The Phagocytic Cell in Host Resistance

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Introduction

The long-intriguing phagocytic c ll came into its own with the work of Metchnikoff a century ago. It was Metchnikoff who established that phagocytic cells serve a protective function, that they assist in resisting infection. This was in direct opposition to the current thought that the mobile phagocytes spread disease by circulating ingested organisms to other parts of the body.

Metchnikoff's work was contrary also to a belief that humoral factors played a greater role in natural resistance than cellular factors. In time, the vital importance of phagocytic cells for host resistance was recognized. Research is currently directed toward both the phagocytic cell and the humoral factors which together form a defense mechanism of great biological value. An appreciation of how this defense mechanism works is gradually being gained.

This publication describes the state of our understanding of the role of the phagocytic cell in host resistance. It testifies to a sophistication of research approach and a developing technology. It also testifies to the blind spots remaining and the concomitant research questions needing answers. The book was occasioned by a conference of leading scientists in both basic and clinical research. The current data were presented on chemotaxis and the phagocytic cell, endocytic events, biochemistry of phagocytic function, and bacteriocidal activity. The role of complement and the lymphokines was also considered to ensure coverage of the role of humoral factors in phagocytosis. One session was devoted to phagocytic defects to permit discussion of defined clinical entities, as well as the biochemical mechanisms involved and the current data on various chemotactic defects in both neutrophils and mononuclear leukocytes. Another session was devoted to maturational defects in leukocyte function, focusing on changes occurring during development in chemotaxis, macrophage antigen processing, and biochemistry of leukocytes.

This is the seventh in a series of publications released on developmental immunology by the National Institute of Child Health and Human Development to investigate the developmental events throughout the lifespan that contribute to effective host resistance. The effort continues to embrace a holistic approach to the development of immunologic competence to facilitate understanding of the totality of the mechanisms involved as well as time-dependent processes.

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Enzyme Activation and the Mechanism of Polymorphonuclear Leukocyte Chemotaxis

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There is general, although not universal agreement, that chemotaxis is a major, if not the major, mechanism by which polymorphonuclear leukocytes and other phagocytic cells are induced to move to the site of infection and thus function in host resistance. In addition, phagocytosis, chemotaxis, and lysosomal enzyme secretion are all manifestations of cell movement: either the movement of the whole cell, as in chemotaxis; movement of a part of the cell membrane and contiguous structures, as in phagocytosis; or the movement of an intracellular organelle from the interior of the cell to either a phagocytic vesicle or the plasma membrane, as in lysosomal enzyme secretion. There are numerous similarities as well as some differences in the mechanisms underlying these diverse functions of the leukocyte (reviewed in ref. 1), so that a consideration of the mechanism of one might be expected to throw light on the mechanisms of the others.

In what follows I shall review the present fragmentary state of our knowledge of the biochemistry of chemotaxis of the polymorphonuclear leukocyte, first paying particular attention to the involvement of the activation of a proesterase, proesterase 1, in the chemotactic process. Then I shall proceed to a very brief and incomplete overview of our knowledge of the other processes involved in the chemotactic response of these cells. (For a more complete review see refs. 1 and 2.) Finally, I shall present a rather diffident general hypothesis of the genesis of the chemotactic process in the polymorphonuclear leukocyte with some heretofore unpublished results of experiments suggested by this theory.

ACTIVATION OF PROESTERASE 1

Di-isopropyl phosphofluoridate and *p*-nitrophenyl ethyl phosphonates are relatively specific irreversible inactivators of a group of enzymes, the so-called serine esterases (serine esterases because they have serine in their active site). These organophosphorous inhibitors depress the chemotactic response of rabbit peritoneal neutrophils if they are present while the neutrophil is being acted upon by the chemotactic agent but not if they are used to pretreat the neutrophil or the chemotactic factor separately. This suggested that the chemotactic response involved the activation of a so-called "activatable esterase" which before contact of the cell with chemotactic factor existed in an enzymatically inert, phosphonate

insusceptible form (3). The various series of *p*-nitrophenyl ethyl phosphonates yield structure-activity relationships, "inhibition profiles" which are characteristic of and serve to identify various serine esterases (4). The inhibition of the chemotactic response by the *p*-nitrophenyl ethyl phosphonates shows characteristic and specific inhibition profiles (3).

Leukocytes incubated with one of the complement-derived chemotactic factors C3a, C5a, or $C\overline{567}$ progressively lose their ability to respond chemotactically to any of the chemotactic factors (5, 6). They are desensitized or as we termed it "deactivated." The deactivation is prevented by the same phosphonates that inhibit chemotaxis and the inhibition profiles are the same for the two processes (5). This indicated that the same activatable esterase is involved in both chemotaxis and deactivation. However, not only phosphonate esters prevent deactivation and chemotaxis; aromatic amino acid derivatives rather specifically do the same. This suggested the hypothesis that the activatable esterase is the precursor of an esterase capable of hydrolyzing aromatic amino acid esters (5). The rabbit polymorphonuclear leukocyte was found to contain an enzyme, esterase 1, which hydrolyzed the aromatic amino acid ester acetyl DL phenylalanine β naphthyl ester, previously used as a substrate for chymotrypsin (7). When the *p*-nitrophenyl ethyl phosphonates were used to inactivate esterase 1, the enzyme gave the same inhibition profiles as found for the activatable esterase in both the inhibition of chemotaxis and the prevention of deactivation. From this, we concluded that esterase 1 was the activated form of the activatable esterase of chemotaxis (7). Esterase 1 is also found in an enzymatically inert, phosphonate insusceptible form which we termed proesterase 1 (8). When the chemotactic factors interact with the polymorphonuclear leukocyte, proesterase 1 is transformed into esterase 1, fully capable of being inhibited by the phosphonate esters and of hydrolyzing acetyl DL phenylalanine β naphthyl ester (8, 9). From this we concluded that proesterase 1 is the activatable esterase of chemotaxis (8).

Both the complement-derived factors $C\overline{567}$, C3a, C5a and a bacterial chemotactic factor activate proesterase 1, although with differing degrees of effectiveness (8, 9). Moreover, there is evidence suggesting that under several different circumstances the level of chemotactic activity attained is related to the degree of activation of proesterase 1 (9).

More recently, I have used the phosphonates to study whether activation of proesterase 1 is involved in the spontaneous motility of the rabbit polymorphonuclear leukocytes. The spontaneous motility of the leukocytes was measured as described previously (9). The rabbit peritoneal polymorphonuclear leukocytes were suspended in Hank's buffer in the top compartment of the modified Boyden chemotaxis chamber and Hank's buffer was placed in the bottom compartment. A 5-μm filter separated the two compartments. The chamber was incubated for 90 min at 37°C (rather than 3 hr as previously described) before counting the number of cells in 5 high-power fields (5 HPF) that penetrated the filter below the upper monolayer. The inhibition profiles were obtained by incubating the *p*-nitrophenyl ethyl alkylphosphonates and the *p*-nitrophenyl ethyl chloroalkyl-

phosphonates with the cells at a final concentration of 0.33 mM. The inhibition profiles so obtained (Fig. 1) are the same as those found for the so-called chemotactic factor dependent inhibition of chemotaxis, for the prevention of deactivation, and for esterase 1. This suggests that the activation of proesterase 1 is also involved in the spontaneous motility of the leukocyte (see below).

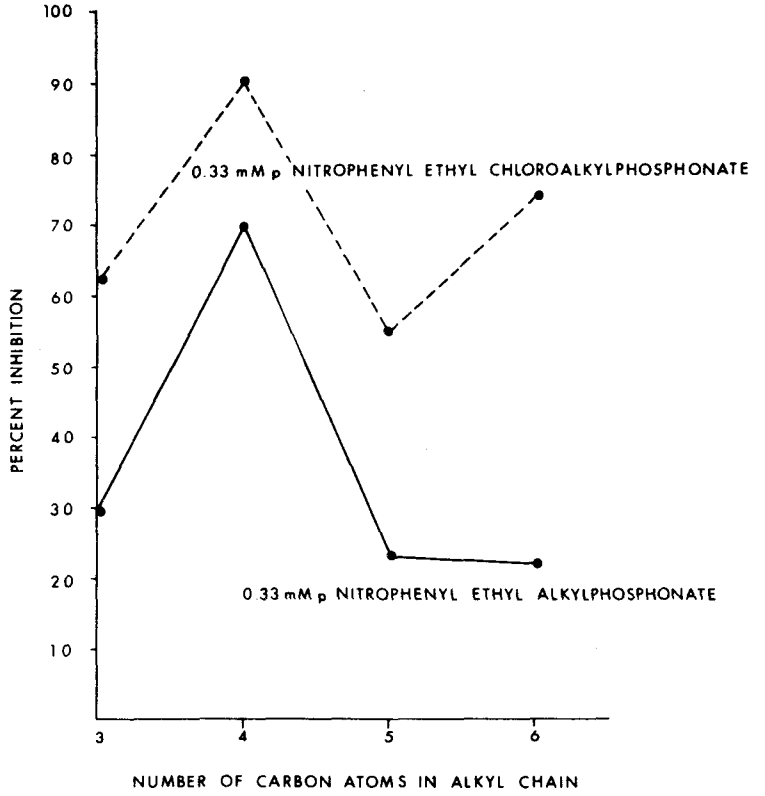

FIG. 1. The profiles of inhibition of spontaneous motility employing the *p*-nitrophenyl ethyl chloroalkylphosphonates and *p*-nitrophenyl ethyl alkylphosphonates.

Very little is known about the properties of proesterase 1. Its activation by C5a does not require divalent cations, implying that the step(s) dependent on divalent cations come after the proesterase activation (10). Dr. Peter Henson and I, on the basis of unpublished work, have estimated that there are at least 500 proesterase 1 molecules per rabbit blood polymorphonuclear leukocyte. We obtained this estimate using radioactively labeled DFP. In this study we first reacted the leukocytes with cold DFP to inactivate all the serine esterases present in an active form, washed the cells thoroughly, activated the proesterase with C5a, and then treated the cells with radio-labeled DFP. The amount of label taken up over and

above that of control cells reacted with C5 was our measure of specific uptake attributable to the proesterase 1 that had been activated by C5a. The results are very preliminary and much more work has to be done, but I believe it does give us an order of magnitude as to the number of proesterase 1 molecules/cell.

We know little about the nature of esterase 1 and nothing concerning its function in the leukocyte. Esterase 1 is found predominately in the microsomal fraction of the neutrophil (unpublished work and 11). Efforts to isolate esterase 1 from the neutrophil have met with great difficulty and little success. This is in part due to the relatively small amount in the cell and its instability and ready breakdown, presumably by the proteolytic enzymes of the cell. When first isolated from cells lysed with Triton X-100, the enzyme has a molecular weight somewhat greater than 150,000. When acted upon by enzymes found in the cell, the activity becomes associated with a fraction of a molecular weight of 40,000 to 60,000.

OTHER ASPECTS OF THE MECHANISMS OF NEUTROPHIL CHEMOTAXIS

Ca^{2+} and Mg^{2+} are both required in the external medium for maximal chemotactic response (12, 13). Maximal spontaneous motility can be attained in the absence of Ca^{2+} by sufficiently increasing the concentration of Mg^{2+} alone, although Ca^{2+} reduces the concentration of Mg^{2+} required (9). However, the reverse is not true: increasing the concentration of Ca^{2+} in the absence of Mg^{2+} does not induce maximal spontaneous motility. Gallin and Rosenthal (13) showed that C5a enhances the efflux of $^{45}Ca^{2+}$ from the cell and it was the efflux which correlated with chemotactic responsiveness. Increasing the K^+ of the medium increases the chemotactic responsiveness; ouabain inhibits chemotaxis, the inhibition being reversed by K^+ (14).

The action of various inhibitors suggests that, as with phagocytosis or lysosomal enzyme secretion, anaerobic glycolysis is the major if not sole source of energy involved in cell motility and chemotaxis (reviewed in 1 and 2). Agents which increase intracellular cyclic AMP levels inhibit the motility of rabbit polymorphonuclear leukocytes and thus depress their chemotactic responsiveness (15). However, neither a bacterial chemotactic factor nor C5a has any effect on the intracellular concentrations of cyclic AMP suggesting that changes in the adenyl cyclase activity of the cell may modulate its motility and thus its chemotactic responsiveness but that adenyl cyclase is not itself a part of the direct biochemical sequence involved in chemotaxis or cell motility (15).

In contrast to β adrenergic agents that stimulate adenyl cyclase causing inhibition of cell motility and chemotaxis (15), cholinergic agents, such as acetylcholine, carbachol, or phorbal myristate acetate, that increase cellular cyclic 3′5′-guanosine monophosphate (cyclic GMP) enhance chemotactic activity (16). The chemotactic enhancement by acetylcholine and carbachol is inhibited by atropine. The effect of the cholinergic agents on spontaneous cell motility was not directly tested. The results, however, were considered as supporting the hypothe-

sis that cyclic GMP promotes cellular events that are antagonistic to those considered to be mediated by cyclic AMP.

I have previously postulated that chemotaxis involves the contractile mechanism of the polymorphonuclear leukocyte (1, 17, 18). The microtubular disaggregating agents, colchicine (19, 20) and vincristine and vinblastine (18) at sufficiently high concentrations (10^{-4}M to 10^{-3}M), inhibit chemotaxis. At subinhibitory concentrations, and with the appropriate level of chemotactic stimulus, these same agents actually enhance chemotaxis and cell motility (21). Cytochalasin B, which is reported to depress microfilament function, reversibly inhibits chemotaxis and spontaneous motility at concentrations of 1 μg and above (22). However, below this level, cytochalasin B enhances the chemotactic response (22). In addition to these paradoxical findings there is accumulating evidence that the microtubular disaggregating agents as well as cytochalasin B have more than one mode of action (1). These findings indicate that more direct evidence than from the use of these inhibitors is necessary to demonstrate the involvement of the contractile elements of the cell.

Actomyosin-like proteins have been isolated from both horse (23) and guinea pig (24) neutrophils. Szent-Gyorgi more than 20 years ago demonstrated that muscle fibers extracted with glycerol contract on the addition of ATP. The ATP-induced contraction of glycerinated cells has been used as evidence for a contractile mechanism being present in a variety of isolated nonmuscle cells. Recently, we have shown that glycerinated rabbit neutrophils when exposed to ATP contract as observed microscopically and also decrease their volume (25). The characteristics of this ATP-induced decrease in volume strengthen the evidence that there exists in neutrophils a contractile system similar to actomyosin. However, the ATP-induced contraction of glycerinated cells does not correlate with the chemotactic responsiveness of the corresponding nonglycerinated cells (26). In fact, chemotactic factors induce an expansion of the volume of nonglycerinated cells (26, 27) and it is this which correlates with their chemotactic responsiveness (25). Thus, more direct and unequivocal evidence of the involvement of the contractile machinery of the cell in motility or any of the other functions of the neutrophil is still required.

AN HYPOTHESIS AS TO THE MECHANISM OF CHEMOTAXIS

Obviously, from the bits and pieces of information available to us, no detailed picture of the molecular mechanism of chemotaxis is possible. The best we can hope for is a generalized and tentative conceptual scheme which will serve as a framework for our present knowledge and a springboard for future experiment. (It should not pass unobserved that a framework may well prove to be a rather shaky springboard.)

Chemotaxis is cell movement directed along and by a gradient of chemical substance, i.e., the chemotactic factor. In this scheme I postulate that one or more molecules of chemotactic factor interact with some sort of receptor site or sites

on the cell, resulting in activation of proesterase 1 to esterase 1. Esterase 1 in turn, probably by proteolytic action, although this is not material in the present context, triggers a complex series of reactions, the end result of which is movement of the neutrophil. The movement is in the direction of chemotactic factor and receptor site interaction and, in moving, fresh receptor(s) is exposed to chemotactic factor and more proesterase 1 is activated, triggering further movement. The net effect is a tendency of the cell to move in the direction of increasing concentrations of the chemotactic factor, that is, along the concentration gradient. However, the activating effect of the chemotactic factor in this scheme is not sufficient in itself to ensure movement along the concentration gradient. It is also required that once the stimulus has acted, the proesterase has been activated, and the subsequent events have been triggered that the site(s) will no longer be capable of being further fired off; it will be deactivated. Thus, the movement engendered by the chemotactic factor(s) acting at *a given site or set of sites* is self terminating and within the time scale in which the chemotactic response occurs is nonrenewable. This ensures that the direction of movement stimulated at one site (or set of sites) will not interfere with the direction of movement stimulated at subsequently reacting site(s).

According to this hypothesis, in the neutrophil, the events occurring in chemotaxis are essentially the same as those responsible for spontaneous cell movement; both are believed to be triggered by the activation of proesterase 1 (see Fig. 1). However, they differ in that the spontaneous movement of the cell is induced by either the spontaneous random activation of proesterase 1 or the activation of proesterase 1 by substances in the medium occurring without a concentration gradient. However, this theory does not preclude, and in fact envisages, that cell migration can occur if the reaction scheme is entered into subsequent to proesterase 1 activation or for that matter at a step or steps bypassing not only the activation step but the step or steps associated with deactivation (assuming the activation and deactivation steps are separable). Some of the implications of this putative ability to bypass the steps associated with deactivation will be dealt with below. In addition, this theory does not take into account the effects of substances released by the cells themselves (30), although this latter effect should be easily accommodated into the theory.

It is obvious from what I have just reviewed that the precise reactions triggered by the activation of proesterase 1 or their sequence is unknown. These reactions apparently involve divalent cations and energy source, and, in addition, one can visualize an ordered and compartmentalized activation and relaxation of the contractile mechanisms of the cell.

This generalized and indefinite scheme has certain implications: one of the first implications is that chemotactic agents should stimulate cell locomotion. Several studies have suggested that rates of movement, in fact, are not altered by a chemotactic stimulus (28, 29). However, as Zigmond and Hirsch (30) observed, in some of these studies the conditions were such that a possible stimulation of movement could have been masked. They considered in their investigation that

some materials that stimulated chemotaxis also stimulated movement. Moreover, Keller and Sorkin (31) showed that cells incubated in the presence of a chemotactic agent increased their random motility.

Although the theory suggests that chemotactic agents increase cell movement, the theory also implies that not all agents that increase cell locomotion are chemotactic. This latter arises from the postulated necessity that a chemotactic agent not only triggers increased movement but that it also be capable of deactivating the sites at which it acts. Thus, substances that can stimulate movement by acting at a step or steps beyond the putative deactivating sequence would not be expected to be chemotactic.

Based on this last idea, and on the idea that, as in muscle, intracellular release of Ca^{2+} activates contraction in the leukocyte and thus presumably acts late in the sequence, I have studied the effects of the ionophore A23187 on spontaneous mobility and also tested it for its ability to serve as a chemotactic factor. A23187 is an antibiotic capable of transporting Ca^{2+} and, somewhat less effectively, Mg^{2+} into cells and isolated organelles such as mitochondria and inducing a release of their sequestered Ca^{2+} (32, 33).

THE ACTION OF A23187 ON THE MOVEMENT OF NEUTROPHILS

The first series of experiments was carried out using Hank's buffer containing the usual 1.7 mM Ca^{2+} and 0.7 mM Mg^{2+}. The techniques for measuring chemotaxis and spontaneous motility were as previously described (2, 9). Briefly, A23187 was tested for its effect on spontaneous motility by placing varying concentrations of the ionophore in the upper compartment with the cells and using only buffer in the lower compartment of the chemotaxis chamber with a filter of 5 or 8 μm average pore size separating the two compartments (10). When A23187 was tested for its possible chemotactic activity, it was placed in the bottom compartment of the chamber separated by a filter of 0.65 μm pore size from the cells in the upper chamber (3).

As seen in Table 1, the only effect of the ionophore was a pronounced inhibition of spontaneous movement at concentrations of 10^{-5} and 10^{-6} M. It had no chemotactic activity. I hypothesized that the reason for the inhibition of spontaneous motility was that in the presence of external Ca^{2+} the A23187 brought so much Ca^{2+} into the cell that the cell went into Ca^{2+} "rigor." In other words, the presence of a high concentration of unbound intracellular Ca^{2+} brought in by the ionophore led to a generalized activation of the contractile mechanism of the cell. Consonant with this interpretation was the microscopic observation that cells treated with concentrations of ionophore that inhibited spontaneous motility did not spread or spread incompletely and slowly on a microscope slide.

On this basis, the experiments were repeated using Hank's buffer containing no Ca^{2+} with the idea that the putative high influx of Ca^{2+} would be avoided and in its place there would be an intracellular influx of Mg^{2+}. Alternatively there could be an intracellular influx of A23187 alone. The Mg^{2+} (or A23187) could

TABLE 1. *Effect of A23187 on spontaneous movement and as a chemotactic agent in Hank's buffer (containing the usual 1.7 mM Ca^{2+} and 0.7 mM Mg^{2+}*

Concentration of A23187 (M)	Spontaneous motility [a] (cells/5 HPF)	Chemotaxis [b] (cells/5 HPF)
0	396	50
10^{-7}	438	79
10^{-6}	216	35
10^{-5}	180	50

[a] A23187 placed with 2 X 10^6 PMN's in upper compartment and Hank's buffer present in the lower compartment of chamber. Incubated for 1½ hr at 37°C. A Millipore filter of 8 μm pore size separated the upper and lower compartment (9).
[b] A23187 placed in lower compartment and 2 X 10^6 PMN's in upper compartment. Incubated for 1½ hr at 37°C. A Millipore filter of 0.65 μm pore size separated the lower from upper compartment (2).

then serve to free the sequestered intracellular Ca^{2+} with, as postulated above, an ordered compartmentalized activation and a corresponding relaxation of the contractile mechanisms of the cell and, thus, an enhancement of cellular movement. If, as indicated above, the effect of divalent cations is late in the sequence, one would expect their site(s) of action to be beyond the deactivation step or steps. Thus, the prediction followed that, in the absence of Ca^{2+}, A23187 should enhance spontaneous motility but not be chemotactic.

Table 2 shows that in a Mg^{2+}-containing, Ca^{2+}-free medium, concentrations of A23187 from 10^{-7} to 10^{-5} M progressively increased spontaneous motility to the level achieved in the presence of 1.7 mM added Ca^{2+}. Despite this distinct enhancing activity, it was not chemotactic.

I also tested the effect of the ionophore on chemotaxis induced by partially purified bacterial chemotactic factor. As expected, in the regular Hank's buffer containing Ca^{2+} and Mg^{2+}, 10^{-5} M ionophore in the upper compartment depressed chemotaxis, presumably through its inhibition of spontaneous motility. In Ca^{2+}-free Hank's buffer containing 0.7 mM Mg^{2+}, utilizing a maximal chemotactic stimulus (1 : 5,000 dilution), 10^{-7}M A23187 gave significant inhibition of chemotaxis. This increased progressively with the concentration of ionophore until at 10^{-5}M A23187 chemotaxis was inhibited more than 60%. However, at a 1 : 20,000 dilution of bacterial factor, giving approximately 20% of the chemotactic activity found with the 1 : 5,000 dilution, the effect of the ionophore varied with its concentration. At 10^{-7} and 10^{-6} M ionophore there was a significant enhancement of chemotactic activity, corresponding to the enhancement of spontaneous motility, but at 10^{-5} M A23187 the chemotactic activity was essentially the same as that of the control without ionophore. The decrease in chemotactic activity at the highest concentration of ionophore was reproducible. Its significance is obscure but suggests some form of interaction between the changes in distribution