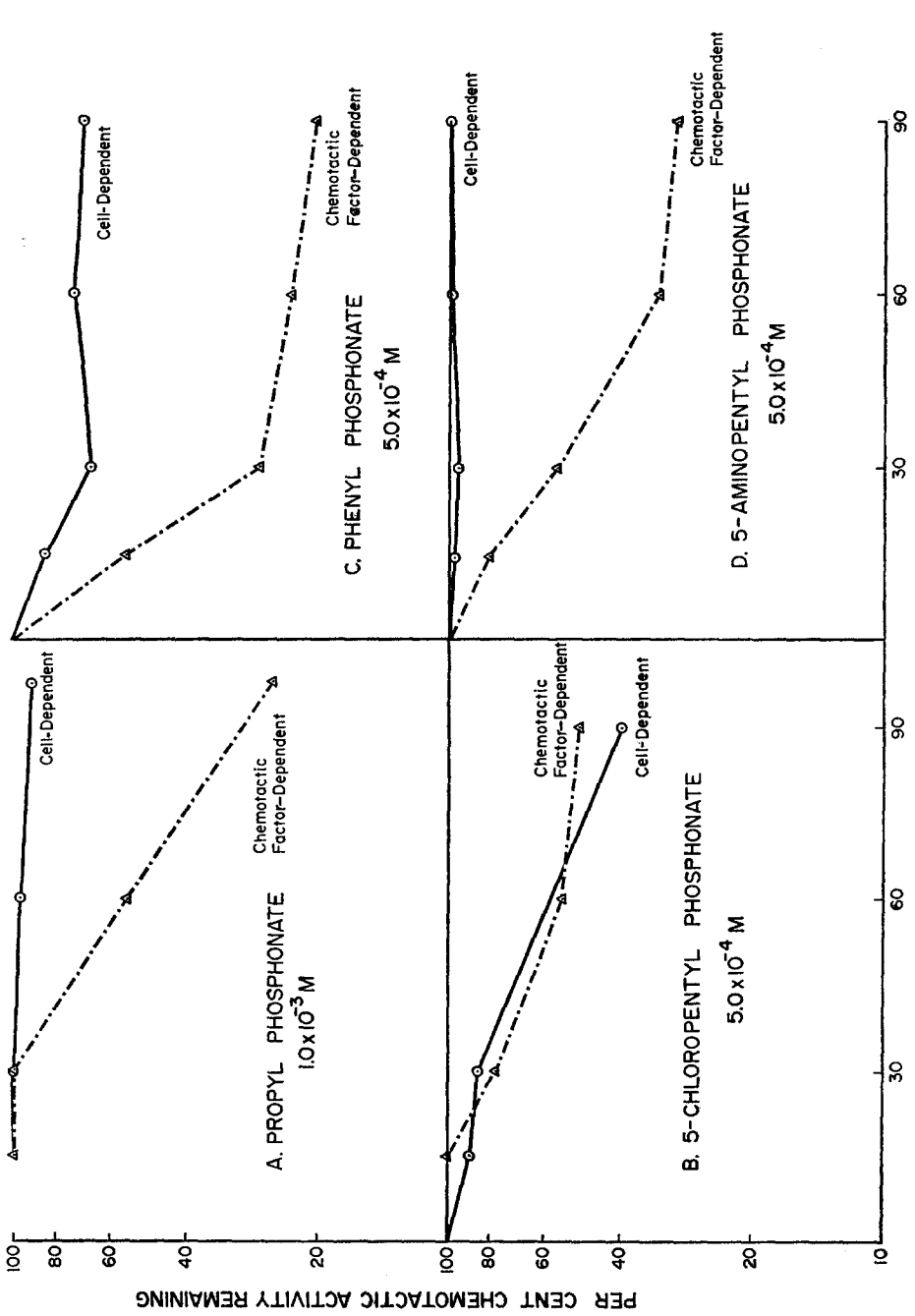

FIG. 2. The time course of cell-dependent and chemotactic factor-dependent inhibition by phosphonates. Results using representatives from each of the four available general classes of phosphonates are pictured. Various patterns of inhibition are evident.

tion as well as serum which had been treated with an immune precipitate. Inhibitors from each of the four available classes of phosphonate esters, alkyl, phenylalkyl, chloroalkyl, and aminoalkyl phosphonates were studied. A 60

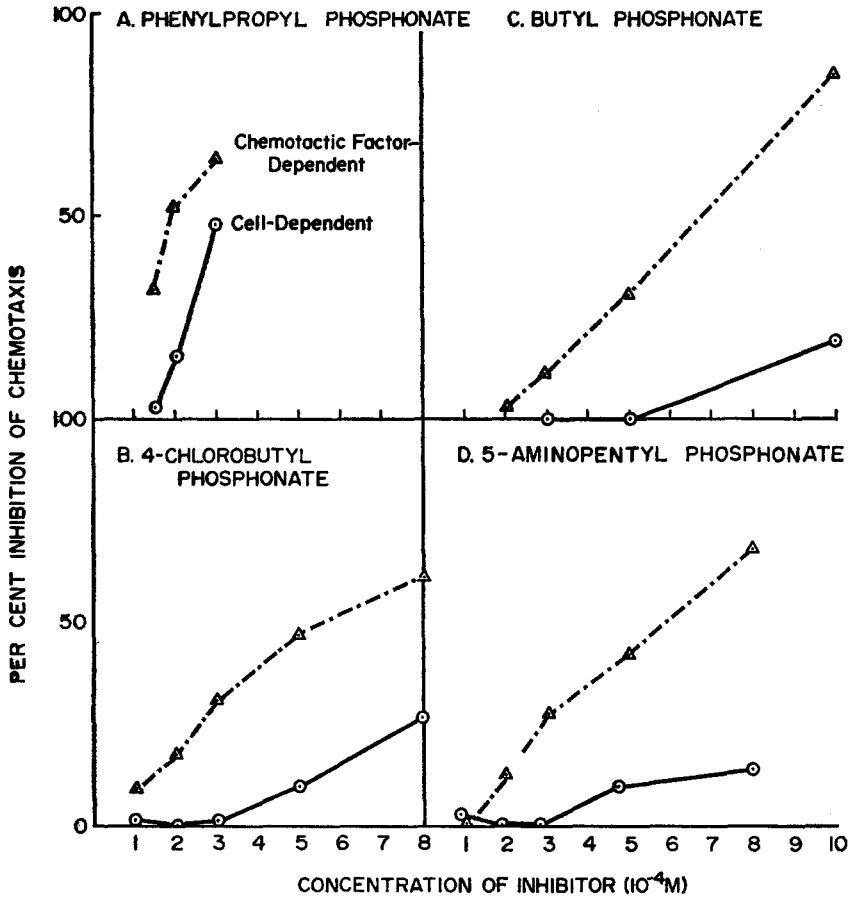

FIG. 3. The concentration dependence of inhibition of chemotaxis by phosphonates. The degree of inhibition is clearly a function of concentration of phosphonate.

min incubation period was used. With a given inhibitor the same batch of cells was employed for both cell-dependent and chemotactic factor–dependent inhibition. With all four inhibitors studied, phenylpropyl phosphonate, 4-chlorobutylphosphonate, butyl phosphonate, and 5-aminopentyl phosphonate, the concentration range was chosen so that the chemotactic factor–dependent inhibition was greater than the cell-dependent inhibition. (Fig. 3, A–D).

The requirement for a minimal concentration of inhibitor before an effect

was discernible is clearly evident with most of the inhibitors in both cell-dependent and chemotactic factor–dependent inhibition.

With the phenylpropyl phosphonate, the curves of inhibition were closely parallel and rose sharply and rapidly in the dose range from 1.5 to 3 × 10^{-4} M (Fig. 3, A).

With the other phosphonates shown (Fig. 3,B,C, and D), there was no cell-dependent inhibition evident over a fairly wide range of concentrations, even though chemotactic factor–dependent inhibition was clearly seen. In the

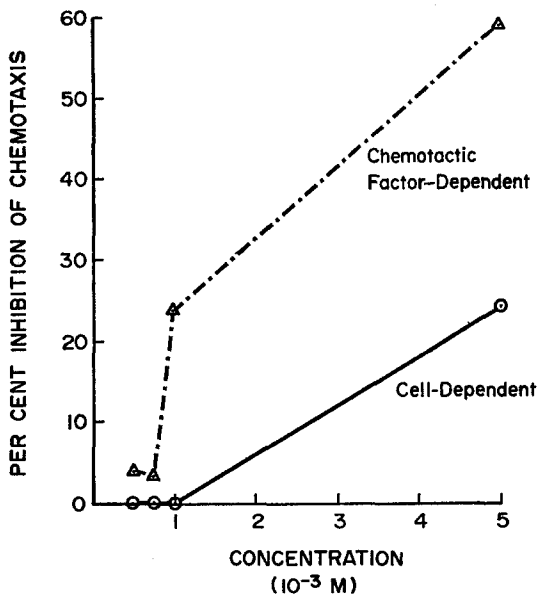

FIG. 4. The concentration dependence of inhibition of chemotaxis by diisopropylphosphofluoridate (DFP).

range of concentration in which both kinds of inhibition were obtainable, the degree of increase in cell-dependent inhibition with increasing concentration of phosphonate was less than in the case of the corresponding chemotactic factor–dependent inhibition.

Concentration Dependence of Inhibition by DFP.—Inhibition of chemotaxis by diisopropylphosphofluoridate (DFP) was studied in order to compare with the inhibitory effects of the phosphonate esters reported above. It was evident that both types of inhibition could be demonstrated with DFP, but substantially higher concentrations were required when compared with the phosphonates (Fig. 4). The dose of DFP required to produce 50% chemotactic-factor–dependent inhibition was 7–20 times greater than amounts of phosphonate esters giving comparable inhibition (see Fig. 3). As with the phos-

phonates, DFP gave distinct chemotactic factor–dependent inhibition at levels where no cell-dependent inhibition was detectable.

Effect of Direct Contact of Chemotactic Factor with Phosphonate Esters on its Chemotactic Activity.—As is evident from Figs. 3 and 4, the organophosphorus inhibitors could induce chemotactic factor–dependent inhibition at concentrations of inhibitor where the cell–dependent inhibition was zero. The question then arose if, under this circumstance, the chemotactic factor–dependent inhibition was caused by the direct inactivation of the chemotactic factor by the phosphonate ester. For this purpose samples of partly purified chemotactic factor were incubated for 1 hr at 37°C with 1×10^{-3} M 4-chlorobutyl phosphonate and with 1×10^{-3} M 5-aminopentyl phosphonate in 1:600 acetone, and with a 1:600 dilution of acetone in medium 199 as a control. At the end

TABLE I

Lack of Direct Effect of Phosphonates on Chemotactic Factor

Material tested	Chemotactic value
Blank (medium 199)	11
200 μl highly purified chemotactic factor in acetone (1:600)*	107
200 μl highly purified chemotactic factor, incubated with 4-chlorobutyl phosphonate (1×10^{-3} M) in acetone 1:600*	109
200 μl highly purified chemotactic factor, incubated with 5-aminopentyl phosphonate (1×10^{-3} M) in acetone 1:600*	117

* Approximately 70 μg N protein incubated for 1 hr at 37°C, followed by overnight dialysis in medium 199.

of the hr the mixtures were dialyzed overnight at 4°C to remove the inhibitor and then tested in the standard manner for their ability to induce chemotaxis of rabbit polymorphonuclear leukocytes.

As can be seen in Table I, treatment of the chemotactic factor with either inhibitor had no effect on the activity of the factor. This was despite the fact that these inhibitors at the concentration used (1.0×10^{-3} M) routinely give 80–100% chemotactic factor–dependent inhibition.

A previous study has shown that the peptide, *N*-carbobenzoxyglutamyl-L-tyrosine inhibited the activity of chemotactic factor by dissociation of the C′5, C′6, C′7 complex into its individual subunits (3). This raised the possibility that the phosphonates might also inactivate the chemotactic factor by causing dissociation of the complex. The lack of any effect of phosphonates on the chemotactic factor observed in the preceding experiment might then be due to reassociation of the complex after removal of the phosphonate. In order to test this possibility the effect of the phosphonate on the chemotactic factor complex was tested directly by means of density gradient ultracentrifugation.

Semipurified chemotactic factor (see Materials and Methods) was ultracen-

trifuged in sucrose density gradient. In one sample, 1×10^{-3} M 4-chlorobutyl phosphonate in 0.5% acetone was present throughout the gradient; in another 0.5% acetone alone was present. Both of these gradients were formed at pH

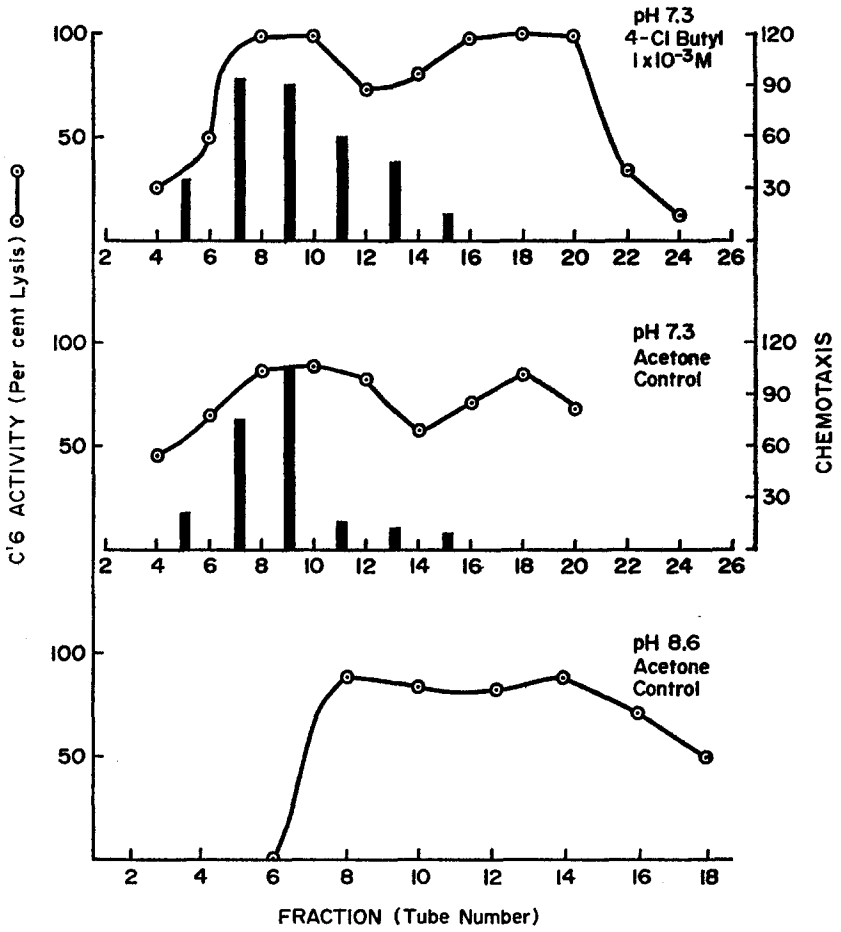

FIG. 5. The effect of 4-chlorobutyl phosphonate on sedimentation of partly purified chemotactic factor in a sucrose density gradient. Neither the rate of sedimentation nor the activity of the chemotactic factor is appreciably altered by the continued presence of the inhibitor at a concentration which is highly inhibitory for cells.

7.3. A third sample of the chemotactic factor was centrifuged at pH 8.6; at this pH most of the complex dissociates (2, 3). After ultracentrifugation, fractions were collected, diluted 1:10 in medium 199 or buffer solution, and tested for C'6 hemolytic activity as well as chemotactic factor activity, as already described.

The results are given in Fig. 5. In the sample in 0.5% acetone (Fig. 5, middle

frame), the C'6 had a bimodal distribution corresponding to the more rapidly sedimenting C'6 bound in the complex and the slower sedimenting, unassociated C'6. The chemotactic factor activity was present in the more rapidly sedimenting fractions as expected on the basis of previous observations (2). In the presence of 1×10^{-3} M 4-chlorobutyl phosphonate, the sedimentation behavior of C'6 and of chemotactic factor activity was the same (Fig. 5, top frame). There was no evidence of any dissociation of the complex by the phosphonate, despite the fact that the concentration of phosphonate used routinely gave high chemotactic factor–dependent inhibition.

The control at pH 8.6 (Fig. 5, bottom frame) showed the anticipated dissociation of the trimolecular (C'5, C'6, C'7) complex. It was obvious that no

TABLE II

Effects of Phosphonates on PMN Response to Highly Purified Chemotactic Factor

Inhibitor*	Concentration	Per cent inhibition of chemotactic response‡	
		Cell dependent	Chemotactic factor dependent
	M		
Butyl	8×10^{-4}	11	64
4-chlorobutyl	8×10^{-4}	29	78
5-aminopentyl	5×10^{-4}	42	78
Phenylpropyl	3×10^{-4}	31	65
DFP	3×10^{-3}	27	70

* Added to cell suspensions.

‡ 200 μl (approximately 70 μg N) highly purified chemotactic factor in medium 199.

such dissociation of the complex occurred in the presence of the phosphonate inhibitor.

Inhibition of Chemotaxis by Phosphonates Using Highly Purified Chemotactic Factor.—In several of the preceding experiments, unpurified or partially purified chemotactic factor had been used, raising the unlikely possibility that the chemotactic factor–dependent inhibition might have been due to an interaction of the phosphonate with some constituent in the preparation other than the chemotactic factor. It was therefore deemed necessary to test the inhibitory action of a representative group of phosphonates on chemotaxis induced by the highly purified factor. Four phosphonates, the butyl, 4-chlorobutyl, 5-aminopentyl, and phenylpropyl phosphonates, and DFP were used. Table II shows that these organophosphorus inhibitors caused the same general degree of cell-dependent and chemotactic factor–dependent inhibition of the response to highly purified chemotactic factor as to the less purified preparations of chemotactic factor.

Profiles of Inhibition of Chemotaxis by Phosphonates.—For each of the four

series of phosphonates (alkyl, phenylalkyl, chloroalkyl, and aminoalkyl phosphonates), the relationship was determined between the length of the alkyl chain and the potency of the phosphonate in giving cell-dependent and chemo-

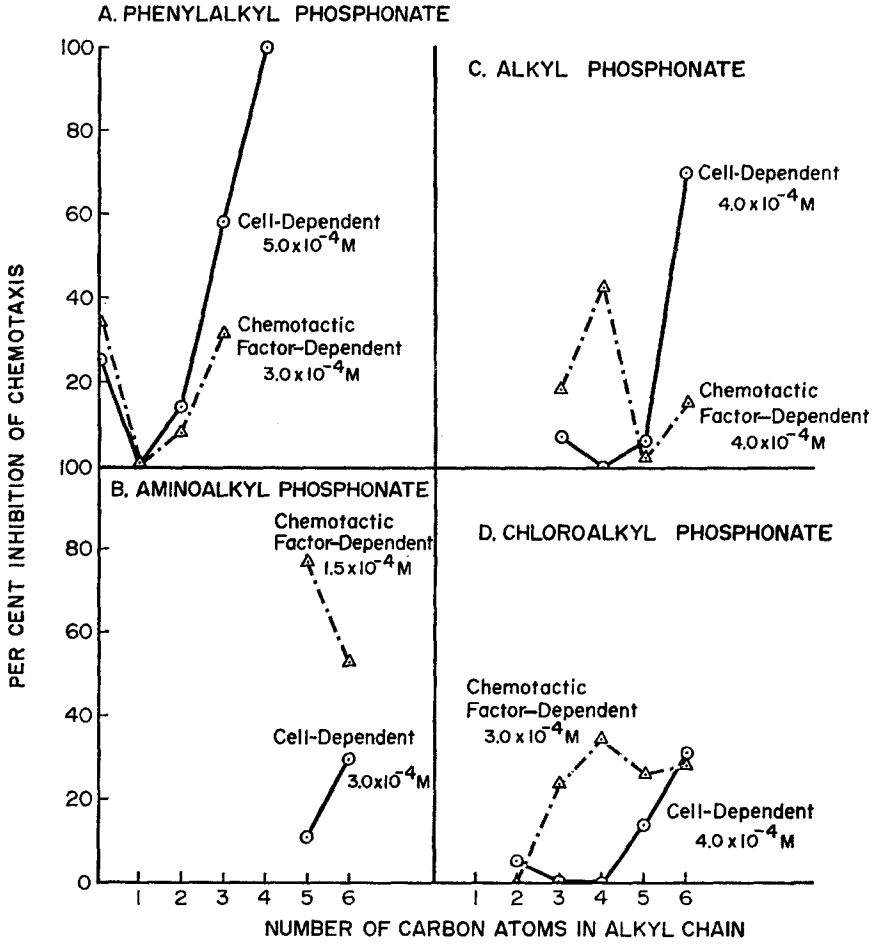

FIG. 6. Structure-activity relationships found for various phosphonates in cell-dependent and chemotactic factor–dependent inhibition. Distinct and divergent patterns for the two types on inhibition are seen for each class of phosphonates except the phenylalkyl series.

tactic factor–dependent inhibition. All members of a given series were tested at a single concentration of phosphonate. Because of the day to day variation in susceptibility of the leukocytes to the phosphonates, a single batch of cells was used each time a series of phosphonates was compared. Activated serum served as the source of chemotactic factor in these experiments. When the chemotactic factor–dependent inhibition was studied, the concentration of the

phosphonate was chosen to be low enough so that in that given experiment there was no cell-dependent inhibition. The lack of cell-dependent inhibition at the chosen concentration was demonstrated in the same experiment. This precaution was necessary in order that a "pure" chemotactic factor–dependent inhibition be obtained, and not one which had superimposed cell-dependent inhibition. Each comparison was repeated at least once.

Fig. 6 shows the results obtained. In the phenylalkyl series (Fig. 6, A), the cell-dependent and chemotactic factor–dependent inhibition profiles were the same, as far as they could be tested. In the phenylalkyl series, interposition of one carbon between the phenyl ring and the phosphorus to form the benzyl

TABLE III

Effect of Substitution of Chloro or Amino Groups on the Activity of Alkyl Phosphonates in Inhibiting Chemotaxis

Phosphonate	Inhibition of chemotaxis*	
	Cell-dependent	Chemotactic factor–dependent
3×10^{-4} M		
Pentyl	0	0
5-chloropentyl	63	55
5-aminopentyl	6	61
Hexyl	96	93
6-chlorohexyl	14	49
6-aminohexyl	25	77

* Expressed as per cent reduction of response compared with untreated cells. Activated rabbit serum (10%) was used as the source of chemotactic factor. As indicated in Materials and Methods, for cell-dependent inhibition PMN's were pretreated with the phosphonate inhibitors. For chemotactic factor–dependent inhibition, the inhibitors were added to cells immediately before addition to chambers.

phosphonate caused a marked reduction in inhibitory capacities. Increasing the chain length by one or two additional carbons resulted in increasing inhibition of both types (Fig. 6, A). The effectiveness of the phenylbutyl phosphonate in chemotactic factor–dependent inhibition could not be studied since at any usable concentration it gave 100% cell-dependent inhibition. In the aminoalkyl phosphonates (Fig. 6, B), the change from the pentyl to the hexyl compound resulted in different effects on the two types of inhibition, there being a reduction in chemotactic factor–dependent inhibition and an enhancement of the cell-dependent inhibition. With the alkyl phosphonate series, the butyl compound was most inhibitory for chemotactic factor–dependent inhibition, whereas the cell-dependent inhibition was strikingly increased going from the four or five carbon phosphonate to the six carbon hexyl phosphonate (Fig. 6, C). In the chloroalkyl series it was found that chemotactic factor–dependent

inhibition was maximal at the four carbon chain, with a slight lessening in inhibitory activities as the chain length increased to five and six carbons (Fig. 6,D). At the concentration employed, there was little or no cell-dependent inhibition in the chloroalkyl series until the pentyl and hexyl phosphonates were reached (Fig. 6,D).

Effect of the Substitution of an Amino or Chloro Group in the Alkyl Chain of the Phosphonates.—The pentyl and hexyl phosphonates and their ω-amino and ω-chloro derivatives were tested at the same concentration in the same

TABLE IV

Uptake of Trypan Blue in Cells Inhibited by Various Phosphonates

Inhibitor used	Concentration	Experiment A		Experiment B	
		Chemotaxis	Dye uptake	Chemotaxis	Dye uptake
	M				
5-chloropentyl	6×10^{-4}	50 (68%)*	0‡	90 (71%)	0
" "	4×10^{-4}			215 (31%)	0
Hexyl	5×10^{-4}	0 (100%)	85	0 (100%)	95
	3×10^{-4}	17 (95%)	21	5 (98%)	34
	2×10^{-4}			106 (66%)	3
	1×10^{-4}			203 (35%)	0
Phenylbutyl	8×10^{-5}	30 (84%)	0	160 (49%)	0
	6×10^{-5}	70 (53%)	0	230 (27%)	0
	4×10^{-5}	118 (16%)	0	310 (1%)	0
None	—	137	0	314	0

* Number of migrating cells determined by standard assay method. Figure in parenthesis refers to reduction of the chemotactic response by comparison to positive controls listed at the bottom of each vertical column.

‡ Per cent of cells (100 counted) with uptake of trypan blue.

experiment for both cell-dependent and chemotactic factor–dependent inhibition (Table III). The substitution of a chloro group for a terminal hydrogen in pentyl phosphonate resulted in a substantial increase in cell-dependent inhibitory capacity, whereas, the substitution of an amino group had little or no effect. On the other hand, the increase in chemotactic factor–dependent inhibition going from the pentyl to the chloropentyl phosphonate could be wholly accounted for by the superimposed cell-dependent inhibition. The latter, however, could not account for the effectiveness of the aminopentyl compared to the pentyl phosphonate.

The cell-dependent inhibition given by the hexyl phosphonate was decreased to about the same extent when either the amino or chloro group was substi-

tuted. The high cell-dependent inhibition made the significance of the high chemotactic factor–dependent inhibition given by the hexyl phosphonate impossible to evaluate. This in turn made it impossible to compare the effect of a substitution of the chloro or amino group in the hexyl phosphonate on chemotactic factor–dependent inhibition.

Trypan Blue Uptake by Leukocytes Treated with Phosphonate Esters.—The exclusion of the dye, trypan blue, was used to determine the extent of cell damage after contact with three phosphonate esters (Table IV). The 5-chloropentyl, hexyl, and phenylbutyl phosphonates were used in concentrations which were known to produce significant cell-dependent inhibition. Incubation of leukocytes with the hexyl compound at the higher concentrations (5×10^{-4} and 3×10^{-4} M) caused significant uptake of trypan blue dye. However, at concentrations below 3×10^{-4} M, no significant uptake of the dye by leukocytes was noted (3% of cells), even though as much as 66% cell-dependent inhibition occurred. With the 5-chloropentyl and phenybutyl phosphonates as much as 84% cell-dependent inhibition occurred in the absence of demonstrable uptake of the dye. As a result of these experiments, it became clear that phosphonate-induced cell-dependent inhibition was not associated with generalized cell damage as defined by the ability of a leukocyte to exclude trypan blue.

DISCUSSION

These studies have clearly established that phosphonate esters and diisopropylphosphofluoridate can inhibit the chemotactic response of polymorphonuclear leukocytes to the complement-associated chemotactic factor. The action of these organophosphorus inhibitors is at least twofold. As is evident from what we term "cell-dependent inhibition" there is a direct, irreversible action of the phosphonate on the cells. In addition to this, there is a "chemotactic factor–dependent inhibition" which requires the interaction of the chemotactic factor and the leukocyte for its manifestation.

The cell-dependent inhibition is progressive; the greater the length of time the cells are in contact with the inhibitor, the greater the degree of inhibition (Fig. 2, A–D). It is also irreversible: removing the cells from the organophosphorus compound does not restore their ability to respond to the chemotactic factor. There is a distinct specificity of the inhibition which is related to phosphonate structure, as is evident from Fig. 6. All of these properties lead to the conclusion that a cell-bound serine esterase existing in an already activated state is necessary for the chemotactic response. Whether this already activated esterase exists as such, is activated during the development of the glycogen-induced peritoneal exudate, or is activated by cells standing at room temperature during the incubation with phosphonate, was not determined.

The inhibition profiles given by the phenylalkyl, alkyl, and chloroalkyl phos-

phonates in the system measuring cell-dependent inhibition are completely different from those obtained by the same three series of phosphonates reacting with chymotrypsin, trypsin, acetylcholine, and C'1a (5, 7). The inhibition profiles found with the 5-aminopentyl and 6-aminohexyl phosphonate in cell-dependent inhibition are dissimilar to the inhibition profiles for C'1a, trypsin, and acetylcholine but similar to chymotrypsin (5). These dissimilarities indicate that the already activated esterase of the polymorphonuclear leukocyte which is blocked by the organophosphorus inhibitors is none of these well characterized enzymes. This conclusion is substantiated by the substrate specificity of the activated leukocyte enzyme reported in the next paper (9).

The inhibition profiles given by these same four series of phosphonate esters when they are reacted with the antigen-antibody–activated esterases of the guinea pig lung (4) and the rat mast cell (6, 7) are distinctly different from the cell-dependent inhibition profiles. These differences indicate that the precursor enzymes of the mast cells of the rat and guinea pig are not the same as the already activated esterase of the polymorphonuclear leukocyte.

In experiments dealing with the release of histamine from rat peritoneal mast cells, an unexpected effect of certain of the phosphonate esters was found. At lower doses the phosphonate ester actually enhanced the release of histamine. It was shown that the few phosphonates giving this behavior did so by virtue of an impurity present in the preparations (6). Most of the phosphonates used here were those shown to be free of the impurity. The three compounds used which did contain the impurity, the hexyl, 6-aminohexyl, and 6-chlorohexyl phosphonates, did not lead to any such anomalous results in the chemotaxis system. Moreover, the results of substrate protection tests using the hexyl phosphonate showed that the inhibition obtained was due to the phosphonate, and not the impurity (9). The rabbit polymorphonuclear leukocyte is apparently similar to the sensitized guinea pig lung in not being susceptible to the action of the impurity (4).

Under some circumstances, (Fig. 2, B), chemotactic factor–dependent inhibition can be completely accounted for by the inhibition of the activated esterase of the leukocyte. This is clearly not the case in all circumstanes. Significant chemotactic factor–dependent inhibition can occur during a period of contact of cell with inhibitor (Fig. 2, A and D) or at a concentration of inhibitor (Fig. 3) where the cell-dependent inhibition is negligible or nonexistent. All the results shown in Fig. 6 on the specificity of the chemotactic factor–dependent inhibition were at concentrations of phosphonates where no significant cell-dependent inhibition was observed over the 60 min incubation period. From Fig. 6 it is also clear that, except for the phenylalkyl phosphonates, the inhibition profiles given by the cell–dependent and "pure" chemotactic factor–dependent inhibition are completely different.

From these results, it is evident that under some circumstances inhibition

of chemotaxis by the organophosphorus compounds can occur only in the presence of the chemotactic factor. A direct irreversible action of the phosphonate on the chemotactic factor itself was eliminated by the experiment reported in Table I in which the chemotactic factor was incubated with the phosphonates in the absence of cells. The experiments illustrated in Fig. 5 showed that the phosphonates do not reversibly dissociate the C'5, C'6, C'7 complex and thus destroy its activity.

In "pure" chemotactic factor–dependent inhibition there is, therefore, a lack of any direct effect of the phosphonates on the chemotactic factor or on the cell, but the chemotactic factor must interact with the cells in order for the inhibition to occur. Since the phosphonates are irreversible enzyme inhibitors, this implies that they inhibit by reacting with an esterase which has been activated by the chemotactic factor. This esterase exists in or on the leukocyte in a phosphonate-resistant precursor form until activated by the chemotactic factor. The term "activatable" esterase will be used to describe this enzyme.

The chemotactic factor–dependent inhibition profile given by the phenylalkyl phosphonates (Fig. 6) is the same as the profile of the cell-dependent inhibition reaction. In work not reported here, the phenylbutyl phosphonate was tested over a concentration range from 4×10^{-5} to 5×10^{-6} M for its activity in cell-dependent and chemotactic factor–dependent inhibition. In this concentration range, the phenylbutyl phosphonate was slightly but consistently more reactive in chemotactic factor–dependent inhibition than in cell-dependent inhibition. This suggests that the phenylbutyl phosphonate at these low concentrations may still be capable of reacting with the enzyme which has been rendered susceptible by the chemotactic factor to inhibition by the phosphonate. If so, this would make the phenybutyl phosphonate more reactive than the phenylpropyl phosphonate in chemotactic factor–dependent inhibition, as well as cell-dependent inhibition. This conclusion is uncertain, however, since it was impossible to find a concentration of the phenylbutyl phosphonate in which there was a clearcut chemotactic factor–dependent inhibition in the absence of cell-dependent inhibition.

The chemotactic factor–dependent inhibition profiles given by the aminoalkyl (Fig. 6, B), alkyl (Fig. 6, C) and chloroalkyl phosphonates (Fig. 6, D) differ in almost every detail from the cell-dependent inhibition profiles. The effect of substituting a chloro or amino group on the pentyl phosphonate on the ability of these phosphonates to give either cell-dependent or chemotactic factor–dependent inhibition is also different (Table III). These differences make it clear that the esterase activated by the chemotactic factor is not the same as the esterase whose inhibition is responsible for the cell-dependent inhibition.

The esterase of the polymorphonuclear leukocyte activated by the chemotactic factor also differs from acetyl cholinesterase, chymotrypsin, and trypsin

(4, 5). The inhibition profiles given by the reactions of the alkyl phosphonates, and the two aminoalkyl phosphonates with C'1a and the activatable enzyme of chemotaxis are essentially the same (4, 6). However, the optimum activity against C'1a in the chloroalkyl phosphonate series is reached by the 3-chloropropyl phosphonate, whereas the peak activity against the activatable enzyme of chemotaxis is given by the 4-chlorobutyl phosphonate (6). Moreover, in the phenylalkyl series the benzyl phosphonate has minimum activity against chemotaxis, but the phenylethylphosphonate is the least active against C'1a (7).

The inhibition profile given by the reaction of the phenylalkyl phosphonates with the activatable enzyme of chemotoxis is distinctly different from that found when the same series of phosphonates inhibits the antigen-induced histamine release from sensitized guinea pig lung (4). However, the changes in reactivity of esterase with phosphonate structure are similar when the responses of the two biological systems to the chloroalkyl, alkyl, and aminoalkyl phosphonates are compared (4).

The distinct similarities found in the various inhibition profiles indicate that the activatable enzyme of chemotaxis, C'1a, and the serine esterase activated during the antigen-induced release of histamine from sensitized guinea pig lung have a similar although not identical specificity; i.e., they are "parazymes" (4).

There is a much greater dissimilarity in the inhibition profiles given by the reaction of the phenylalkyl and chloroalkyl phosphonates with the activated esterase of chemotaxis, and the esterase involved in the previously studied allergic release of histamine from rat peritoneal mast cells (6, 7).

The present work brings out a distinct resemblance between the chemotaxis of polymorphonuclear leukocytes induced by the chemotactic factor, and the release of histamine from rat and guinea pig mast cells induced by the antigen-antibody reaction (6, 7). In all cases, the activation of a precursor esterase is required for the specific response. The possibility that an already activated esterase is also needed for the antigen-antibody–induced release of histamine from guinea and rat mast cells has not been investigated under conditions similar to those used here. In view of the present findings in regard to the cell-dependent inhibition of chemotaxis by phosphonates, a similar study of the antigen-antibody–induced release of histamine is indicated. Lichtenstein and Osler (10) have already obtained cell-dependent inhibition by DFP of antigen-induced histamine release from sensitized human leukocytes. In this mode of histamine release, however, no evidence for a precursor esterase could be obtained (10). This latter point also needs restudy.

Finally, it is important to point out the data which emphasize the specificity of inhibition of chemotaxis by various phosphonate esters. The fact that no uptake of the dye trypan blue was found in leukocytes treated with phosphonate esters under conditions resulting in a high degree of cell-dependent inhi-

bition indicates that the effect on the cells is not one of generalized cell damage. Cells so treated still retain the ability to exclude the dye. This is in keeping with the idea that the phosphonates inhibit cell responsiveness to the chemotactic factor by blocking a specific enzyme system.

SUMMARY

Studies in the time course of the response of rabbit polymorphonuclear leukocytes (PMN's) to the complement-associated chemotactic factor have revealed that the response is virtually complete by 60 min with less than 15% additionally responding cells thereafter.

Phosphonate esters with a well defined capacity to inhibit serine esterases have been used to study the cell-associated enzymes of the rabbit PMN required for the chemotactic response. Two types of inhibition of the cell response to the chemotactic factor have been found: (*a*) *cell-dependent inhibition* occurring as a result of pretreatment of PMN's with phosphonate esters; and (*b*) *chemotactic factor-dependent inhibition* demonstrated only when the phosphonate ester is present during the chemotactic response.

Differences were found in these two modes of inhibition caused by various phosphonates, in terms of their time course of inhibition, in the dose response curves, and in the structure-activity relationships.

It has been conclusively demonstrated that the phosphonate esters have no direct inhibitory effect on the chemotactic factor. This has been shown by retention of activity of the chemotactic factor following incubation with phosphonate esters and subsequent removal by dialysis. In addition, the activity of the chemotactic factor and its physical-chemical characteristics in density gradient ultracentrifugation were unaltered in the continued presence of a potent phosphonate inhibitor of chemotaxis.

The uptake of the dye trypan blue was studied in cells treated with phosphonate in such a manner to induce cell-dependent inhibition of chemotaxis. Even when 84% cell-dependent inhibition of chemotaxis occurred, no uptake of the dye by leukocytes was found. Thus, phosphonate-induced inhibition of cell responsiveness in chemotaxis was not associated with generalized cell damage as defined by exclusion of the dye.

It is concluded that *cell-dependent inhibition* is due to the presence of a cell-bound esterase which is already activated and thus susceptible to inhibition by phosphonate esters *before* contact of the cell with the chemotactic factor. The second type of inhibition, *chemotactic factor-dependent inhibition*, is considered due to a cell-bound esterase which becomes susceptible to inhibition by phosphonate esters only *after* contact of the PMN with the chemotactic factor. It is postulated that the chemotactic factor activates this phosphonate-resistant precursor making it susceptible to the inhibitory action of the phosphonate ester.

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