EFFECTS OF COXSACKIE B5 VIRUS AND VIRUS INFECTED MEDIA UPON HUMAN NEUTROPHIL POLARITY IN VITRO

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ABSTRACT

EFFECTS OF COXSACKIE B5 VIRUS AND VIRUS INFECTED MEDIA UPON HUMAN NEUTROPHIL POLARITY *IN VITRO*

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In previous studies, viruses depressed neutrophil (PMN) chemotaxis; whereas, lysates from virally infected media induced a chemotactic response. Coxsackie B5, an uninvestigated virus, and virally infected culture media (VIM) were examined for PMN influence using one sensitive assessment of chemataxis, shape change (SC). Virus was grown and titered in HeLa cell culture, and human PMNs were isolated by established procedure. PMNs were incubated with media, fixed with glutaraldehyde, and examined for SC. Media used to support cell growth and media for growing cells produced minimal SC. VIM alone and supernatant from ultracentrifuged VIM caused significant responses. The VIM response was dose and concentration dependent. Maximum SC produced was 75% of maximum achieved with the chemotactic factor f-Met-Leu-Phe. Media from nonspecifically injured HeLa cells did not evoke a SC response. VIM exerts chemotactic-like activity on PMNs *in vitro*. Presumably, virus-specific attack on HeLa cells causes release of a SC-inducing substance.

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INTRODUCTION

Polymorphonuclear leukocyte (PMN, neutrophil, granulocyte) chemotaxis has been the subject of frequent and consistent inquiry. The ability of granulocytes to migrate towards foreign substances is instrumental in initiating a host immune response. *In vitro* analyses have focused on cataloguing and defining this activity via several modes. One crucial component of the chemotactic response is assumption of polarity by the neutrophil prior to movement (19,43,48,50). Neutrophils elongate, develop a pseudopod anteriorly, and manifest a uropod posteriorly (24). A visual determination of a change in shape from a spherical to a polar form provides a quantifiable assay of chemotactic responsiveness. Using this design, Smith *et al.* observed and defined responses for several chemotactic agents including C5a, bacterial chemotactic factor, and the synthetic peptides f-Met-Phe and f-Met-Leu-Phe (20,42).

Research concerning viral influences on PMN chemotaxis is limited, and, to date, there has been no examination of virus effects on neutrophil polarity. Usually, the virus employed has been influenza (56,57, 59,60,61,63,64,65), although recently work has been done with ECHO virus (75,89), an enterovirus, among others. Where studied, viruses depress neutrophil motility. Studies focusing on the products of virally infected culture media, however, demonstrate a chemotactic response

(83,84,85). Apparently, viruses can act either directly or indirectly, with differing effects.

The Coxsackie viruses (genus Enterovirus, family Picornaviridae) have received little attention in this field. Except for studies of phagocytosis (87,90,91) and bactericidal capacity (92), their effects on neutrophil function are unknown. They do infect humans, however, especially children. Coxsackie B5 virus, one member of the group, is associated with aseptic meningitis, herpangina, myocarditis, and pleurodynia. Investigations of Coxsackie B5 virus effects upon neutrophil behavior could prove clinically invaluable. The present study deals with the *in vitro* influence of Coxsackie B5 virus and virally infected media, compared with appropriate controls, upon human neutrophil shape.

REVIEW OF LITERATURE

A. Polymorphonuclear Leucocyte Chemotaxis

Biologists have been fascinated for over 100 years by a cell's or an organism's ability to respond directionally to chemical stimuli. Pfeffer coined the term "chemotaxis" in 1884 to describe such movement (1). Many lower organisms such as bacteria, protozoa, and slime molds show chemotaxis, but, in vertebrates, only leukocytes express this ability definitively. Polymorphonuclear leukocyte (PMN) chemotaxis has received considerable attention in mammalian studies, presumably because of the influence on inflammatory responses in an individual; PMNs are usually the first leukocytes to appear at sites of infection or injury. Attempts to characterize leukocyte chemotaxis, though, have yielded a plethora of information. Several classes of chemotactic agents have been defined under a myriad of experimental conditions, and various techniques have been utilized to evaluate chemotaxis. Chemotaxis, however, is not one event per se, and to suggest that the phenomenon is multifaceted implies a necessity to delineate the components. Hence, recent researchers confined their *in vitro* investigations to specific aspects of the neutrophilic response; namely, receptor-ligand interaction, cellular polarity, random migration, and orientation within a gradient and "corrected" undirectional migration. Careful study of each of these components may

enable us to formulate a model for *in vitro* PMN chemotaxis to stimulants of inflammation or infection.

Active locomotion of cells requires intrinsic mechanisms of response to environmental stimuli. Directed orientation response is taxis; if chemically mediated, the response is chemotaxis. Keller *et al.*, in an effort to differentiate among the terms describing cellular locomotion and so to standardize their usage, defined chemotaxis as "a reaction by which the direction of locomotion of cells or organisms is determined by substances in their environment". Chemokinesis, on the other hand, is "a reaction by which the speed or frequency of locomotion of cells and/or the frequency and magnitude of turning (change of direction) of cells or organisms moving at random is determined by substances in the environment". Specific locomotory terms should be supported by precise experimental data; otherwise, noncommittal descriptive terms should be employed, for example, "stimulated movement" (2).

Several techniques have been developed, modified, and adopted for the analysis and quantification of leukocyte migration. In 1962 Boyden pioneered the work with a bicompartmental chamber separated by a millipore filter. PMNs were placed in the upper compartment and a test factor was placed in the lower compartment. By varying the solution in the two compartments, a gradient of the factor was established across the filter. After three hours incubation, the degree of chemotactic stimulation by the material was assessed by counting the number of PMNs that had migrated through the filter (3).

Boyden chamber chemotaxis, though an admirable first step in evaluation, is fraught with problems. Accelerated movement of cells through the

filter might represent true chemotaxis, or chemokinesis, or both. Furthermore, as Zigmond points out, Boyden results may be affected by adhesiveness of cells to the filter material, tortuosity and size of the pore channels, detachments of cells from the bottom surface, and the ability of some factors to stimulate migration at low concentrations and to inhibit movement at high concentrations (4). Another system, the agarose assay, developed by Nelson (5), also hinders clear distinction between chemotaxis and chemokinesis.

To more clearly demonstrate a chemotactic influence, researchers modified the Boyden chamber or developed other tests. Zigmond and Hirsch, using the Boyden System, for incubations up to 90 minutes, determined chemokinetic effects and then corrected for them when evaluating the chemotactic response. They determined random locomotion by measuring the distance into the filter that the two front cells moved when the same material was present in equal concentrations on both sides of the filter. The distance moved increased linearly with time; whereas, the number of cells on the bottom of the filter reached a peak and declined with time. They showed that the distance moved by the two front cells was representative of the whole population by noting that the distribution of cells in the filter at various incubation times approximated that expected from a uniform population exhibiting random-walk behavior. Given a standard for ascertaining random movement, they could test directional responsiveness of cells: if the actual movement of cells in a positive gradient of substance was greater than that predicted on the basis of random locomotion alone, the substance would be considered chemotactic. Accordingly, Zigmond and Hirsch plotted the data in an easily readable checkerboard graph (4).

Millipore chamber assays allow the measurement of changes in the distribution of populations of cells only; visual microscope assays must be employed to analyze movement of individual cells. Additionally, observation of individual cells provides more direct evidence for non-random locomotion towards a gradient. Zigmond and Hirsch developed a slide-coverslip method for testing directionality of locomotion: cells were scored in regard to their orientation in the 180° sectors towards or away from the line of test material, as indicated by cell morphology (4). In further studies, they determined the precision of orientation by comparing the orientation of movement of individual cells with the most direct possible path to the chemotactic factor, and they computed the degree of chemotaxis by calculating the ratio of the most direct path length to the actual path length (6). They evaluated also the frequency and magnitude of turns: distance between turns did not alter as a function of orientation, but there was a significant correlation between the magnitude of a turn and the orientation of a cell immediately prior to the turn (6). Furthermore, direction of turn was correlated with orientation: the next turn of a cell moving at an angle of 30° or more from the direct path to the chemotactic material was towards the material (6). From these analyses, Zigmond and Hirsch concluded that PMNs sense, and respond with accuracy to, a chemical gradient (6).

In 1977 Zigmond described another method for visually quantitating PMN chemotactic response, the orientation chamber. The chamber consisted of a Plexiglass slide containing two wells separated by a bridge. Chemotactic factor was placed in one well and buffer solution in the opposite well; cells were situated on the bridge, held in place by a cover slip. With this system, she viewed cells by phase microscopy

during exposure to controlled concentrations and concentration gradients of chemotactic agents. She demonstrated that PMN orientation is dependent upon both the mean concentration of the agent and the concentration gradient of the factor. In the optimal range, PMNs oriented with a 1% concentration difference across their dimensions. Orientation response was reversible. Moreover, at high cell concentrations, and with equiconcentrations of chemotactic factor in each well, PMNs on each side of the bridge oriented towards the nearest well (edge effect). Presumably, this effect results from inactivation of chemoattractant by the PMNs, possibly via absorption, digestion, or ingestion. Finally, this visual assay showed optimal responsiveness at approximately 10-fold concentrations higher of chemotactic factor than the millipore assay did. The disparity may be due to differences in parameters measured. The orientation chamber method measures cell orientation; whereas, the millipore filter assay measures oriented movement. Nonetheless, the Zigmond chamber may greatly simplify the determination of the chemotactic influence of a substance (7).

Using the various assays, several compounds were identified as chemoattractants for neutrophils. Boyden showed that antigen-antibody complexes in the presence of fresh serum generated chemotactic activity (2). Researchers surmised that the complement system might be involved, as heating serum at 56°C for 30 minutes abolished the chemotactic effect. Shin et al. identified C5a, a cleavage product of the fifth component of complement, as the major chemoattractant in serum (8). Human C5a is a glycosylated peptide composed of 74 amino acids with a C-terminal arginine. C5a is rapidly hydrolysed by carboxypeptidase B,

which cleaves the arginine to leave $C5a_{desarg}$, chemotactic at 10^{-8} M (10-fold higher than C5a) (9). Wilkinson noted that many compounds are cytotaxigenic (i.e., generate chemotactic activity by activating serum components, particularly complement) including cellular products (9) and bacterial proteases or endotoxins (10). Cytotaxigenic systems are useful in chemotactic analysis for the following reasons: 1) A solid source, complement, produces gradients of soluble, diffusible chemotactic factors. Some bacteria, for example, simply do not release these soluble, diffusible molecules necessary for chemotaxis. 2) A gradient of a factor generated continuously (i.e., by complement activation) will not decay rapidly (10).

There are other chemotactic factors. Crystal induced factor (CCF), a glycoprotein with molecular weight 8400, is produced by human neutrophils after ingestion of sodium urate crystals or of crystalline calcium pyrophosphate and stimulates neutrophils. Lymphokines, which are effector molecules released by sensitized lymphocytes upon incubation with the sensitizing antigen, are chemotactic, especially for mononuclear phagocytes. Additionally, some LDCFs (lymphocyte-derived chemotactic factors) activate neutrophils and eosinophils. Lectins such as concanavalin A and leucoagglutinin induce chemotaxis and chemokinesis in mononuclears and to some extent in neutrophils. Denatured human serum albumin, hemoglobin, and myoglobin are chemotactic as measured by checkerboard assays. Chemotactic properties exhibited by this group probably result from the exposed hydrophobic ions, which are packed into the interior of the molecule in the native state. Arachidonic acid derivatives activate PMNs. 12-L-hydroxy-5,8,10, 14-eicosatetraenoic acid (HETE), generated from

arachidonic acid by lipoxygenases present in neutrophils or platelets, attracts neutrophils at 1 μg/ml. 12-L-hydroxy-5,8,10, heptadecatrienic acid (HHT), a cycloxygenase-mediated arachidonic acid derivative, is chemotactic for eosinophils in the range 2-50 μg/ml. Dihydroxy HETE products attract leukocytes at lower concentrations than the mono-HETE's do; the most potent is leukotriene B4 (5,12 diHETE) which attracts neutrophils at 1 ng/ml. Incorporation into PMN membrane phospholipids is the presumed mechanism of action for these lipid substances (9).

Peptide factors from E. coli, or factors presumably related to those from E. coli, are of especial chemotactic interest. Heterogeneous E. coli extracts, of anionic character, with blocked N-termini, are chemotactic for neutrophils (11). E. coli (bacterial) protein synthesis commences with N-formyl methionine, which is later cleaved from the peptide chain (10). Since eukaryotic cells do not begin protein synthesis with formyl methionine (except for mitochondrial cells), this initiating amino acid could be a signal for stimulating phagocytic cells into action against the foreign prokaryotes (10). Such a hypothesis remains speculative and awaits critical examination: demonstration that the E. coli peptide extracts are N-formylated methionine peptides has not yet been achieved. Nevertheless, such rationale facilitated the discovery and subsequent utilization of synthetic formyl-methionyl peptides as chemoattractants for PMNs and mononuclears (10).

Schiffman et al. demonstrated that a group of formyl methionyl di- and tri-peptides are potent attractants for neutrophils and macrophages; whereas, the corresponding nonformylated compounds are not chemotactic (12). They made the following inferences regarding requirements

for activity in these compounds:

1) Requirement for N-acylation: nonacylated peptides and free methionine show no activity;
2) Specificity for formylation: f-Met-Leu is more active than Ac-Met-Leu;
3) Requirement for methionine: of the formyl amino acids tested, only formyl methionine is active;
4) Requirement for minimum size: most active f-Met peptides contain at least two residues (12).

Showell et al. demonstrated that formyl tripeptides are more active than dipeptides, e.g., f-Met-Leu-Phe gives maximal chemotactic response at concentrations of 10^{-9} to 10^{-8} M, but f-Met-Phe is maximally active at 10^{-6} to 10^{-5} M (13). Furthermore, the order and constitution of the peptides is important:

1) Optimal activity is achieved with methionine in the terminal position, although an aliphatic amino acid of similar hydrophobicity such as leucine or norleucine also can confer some activity.
2) The second amino acid should be neutral and polar, such as leucine, for maximal activity.
3) In tripeptides, phenylalanine in the third position greatly increases activity.

Thus, the most potent chemotactic peptide synthesized is N-f-Met-Leu-Phe (13). The development of these peptides is a blessing for chemotactic research.

Several investigators explored the binding of peptides to neutrophils. Binding is specific, saturable, of high affinity, and displaceable by other peptides. Aswanikumar et al. demonstrated that rabbit neutrophils have approximately 100,000 binding sites for f-Norleu-Leu-Phe (10^{-6} M) with affinities (KD) of 1.5×10^{-9} M (14). Similarly, Williams et al. described the binding of f-Met-Leu-Phe to human neutrophils and determined 2000 binding sites per cell and a K_D of $1.2\text{-}1.4 \times 10^{-8}$ M (15). Both groups found that the binding constants of the peptides are similar to the concentrations of peptides which give maximal chemotactic response (14,15). Indeed, Zigmond noted, by determining the concentration at which the cell exhibits optimal chemotaxis in a gradient, one can predict the binding constant of that agent (7).

The aforementioned characteristics of peptide binding indicate that neutrophilic response to chemoattractants is a receptor-mediated event. The peptide receptors are likely plasma membrane components (14) and are probably proteins of 55,000-70,000 molecular weight, as suggested by affinity labeling techniques (16). Furthermore, C5a does not compete for the oligopeptide receptors, even at concentrations above those which induce chemotaxis (14,15). Chenoweth and Hugli later demonstrated a specific C5a receptor (17). Spilberg and Mehta isolated a receptor for CCF on human PMNs (18). Several nonidentical receptors, therefore, are present on neutrophils. Specific receptor-ligand interaction is probably essential for generating the chemotactic response. Studies with formylated peptides indicate that, besides directing locomotion, these agents cause changes in neutrophil adhesion to substrata (20), induce lysosomal enzyme release (13), and activate cells metabolically by

increasing hexose monophosphate shunt activity, oxygen consumption, and superoxide and hydrogen peroxide production (21,22). Probably these cellular functions occur in a prescribed sequence, though a single ligand-receptor binding event is sufficient to initiate the process.

Zigmond delineated the chemotactic phenomenon into three parts: 1) a sensory mechanism whereby the cell detects the presence of a stimulant and the direction of a gradient; 2) a transducer mechanism by which the directional information is transformed into cellular messengers; and 3) an effector mechanism which mediates the mechanical and motile changes which produce cell polarity and locomotion (19). To respond appropriately to external substances, neutrophils must detect the stimuli. Neutrophils differentiate gradients of as little as 1% across their surface. This sensitivity to differences in concentrations of chemoattractants probably reflects an ability to detect differences in receptor occupancy (19). Hence, modulation of receptors, through changes in number, affinity, or distribution, may effect changes in cellular response.

Neutrophils adapt to changes in the concentration of peptide chemotactic factors. Adaptation, as defined by Zigmond et al. is the "reversible extinction of a cell's responsiveness to a stimulus caused by an adjustment of the cell's sensitivity". Concentration jump experiments with f-Norleu-Leu-Phe (fNLLP) demonstrated transiently altered aspects of neutrophil morphology and locomotory behavior. Rapid increases in peptide concentration caused PMNs to stop locomotion and to develop ruffles over most of their surface. After a short delay (few minutes), the cells withdrew all but the anterior ruffles and resumed locomotion. Sudden decreases in concentration of peptide induced cells to stop locomoting,

round up, and form small blebs over their surfaces. After a delay, the blebs disappeared and the cells renewed their locomotion. In both cases, the transient nature of the response indicated that the cell adapts to a new concentration. Moreover, the duration of these responses was roughly proportional to the change in receptor occupancy that would be expected to occur for a given concentration change. The role, if any, that adaptation plays in chemotactic responsiveness, such as in gradient detection or in in vivo situations, is unknown (23).

The reversibility of the adaptation phenomenon contrasts with the irreversibility of deactivation. Exposure of cells to high concentrations of chemotactic factors renders them unresponsive to chemoattraction by the same factors (24). Ward and Becker initially categorized the phenomenon when they noticed that if neutrophils were incubated with a chemotactic factor, then washed, and then re-exposed to a gradient of the same factor, they failed to locomote (25). Becker later showed that C5a could induce self-deactivation (deactivation of cells to itself), but could not cause deactivation to bacterial chemotactic factor (derived from E. coli) (26). As Gallin et al. showed, deactivating doses of chemotactic factors (higher than optimal chemotactic dosage) induced optimal lysosomal enzyme secretion and led to a reduction in the binding of f-Met-Leu(^{3}H)-Phe to the cells (27). The reduction in peptide binding as well as factor specificity may indicate a receptor dependency for deactivation.

Receptor number on PMNs changes in response to chemotactic factors. Sullivan and Zigmond noticed "down regulation", that is, ligand-induced loss of plasma membrane receptors. Incubation of cells with unlabeled

fNLLP at 37°C for various times produced a decrease in receptors available for binding upon subsequent exposure (after 5 minute washing) to tritiated peptide at 4°C. The rate and extent of receptor loss were dependent on the concentration of fNLLP present during preincubation; a dramatic decrease occurred at 3×10^{-7} M. Most receptors disappeared during the first five minutes, and a plateau level was achieved within fifteen minutes with all concentrations. Dose response correlated with fNLLP binding, as half-maximal loss of receptors occurred at a concentration near the K_D of binding. Additionally, down regulation was temperature-dependent, occurring more definitively at 37°C. Affinity of receptors before and after loss, however, was unchanged (28).

Even after down regulation is complete, that is, after equilibration of receptors, peptide uptake continues. Increased peptide accumulation represents irreversible binding and probably is receptor-mediated, although a pinocytic mechanism also may be involved. Pinocytosis is suggested by the stimulation of the uptake of tritiated sucrose from the fluid medium by fNLLP at varied concentrations. Nonetheless, after a 90 minute incubation (37°C) in 10^{-7} M fNLLP, the amount of nondissociable peptide that accumulated exceeded the initial number of receptors by 300%. Assuming that receptor loss occurs by internalization of a receptor-ligand complex (in contra-distinction to a receptor affinity alteration), a mechanism for surface receptor resupply must exist (28).

Receptor-mediated peptide uptake may be the catalyst for the expression of new receptors on the plasma membrane. On the basis of kinetic analysis, Zigmond et al. proposed that the rate of receptor internalization equals the rate of receptor recovery for any peptide

concentration, that is, a steady-state receptor level is maintained (the plateau receptor number) (29). Experimentally, the rate of receptor recovery was approximately twice the rate of loss (not considered significant); possibly there exists a limited pool of extra receptors that become available for membrane insertion upon addition of peptide (29). Further support for this pool of spare receptors comes from the "super-recovery" noticed when concentrations of peptide close to the K_D are used (29). At concentrations much greater than the dissociation constant, however, the amount of recovery decreased, possibly due to occupancy of an increased proportion of the receptors (29). Recovery is efficient and rapid: upon removal of peptide from the milieu, cells regained 80-100% of initial binding capacity within twenty minutes (28). The recovery rate constant diminished, though, with increased peptide concentrations (29).

To summarize, receptors are in a state of flux. Receptors bind peptides, and the complex is internalized. Niedel et al. used a fluorescent (rhodamine conjugated) synthetic peptide to document visually specific binding, rapid aggregation, and subsequent internalization within endocytic vesicles (30). Nonsaturable uptake occurs concurrently in direct proportion to the concentration of peptide in the medium and represents an irreversible component of binding. Pinocytosis contributes to both types of uptake. The internalized peptide is partitioned into a nonreleasable (storage) pool or is released for subsequent digestion; whereas, the receptors are recycled to the plasma membrane and reinserted to accommodate new peptide (29). Thus, peptide uptake and release approaches steady state as does receptor loss and recovery. Implications

of receptor modulation for the chemotactic response are unclear. Down-regulation decreases sensitivity to gradients at high concentrations of peptide (greater than K_D), that is, orientation diminishes (31). PMNs apparently sense gradients by discerning differences in receptor occupancy; if so, loss of receptors could be integral to the cellular response. Furthermore, adaptation and deactivation, chemotactic factor-related phenomenon, may be expressions of receptor modulation.

Directed locomotion, as well as other neutrophil responses, requires that the information perceived by the cell's sensory apparatus be translated to the motile machinery, that is, a signal transduction mechanism must exist. Binding of chemotactic factors to the cell surface initiates a series of events which involves metabolism of arachidonate and involves changes in ion levels, membrane potentials, cyclic nucleotides, and lysosomal enzymes. Inhibition studies suggested roles for the lipoxygenase products of arachidonic acid metabolism (HETE's) in PMN migration, hexose transport, aggregation, and lysosomal degranulation (24).

Leukocyte chemotaxis persists in media of varied ionic composition, but chemotactic factors cause changes in the fluxes of Na^+, K^+, and Ca^{++} across the membrane. F-Met peptides induce an immediate influx of Na^+, followed by a delayed increase in Na^+ efflux and K^+ influx, probably attributable to Na^+K^+ATPase (7). Chemotactic factors increase influx and efflux of Ca^{++}, increase membrane permeability to calcium, and stimulate release of membrane-bound calcium (as deduced from chlortetracycline and from potassium pyroantimonate experiments) (9). Sha'afi and Naccache proposed the following model regarding the role of calcium: Released membrane-bound calcium associates with calmodulin, and the complex causes

release of arachidonic acid via phospholipase A_2 activation. Lipoxygenase metabolites of arachidonate may amplify the release of membrane-bound calcium and magnify the permeability of the plasma membrane to calcium and other ions (32,33).

Alterations in transmembrane potential might accompany changes in membrane permeability. Microelectrode studies with mononuclear leukocytes showed that chemotactic peptides and C5a produce a brief depolarization followed by a more prolonged hyperpolarization (24). PMNs responded to chemotactic factors with altered surface charges and hyperpolarization of the plasma membrane (24). Seligmann et al., using a fluorescent dye when examining f-Met-Leu-Phe influence on human neutrophils, noted a decrease in cell-associated fluorescence followed by an increase in fluorescence--a scheme of events corresponding with the changes in polarization associated with the mononuclears (34). The significance of electrical events in chemotactic responses is presently undetermined, but changes in membrane potential may be simply indications of the occurrences of other cellular events (9).

Cyclic nucleotides serve as second messengers in many cell activation systems; consequently, a role for them in neutrophilic activation is not unlikely. Cyclic AMP (cAMP) and cyclic GMP (cGMP) apparently do not stimulate chemotaxis, though cGMP may influence random migration. Agents that alter cyclic nucleotide levels within neutrophils, however, have more demonstrable effects on chemotaxis. For instance, chemicals which increase intracellular cAMP, such as theophylline, caffeine, epinephrine, or prostaglandin E1, decrease neutrophil motility; whereas, depressants of intracellular cAMP, for example imidazole, tend to

stimulate neutrophil locomotion. To the contrary, substances which elevate intracellular cGMP levels, such as phorbol myristate acetate, phenylephrine and prostaglandin F_2, enhance the neutrophilic locomotor response. Levamisole stimulates both chemokinesis and chemotaxis while maintaining cGMP levels, in the presence of chemotactic factor. These observations, although interesting, do not necessarily implicate cyclic nucleotides in the chemotactic response. Drugs may alter nucleotide levels, and they may modulate locomotion; the two events might be mutually exclusive. Still, in many cells, cyclic nucleotides are calcium-dependent enzymes. An intimate relationship between the ion and the enzyme may be essential for leukocyte locomotion. Further investigations will substantiate or dismiss a significant role for cyclic nucleotides in transduction (9).

Chemoattractants induce the extracellular release of neutrophil granules, especially in the presence of cytochalasin B. Neutrophil granules are of two types: 1) azurophil, or primary, granules contain hydrolytic enzymes such as myeloperoxidase, β-glucuronidase, and proteases; 2) specific, or secondary, granules contain lysozyme, lactoferrin, collagenase, and proteases. Gallin et al. determined that higher doses of chemotactic factor are necessary to generate enzyme release than to direct locomotion and that such doses, in fact, inhibit locomotion (35). Chemotactic levels of the factor induce nominal secretion of enzymes and simultaneously enhance the number of receptors on the neutrophils. Gallin's model suggests that specific granule membranes bear possibly one-third of the formyl methionyl peptide receptors in PMNs and that these membranes fuse with plasma membranes during exocytosis (granular

content release) (36). Venge demonstrated that casein causes release of a chymotrypsin-like protease from human granulocytes (37). Neutrophils accelerated at the time of protease release, possibly as a result of simultaneous release of a cell-derived chemotactic factor (37). Zigmond and Hirsch also described the presence of a cell-derived chemotactic factor upon neutrophil incubation with aggregated gamma globulin (4). Ward and Becker, in studies with rabbit PMNs, demonstrated the existence of a serine esterase, which is convertible into an active form (esterase 1) upon contact with chemotactic agents (38,39). Similar inhibition profiles of esterase 1 activity and neutrophil locomotion by phosphonate esters indicated that the esterase might be essential for chemotaxis (38,39). More recently, Aswanikumar et al. showed that rabbit neutrophil chymotrypsin-like peptides cleave and digest formyl methionyl peptides (40). Since digestion of peptides correlates with locomotion of neutrophils, as indicated by enzyme inhibition data, digestion might be necessary for peptide-cellular interaction (40). Transduction of information derived from ligand-receptor reaction may be dependent upon any one, or all, of these enzymes, and/or upon any of the other processes (arachidonate metabolism, ionic events, membrane polarization, cyclic nuncleotides) discussed above.

Although transductional events are ill-defined, sensory information is processed, and cells move. Leukocytes crawl on substrata; adhesion to surfaces, therefore, is probably a critical component of movement. In vitro studies have been concerned with the effects of substances such as glass, plastic, serum, or albumin upon neutrophil adhesiveness (9). Cells that adhere to uncoated glass or plastic cannot move; if high

concentrations of protein are added to such substrata, however, cells float off the substratum (9). Nonetheless, cells placed on uncoated glass respond to chemotactic stimuli, as judged by their ability to orient and by biochemical activity (41). If neutrophils are placed in the same gradient in a medium containing serum albumin, they locomote trenchantly towards the factor; ostensibly, the albumin coat provides a suitable substratum for motion (41). Smith et al. demonstrated increased adhesiveness of human neutrophils to glass pretreated with bovine serum albumin or 10% serum in the presence of f-Met-Phe, C5a, or bacterial chemotactic factor (from E. coli), as compared to controls not incubated with a chemotactic factor (20). In the absence of chemotactic agents, though, neutrophils adhere less to coated glass than to uncoated glass (20). Furthermore, the enhanced adhesiveness stimulated by the chemotactic factors did not diminish to control levels 45 minutes after removal of the factor (except for bacterial factor) and remained unaltered upon subsequent exposure to a chemotactic factor (20). The same conditions, however, in concurrent experiments, induced chemotactic deactivation in terms of Boyden chamber chemotaxis response (20).

In later studies, Smith et al. examined the effects of stepwise increases in the concentration of f-Met-Phe on neutrophil attachment to and detachment from serum-coated glass and on the distribution of surface binding sites for albumin-coated latex beads. Initial exposure of PMNs to f-Met-Phe resulted in increased neutrophil adhesiveness and in random binding of latex beads to the cell surface. The second higher dose of f-Met-Phe caused diminished adherence, detachment of cells from front to back, and transport of latex bead binding sites from the head to the

tail. After a third exposure to f-Met-Phe, beads bound to the front if the peptide concentration was higher than the previous dose and bound to the rear if the concentration was lower than the second dose. They hypothesized that the decreased adherence to protein-coated glass associated with sequential increases in chemotactic factor concentration results from redistribution of adhesion sites. Migration of adhesion sites from head to tail may be important in orientation of cells in a gradient (42).

Additional adhesion studies supplied support for the above hypothesis. Anderson et al. described a patient with glycogenosis type 1b: the patient's neutrophils failed to demonstrate enhanced adherence after initial exposure to chemotactic stimuli or decreased adherence after sequential exposure to increasing concentrations of the agent and failed to redistribute adhesion sites from front to rear (as demonstrated by the latex bead technique) (43). Patient neutrophil motility also was markedly reduced (43). In consequence, the researchers suggested that redistribution of adhesion sites is intrinsically related to the mechanism of cell locomotion (43). In another study, Anderson et al. observed diminished motility of neonatal PMNs concomitant with adherence abnormalities similar to the glycogenosis 1b individual and with an inability of the PMNs to redistribute adhesion sites for latex beads (44). They inferred, again, that motility is functionally linked to adherence, possibly in an interdependent fashion (44).

Adhesion is a relative phenomenon, invariably dependent upon both the cell and the substratum. In vivo, presumably, neutrophils interact with endothelial cells during margination, though physiological situations

are difficult to reproduce *in vitro*. Lackie and de Bono showed that rabbit neutrophils attach well to aortic endothelial cells and locomoted freely when in contact with them (45). Smith *et al*. noted chemotactic factor time- and concentration-dependent changes in adherence of rabbit peritoneal neutrophils to pig aortic endothelial cells grown as tissue culture monolayers, though the changes in adhesiveness were restricted to neutrophils (46). When the endothelial cells were treated with the chemotactic factors, no change in neutrophil adherence resulted (46). Hoover *et al*., however, observed binding of f-Met-Leu-Phe to calf endothelium and a concomitant increase in neutrophil adherence to the endothelial cells (47). *In vivo*, then, endothelial cells may be a critical determinant in efficacious adherence of neutrophils, possibly via chemotactic factor binding sites. Wilkinson proposed the following model for neutrophil adhesion *in vivo*: A transient increase in adhesiveness of neutrophils enables attachment to, and then detachment from, vascular endothelium after which the cells migrate through the tissues to the source of the gradient whereupon they become strongly adherent and perform appropriate phagocytic functions (9).

Chemotaxis depends on active cellular movement, which requires the assumption of polarized morphology by the cell. Cells circulating in the bloodstream or incubating in nonstimulatory medium are round (19). When stimulated by chemotactic factors, PMNs develop a broad lamellipodium anteriorly, a midregion containing a nucleus and a centriole, and a constricted tail (uropod) (19). Granules and mitochondria are excluded from the lamellipodium, or pseudopod, but are found in the midsection and tail (19). A complex arrangement of actin microfilaments are present in

the lamellipodium and uropod (24). Microtubules originate from the centrioles found within nuclear folds and extend toward the filamentous ends (24). Retraction fibers, microfilament-filled strands of cytoplasm, extend distally from the tail and are stretched out as the neutrophil translocates the substrate (19). Eventually, the fibers are pulled up from the surface and resorbed, or they break, leaving remnants to indicate the attachment sites (19).

Morphological polarity is not restricted to locomoting cells. Smith et al. demonstrated that polarization of neutrophils occurs in suspension within a minute of exposure to chemoattractants (20). They quantified this polarization by categorizing the neutrophils according to degree of shape change; spherical, including round cells and round cells with ruffling; and nonspherical, including polarized cells and polarized cells with uropods (20). Nonetheless, investigations of neutrophilic movement fortify the contention that locomotion is linked to polarity. Cytochalasin B prevents the change in shape to a bipolar form (20) and inhibits locomotion of neutrophils (48). Furthermore, depression of cellular motility with concomitant lack of shape change to chemotactic stimulants occurred in a glycogenosis type 1b patient (43).

Acquisition of polarity may depend upon an intrinsic cellular motile apparatus. Microfilaments consisting of actin, myosin, actin-binding protein, and assorted contractile proteins probably are important for leukocyte motion (10). In locomoting cells, microfilament networks are most concentrated in the pseudopod, and uropod; chemotactic stimuli may induce filament network formation (9). Senda et al., in examining contractional leukocytic movement, noted thick and thin filaments when

leukocytes were incubated with heavy meromyosin; thick filaments seemed to correspond to myosin aggregates and thin ones to F-actin (49). They hypothesized that the motive force for the movement of leukocytes is due to association and dissociation of myosin A and F-actin (49). Microtubular involvement in chemotaxis also has been considered, although support for a role relies on indirect studies using depolymerizing drugs, such as colchicine and vinblastine (9). Wilkinson, after several investigations, believes that microtubules are not obligatory for gradient detection nor for directed locomotion, but are essential for accurate turning and for the maintenance of shape and polarity (9).

Zigmond et al. identified a behavioral polarity inherent in locomoting PMNs in association with the morphological polarity previously described. Cells locomoting in homogeneous solution persisted in a given direction and made turns of small angles, while maintaining a single pseudopod at the front. Increasing the concentration of chemotactic peptide (fNLLP) enhanced anterior pseudopod production and ensured directionality. Even reversing the gradient did not alter inherent polarity: cells responded by walking around in a circle in a series of small turns. Furthermore, when a micropipette containing chemoattractant was placed directly behind locomoting cells, none of the cells responded by forming a pseudopod at the tail. Zigmond et al. suggested that behavioral polarity may result from an assymmetric distribution of a component required for lamellipodium formation, such as chemotactic receptors, transducers, or elements of the motile apparatus. Polarity, once established, stabilizes orientation in the gradient and amplifies the directional signal. Ostensibly, the cell becomes visibly committed to a particular

orientation upon chemotactic stimulation; the commitment may underlie cellular responsiveness (50).

The chemotactic response is dependent on molecular organization and coherence of the motile machinery. Resulting coordinated locomotion includes: extension of lamellipodia; presence of a cortical gel in pseudopods and cortex with constriction at the uropod; flow of cytoplasm into advancing pseudopods from the tail; formation and breaking of attachments to the substratum; and the orientation of the cell in the chemical gradient (19). Neutrophilic movement, thus, reflects a unified, integrated effort.

In spite of intensive study and observation of neutrophil chemotaxis, the mechanism for such directed movement is a topic of debate. Two theories for gradient sensation exists: 1) spatial; and 2) temporal mechanisms. The spatial model requires multiple cellular receptors integrated such that the neutrophil detects differential receptor occupancy across the surface and determines which side experiences the highest concentration of chemoattractant (24). In accordance with this model, Zigmond demonstrated: 1) in a gradient, leukocytes turn toward the higher concentration of chemotactic factor without depending upon frequency or magnitude of the turns for the orientation of movement (6); 2) stationary leukocytes sense the gradient across its dimensions (even a difference in concentration of 1%) and orient in that gradient without translocating (7); 3) the cell exhibits optimal chemotaxis at the concentration of the binding constant (K_D) of the peptide interacting with the cell receptors (7).

Several aspects of neutrophil behavior, however, support a temporal mechanism. Temporal sensing implies that the cell perceives a concentration of chemotactic factor at one time, moves linearly for a fixed time interval, stops, and perceives a new concentration before resumption of locomotion--a mechanism that describes adeptly bacterial movement (24). Support for the model in leukocytic movement is as follows: 1) increasing the concentration of a chemotactic stimulant transiently induces pseudopod formation, and decreasing the concentration causes pseudopod withdrawal (23); and 2) turning occurs by extending a region of an already existing pseudopod (50). Additionally, a complex temporal mechanism in which cells have the ability to remember previous concentration levels and prior direction may prevail (6). Recently, Zigmond suggested that the two mechanisms may be working in concert. For instance, a stationary cell in a stable gradient may detect a spatial signal; whereas, a moving cell in a changing concentration of a chemotactic factor may respond to a temporal signal (50). Cell response in a gradient, Zigmond theorized, is intrinsically dependent upon adoption of polarity, regardless of the mechanism of sensing that gradient (50). In fact, polarity (behavioral) exists in the absence of any gradient (20) and may be the major determinant in the chemotactic response.

Chemotaxis is a multifaceted process, consisting of an array of components working in parallel and/or successive harmony to achieve a desired end--cell locomotion towards an attractant. Receptor binding of stimulating ligand and consequent modulation of the receptor unit indubitably trigger a transductional mechanism which transfers external information to the cell interior. The motile machinery of the neutrophil

responds to the message by polarizing morphologically (probably in accord with a predetermined behavioral polarity) via extension of an anterior pseudopod, cortical and body contraction, and uropodal construction. Sensing the gradient, via a spatial, a temporal, or a combined mechanism, the cell translocates across the substrate by crawling and by attaching and detaching from adhesion sites. Invariably, the neutrophil moves up the gradient towards the highest concentration of the attractant. The chemotactic response appears biologically well-organized and purposeful. Such a logical process is desirable in a physiological milieu susceptible to injury, infection, and inflammation.

B. Viral Influences on Polymorphonuclear Chemotaxis

Polymorphonuclear leukocytes (PMNs, neutrophils, granulocytes) are instrumental in host defense against invading micro-organisms, as exemplified by their rapid appearance at sites of infection and inflammation. Defects in PMN responsiveness are associated with a number of disease syndromes and with both bacterial and viral infections in individuals with noticeably unimpaired defense systems. Virus-PMN interactions are of paramount investigational importance as a result of an increased tendency towards bacterial superinfection upon viral invasion and because recurrent infections are prevalent with many viruses. Many viruses alter phagocyte (PMNs and mononuclears) functions, such as oxidative metabolism, phagocytosis, bactericidal activity, and chemotaxis (51). Among these functions, chemotaxis shows consistent depression in response to direct virus exposure, whether *in vitro* or *in vivo*. Virus effects on PMNs may be a direct one, via attachment and intracellular localization, or an

indirect one, mediated through host tissue products released upon viral infection. Interestingly, these tissue products are leukotactic rather than leukodepressive (9). Regardless of their mechanism of action, still subject to speculation, viruses influence PMN function and, thus, are an important target for immune research.

In early viral leukocyte research the term "leukocyte" was often used indiscriminately, and phagocytosis was the focus of investigation. With the advent of the Boyden Chamber and later measures for the assessment of leukocyte chemotaxis, concomitant with virus localization within PMN fractions, more attention was devoted to neutrophil-virus interactions. Most chemotaxis studies utilized influenza virus, as this virus was frequently associated with malady and was implicated in phagocytosis inhibition (52,53,54). Prior to the development of chemotactic techniques, however, Voisin et al., using phase contrast microcinematography, observed a reduction in motility of guinea pig PMNs and gross abnormalities in PMN morphology after intraperitoneal injection of three types of influenza virus (in three different groups of guinea pigs) (55). Influenza B Lee virus exerted the most profound effects (55). Immunization with Influenza B Lee virus, however, insured protection towards subsequent inoculation of virus: PMN motility was normal (55).

The Boyden Chamber is the most prominent method of analyzing influenza virus effects upon PMN chemotaxis. In 1976, Larson and Blades, using influenza B virus, noted a 65% reduction in the number of neutrophils migrating to a casein stimulus, an effect abrogated by heating the virus or by neutralizing the virus with specific antiserum (56). Using similar techniques, Schlesinger et al. demonstrated that two influenza A strains

impaired neutrophil chemotaxis with fewer than ten viral particles per cell (57). Craft et al., however, found no significant difference between influenza-infected children and controls (normal adults and normal children) in chemotactic response, although the motility of virus-infected PMNs was depressed (58).

In a pair of influenza studies, one with human neutrophils and the other with rat neutrophils, Ruutu et al. noted depressed motility (59,60). After a 60 minute incubation of human neutrophils with purified influenza A virus, random locomotion, chemokinesis, and chemotaxis all were markedly reduced; the reduction was more pronounced in the presence of human serum devoid of antiviral antibodies (59). Inactivation of virus infectivity with Tween 80 and ether did not alter the viral effect, but anti-influenza serum repressed the inhibitory activity (59). Influenza B virus behaved differently: at concentrations sufficient to produce inhibition of motility with influenza A virus, influenza B virus did not disturb neutrophils; at higher concentrations of influenza B virus, the neutrophils agglutinated (59). The authors suggested that different strains of influenza may possess different potentials for inhibiting neutrophil motility (59). In the rat study, however, both strains of influenza virus depressed neutrophil random locomotion, chemokinesis, and chemotaxis (60). The difference in effect of influenza B virus between human and rat neutrophils suggests that a difference in neutrophil sensitivity may exist among species. From their studies, the authors would not conclude that influenza virus has a selective depressive effect on chemotaxis; rather, the virus probably depresses motility in general (60).

Larson and colleagues demonstrated influenza virus-specific inhibition of chemotaxis of human PMNs, as evidenced by using a sucrose gradient purified virus preparation. Furthermore, they observed a correlation between the number of viral particles per PMN and the effect on PMN function (61).

Baum and Douglas challenged human subjects with influenza virus and noted a defect in PMN chemotaxis that persisted three weeks. Immunizing another group of subjects six weeks prior to viral challenge produced a normal chemotactic response within seven days of the challenge. Consequently, the authors argued for influenza immunization as a clinical prophylactic (62).

Using the Nelson agarose technique, Debets-Ossenkopp et al. measured a greater than 50% reduction in migration of human PMNs incubated with influenza A virus compared to controls (63).

In another assessment of PMN chemotaxis, Larson et al. noted a significant impairment in patients suffering influenza virus infection. Additionally, they infected two groups of volunteers with different influenza virus strains and found depressed PMN chemotaxis in one group and normal chemotaxis in the second group. Again, differences in virus strain may account for the disparity in responsiveness. Variability in host resistance also may be a contributing factor in neutrophil behavior (64).

During 1971-1977, Martin and colleagues performed a series of functional tests on the PMNs of volunteers inoculated intranasally with influenza A virus and found dysfunction in the chemotactic response only (65).

In two studies with chincillas, Abramson et al. demonstrated depressed PMN chemotaxis (66,67). Six days after intranasal inoculation

with influenza A virus, maximum reduction in chemotaxis (37%) occurred (66). In the subsequent study, they demonstrated a relationship between influenza-induced middle ear changes and PMN dysfunction in the development of pneumococcal otitis media: the frequency of otitis media increased as the time of pneumococcus inoculation approached the time of virus-induced PMN dysfunction and the development of eustachian tube dysfunction (67).

Other viruses have been studied in regard to their influence on PMN chemotaxis. A child suffering from recurrent bacterial infections and disseminated cytomegalovirus (CMV) infection showed defective neutrophil motility, though the influence of the virus was questionable (68). In a later study with patients harboring CMV, Rinaldo et al. found no inhibition of PMN migration in response to chemotactic stimuli (69). In an in vivo study with CMV in guinea pigs, however, Yourtee and co-workers noted granulocytopenia concomitant with a decreased peritoneal neutrophil exudate response to an inflammatory stimulant, intraperitoneal casein (70). The depressed neutrophil response was associated with acute CMV infection, but this deficiency may not represent a chemotactic defect. From these reports, CMV has little apparent detrimental effect on PMN chemotaxis.

Measles virus has been investigated with respect to PMN chemotaxis. Anderson et al., using a Boyden system, found significantly depressed chemotaxis in children with measles infections as compared to controls without virus and to controls with uncomplicated virus infections (71). They suggested that the defect is intrinsic to the neutrophils since endotoxin-activated patient serum was chemotactic for control

neutrophils (71). In a later similar study, the same researchers noted gross impairment of PMN random migration and chemotaxis to endotoxin-activated serum and hydrolyzed casein in patients suffering measles virus infections, as compared to normal controls (72). Furthermore, they observed a marked reduction in PMN accumulation *in vivo* using Rebuck skin windows (72). Again, they concluded that the defect is intrinsic to the neutrophil and not serum-related (72).

Magliulo *et al.* demonstrated an impaired leukotactic response in the leukocytes of acute hepatitis B patients; the effect, they postulated, is due to an intrinsic leukocyte defect (73).

Rabson and co-workers found mildly reduced random migration and markedly depressed PMN chemotaxis to normal endotoxin-activated serum, but not to casein, in individuals infected with herpes simplex virus. They attributed the difference in response to chemotactic stimuli to blockage or destruction of "specific receptors" on the PMNs for activated serum. *In vitro* levamisole treatment of neutrophils restored normal chemotaxis, and *in vivo* administration of levamisole improved chemotaxis in half of the patients (74).

Bültmann *et al.* incubated human neutrophils with various concentrations of infectious ECHO virus and found dose dependent selective inhibition of neutrophil chemotaxis using a Boyden assay. A virus-granulocyte ratio of at least 1:1 was necessary to insure a significant difference between experimental and control groups. They offered no mechanism of virus action, but eliminated viral-induced cytotoxicity and competitive inhibition of granulocyte surface receptors (75).

In a study with children harboring respiratory syncytial virus (RSV), Craft and colleagues noted a depression in neutrophil chemotaxis (58).

Park et al. examined the effects of several live viruses (measles, RSV, mumps, varicella zoster, and CMV) on human neutrophil chemotaxis in vitro and found impairment in all instances, ranging from 50% reduction with measles virus to 95% reduction with varicella zoster virus. Inactivated virus preparations also produced a depression in neutrophil chemotaxis from 15 to 30% (76).

Finally, Solberg et al. described reduced PMN chemotaxis and spontaneous migration in patients with viral infections, including mumps, Coxsackie B, influenza, infectious mononucleosis, morbilli, varicellae, and rubella. They suggested that the viruses act on a cytoplasmic cellular function to induce an inhibitory response (77).

Viral mechanism of action on PMNs remains undefined, but there are several hypotheses. The prevailing opinion that viruses alter directly cell function rests on experimental evidence of attachment to virus to cells and on intracellular localization of viral particles. Ginsberg and Blackmon demonstrated that influenza virus adsorbs to guinea pig PMNs and that the rate and amount of adsorption correlates with the number of leukocytes and the quantity of virus (52). Larson et al. suggested that viral attachment, replication, and/or neuraminidase activity may influence the chemotactic response (64).

Schlesinger et al. claimed that penetration of the virus into the cell is necessary to effect a depression of neutrophil chemotaxis since pretreatment of the cells with amantadine hydrochloride (in concentrations

known to inhibit viral penetration) eliminates the depressed chemotactic phenomenon (57). In Ruutu's studies, however, prior amantadine treatment did not abrogate neutrophil chemotactic inhibition attributable to influenza A (59). In earlier work, Inglot and Davenport demonstrated adsorption and localization of influenza virus within guinea pig leukocytes after exposing the leukocytes to fluorescent viral antibody (78). Additionally, Sommerville and Macfarlane, localized Coxsackie virus type B within PMNs of patients with aseptic meningitis, Bornholm's disease, and pyrexia (79). Fluorescent antibody techniques demonstrated specific fluorescence in the polymorphonuclear fraction; the number of positive cells depended upon time in relation to onset of illness, severity of illness, and amount of virus present (79). Using electron microscopy, Kańtoch and Zapf observed that Coxsackie virus type A4 penetrates into guinea pig leukocytes (80).

Chemotaxis requires energy. Anderson et al., in analyzing their measles virus studies, suggested that virus infection could interfere with essential energy-producing processes and thereby diminish the chemotactic response (71,72). Mills and co-workers showed that influenza A virus stimulates oxidative metabolism (81). They suggested that virus-initiated generation of toxic radicals could cause cellular changes which impair essential PMN defense responses (81).

In contradistinction to the above studies illustrating virally-induced chemotactic response depression, there are reports of chemoattractant effects of viruses, mediated through tissue cells that serve as substrates for viral infections. As a prelude to discussing these investigations, Blackman and Bubel described polio virus induction of the

release of lysosomal β-glucuronidase and the subsequent leakage of cellular proteins (lactic dehydrogenase and glutamic oxalacetic-transaminase) and virus from HEp-2 cells into the external milieu (82).

Neutrophil chemotaxigenic studies became a focus of interest. Brier et al. showed that primary rabbit kidney (PRK) cells infected with herpes simplex virus (HSV) release a cell factor that, upon incubation with serum complement, generates PMN chemotaxis. Even lysates from uninfected PRK cells (obtained by sonication) generated PMN chemotactic activity upon incubation with serum, suggesting that nonspecific cell injury can stimulate release of the chemotaxigenic substance. The authors postulated the following sequence of events: 1) infection with HSV, a lytic virus, produces cellular damage which results in the release of a chemotaxis-generating factor; 2) this factor then interacts with serum complement to cleave C5a from C5; 3) C5a, demonstrably chemotactic, induces PMNs to migrate to the locus of viral injury (83).

Ward et al. infected chick embryos with Newcastle disease virus (NDV) or with mumps virus (MV), Enders strain, and infected BGM cell monolayers (African green monkey cells) with mumps virus, ABC strain. Allantoic fluids from NDV-infected and from MV-infected embryos attracted rabbit neutrophils, as did fluids from MV-infected cell cultures, the activity residing in an ultracentrifuged virus-free supernatant fraction. Lysates of uninfected allantoic membranes or cells, as derived by freeze-thawing, were not chemotactic, suggesting that nonspecific cell injury does not induce the release of chemotactic factors. Besides this serum-independent activity, virally infected chick embryos also released a substance which, when incubated with purified C3 or C5, generated chemotactic activity (84).

Snyderman and colleagues, using the PRK-HSV system, showed that addition of antiviral antibody and complement, when newly synthesized HSV antigens were noticeable on the PRK cell surface, resulted in injury to the cells and produced a material with chemotactic activity for rabbit neutrophils. The chemotactic activity resided in the cell-free supernatant obtained via centrifugation. Uninfected cells, exposed to the same conditions, failed to generate chemotactic activity. Furthermore, incubation of antibody and complement with viral particles adsorbed to the cell surface (prior to penetration) generated chemotactic activity in the absence of cellular injury. The researchers suggested four possible mechanisms of chemotactic response to viral infection: 1) viruses destroy cells, which then release enzymes that cleave chemotactic substances from complement components; 2) interaction of antibody with virally-induced antigen on the cell surface could lead to activation of the complement system; 3) interaction of antibody with virus antigen adsorbed to the cell surface could activate the complement cascade; and 4) lymphocytes stimulated by virus might liberate chemotaxins (85).

Many virus infections predispose a host to future bacterial infection, presumably by altering the host's immune network. Subjects harboring viruses show a depressed neutrophil chemotactic response as well as modifications in other phagocytic functions, such as particle engulfment and bacterial killing. Additionally, in <u>in vitro</u> experiments where viruses interact with PMNs, reduced PMN motility results. Viruses interacting with tissue cells in culture, however, induce the release of chemotactic factors and chemotaxigenic substances, leading to the accumulation

of granulocytes at areas of infection. The story is confusing and probably complex: on one hand viruses appear to mitigate the immune response, while on the other hand, they ostensibly contribute to inflammatory enhancement. Continued investigation, hopefully, will unravel the mysteries of viral invasion.

MATERIALS AND METHODS

A. Cell Cultures

HeLa cell (Flow Laboratories, Inc., McLean, Virginia) growth-supporting media was Eagle's Minimal Essential Media with nonessential amino acids and Earle's Balanced Salt Solution Formula #78-5470 (Gibco, Grand Island, New York); 2% heat inactivated (56°C, 30 minutes) fetal Calf Serum (Gibco Laboratories); and antibiotics: Streptomycin Sulfate USP 100 mg/ml (Eli Lilly and Company, Indianapolis, Indiana) or Gentamicin 50 mg/ml (Gibco Laboratories), Penicillin G. Potassium 100,000 units/ml (Eli Lilly and Company). 1 ml antibiotics per 1 liter media was used.

Cells grew in 75 cm^2/polystyrene tissue culture flasks (Corning Glass Works, Corning, New York) in a 5% water jacketed CO_2 incubator (National Appliance Company, Portland, Oregon), maintained at 37°C. Upon monolayer confluence (approximately 6 x 10^6 cells), the cells were trypsinized (5 ml 0.1% trypsin (Gibco Laboratories), 0.05% EDTA in Dulbecco's Phosphate Buffered Saline (PBS), pH 7.3). After a 5 minute incubation in trypsin, 10 ml fresh media (FM) was added to the flask to neutralize the trypsin's activity. The cells were distributed into new flasks and FM (10-15 ml) was added for growth.

B. Virus Growth

Coxsackie B5 virus (initially obtained from a clinical specimen, typed and passaged multiple times in HeLa cell culture at Michigan Department of Public Health) was passaged in HeLa cell monolayers sustained in media and was harvested upon 48 hours incubation. Viral cytopathogenicity (CPE) was indicated by the rounded appearance of HeLa cells and subsequent detachment from the polystyrene substratum. Viral effect was visualized in serial passage over time in HeLa cell culture and could be neutralized by the addition of specific Coxsackie B5 virus antisera (obtained from National Institute of Health, Bethesda, Maryland).

C. Virus Titration

Coxsackie B5 virus was titrated according to the method of Reed and Muench (86). Upon monolayer confluence of uninfected HeLa cells in a 75 cm^2 flask, the cells were trypsinized and redistributed into Pyrex® glass tubes, 16 x 125 mm (American Scientific Products, McGaw Park, Illinois). Approximately 1 x 10^5 HeLa cells were added to each tube and an additional 1 ml FM was added per tube to insure cell growth. After a suitable incubation period (24 hours), virus infected media (VIM) was serially diluted and 0.2 ml was added to each tube. Three titration tubes were used per dilution. Tubes were examined daily for the presence of CPE. Repeated experiments showed that maximal viral CPE occurred at 48 hours post inoculation. Accordingly, on the second day after inoculation (3 days past seeding of cells), calculation of virus titer was done as follows:

1. Proportionate distance (PD) =

$$\frac{\%CPE\ @\ dilution\ above\ 50\% - 50\%}{\%CPE\ @\ dilution\ above\ 50\% - \%CPE\ @\ dilution\ below\ 50\%}.$$

2. Multiply PD by dilution factor (= -1 for serial 10-fold dilution).

3. Add negative logarithm of dilution above 50% and product of PD and dilution factor. This number is $\log TCID_{50}$.

4. Virus titer is reciprocal of $TCID_{50}$.

A BIOSTAR® inverted biological microscope (bright field, American Optical Series 1820, Buffalo, New York) was used to assess HeLa cell culture growth and to determine viral titers.

D. Neutrophil Isolation

Neutrophils (PMNs) were isolated according to the method of Smith and Hollers (20). Whole blood (4.5 ml), obtained by venipuncture from healthy adult donors, was drawn into a citrate-coated tube (0.5 ml of 0.105 M buffered citrate solution) and then diluted with an equal volume of Ca^{++} free PBS in a 16 x 100 mm borosilicate tube. The blood was gently mixed with the PBS, layered onto 4 ml Ficoll-Hypaque solution (2.16 gm Ficoll, Sigma Chemical Company, St. Louis, Missouri; 3.39 gm Hypaque (Sodium diatrizoate), Sigma Chemical Company; and 34 ml distilled water) in a siliconized tube (16 x 100 mm), and centrifuged at 1500 rpm (500 x g) for 20 minutes at 10°C (Sorvall RC-5 Superspeed Refrigerated Centrifuge, DuPont Instruments, Dover, Delaware). After centrifugation, the layers of plasma, PBS, monocytes, lymphocytes, platelets, and Ficoll-Hypaque were removed by pipette aspiration; the PMNs and the red blood cells (RBCs) were transferred to a test tube (16 x 100 mm), which had been serum-coated and

washed twice with PBS. The blood cells were diluted with Ca^{++} free PBS to 1.5 times the original volume, and 1.7 ml Volex 6% hetastarch (Lansing Chapter American Red Cross) was added as an RBC sedimenting agent. After a gentle mixing, the cells sedimented approximately 30 minutes at room temperature. The PMN-rich supernatant was removed and transferred to a serum coated tube washed twice with PBS, and an equal volume of Ca^{++} free PBS was added to the solution. The diluted cell solution was centrifuged at 200 x g for 15 minutes (International Clinical Centrifuge, Model CL International Equipment Company, Needham, Massachusetts). The supernatant was discarded, and the cell pellet was resuspended in 1 ml PBS (with Ca^{++}). The PMNs were counted with a Coulter Counter (Model B, Serial Number B3475, Coulter Electronics, Hialeah, Florida): After mixing 0.020 ml cell suspension with 10 ml diluent (Repipet Jr.®, Labindustries, Berkeley, California) and two drops RBC lysing agent (American Scientific Products) in a container, the container was attached to the counter. Cells collected were demonstrated to be greater than 95% neutrophils. PBS (with Ca^{++}) was added to adjust the concentration of neutrophils to 2.3×10^6 per ml.

E. Chemotactic Factor

Stock solutions of N-formyl-methionyl-leucyl-phenylalanine (fMLP) (Sigma) were prepared in dimethyl sulfoxide (DMSO) (Mallinckrodt, Incorporated, Paris, Kentucky) and were stored at -20°C. Viability of neutrophils in a given experiment was demonstrated by morphological response to 6×10^{-10} M fMLP.

F. Freeze Thawing of Media

Spent media (SM) and HeLa cells in fresh media (FT) were subjected to three rounds of freeze-thawing. SM was transferred from tissue culture flasks into a Pyrex® tube. The tube was agitated in a dry ice-acetone bath until the media was frozen; the tube was then agitated in a hot water bath until the media was melted. The process was repeated twice. After the last thawing, the solution was spun in an International Clinical Centrifuge at 500 x g for 5 minutes to pellet debris. The supernatant was aliquoted and stored at -70°C until tested.

The HeLa cell monolayers were disrupted with trypsin solution, and FM was added to neutralize the trypsin. The cells were spun at 500 x g for 5 minutes, the supernatant containing trypsin and FM was discarded, the cell pellet was resuspended, and FM was added. After three cycles of freeze-thawing, the solution was spun (500 x g, 5 minutes), and the supernatant was aliquoted and was stored at -70°C until tested.

G. Hypotonic Lysis of HeLa Cells

Upon confluence of the monolayer, SM was discarded, the cells were washed with PBS, and 7 ml sterile water pH 7.2 was added to the flask. After sitting at room temperature for 15 minutes, the cells were suffused with 7 ml double-strength PBS to restore isotonicity. The supernatant was aliquoted and was stored at -70°C until tested.

H. Centrifugation of Viral Specimens

1. Clarification

VIM in 5 ml samples (duplicates) was placed in 15 ml Corex® glass tubes (18 x 100 mm) and centrifuged at 10,000 rpm (12,100 x g) at 4°C for 10 minutes (Sorvall Superspeed RC2-B automatic refrigerated centrifuge, Rotor Sorvall Type SS34, Newtown, Connecticut). The supernatant (approximately 4.8 ml) was transferred to 10 ml polycarbonate ultracentrifuge tubes (Beckman Instruments, Palo Alto, California). The cell debris was washed three times with PBS and recentrifuged each time at 10,000 rpm for 10 minutes at 5°C. After the third washing, the pellet was resuspended in 1 ml PBS and maintained at 4°C (approximately one hour) until tested against neutrophils.

2. Ultracentrifugation

The 4.8 supernatant obtained from clarification was ultracentrifuged at 40, 500 rpm (100,000 x g) at 4°C for 60 minutes (Beckman Preparative Ultracentrifuge Model L8, Beckman Instruments, Beckman Fixed Angle Rotor Type 50 Ti, radius:37.4 mm-80.8 mm, Serial Number 1622). The supernatant was transferred to a Falcon plastic test tube (12 x 75 mm, Falcon, Oxnard, California), and the viral pellet was resuspended in 1 ml PBS and then transferred to a Falcon tube. The solutions were maintained at 4°C until tested against neutrophils.

I. Experimental Protocol

1. Large Volume

a. Neutrophils

Glass tubes (12 x 75 mm) were coated with normal human serum and washed two times with PBS, after which 0.1 ml neutrophils (approximately 230,000) were pipetted into them. The neutrophils were incubated for 15 minutes in a 37°C 5% CO_2 incubator.

b. Media

VIM, SM, or FM in 0.2 ml quantities were added to 0.7 ml PBS, and the solution was incubated for 15 minutes at 37°C, 5% CO_2.

c. Controls

A PBS (negative) control (0.9 ml) and an fMLP (positive) control (0.8 ml PBS plus 0.1 ml 6 x 10^{-9} M fMLP) were performed with each experiment.

d. Reaction

The neutrophils were added to the warmed media, and the mixture (1 ml total volume) was incubated for 5 minutes at 37°C, 5% CO_2. The cells in suspension were then fixed with twice the volume of buffered (0.4 M PO_4 buffer) 2% glutaraldehyde (Sigma Chemical Company). The fixed suspensions were maintained at 4°C until analyzed.

2. Small Volume

a. Neutrophils

Glass tubes (12 x 75 mm) were coated with normal human serum and washed twice with PBS, after which 0.1 ml neutrophils (approximately 230,000) were pipetted into them. The neutrophils were incubated for 15 minutes at 37°C, 5% CO_2.

b. Media

FM, SM, VIM, FT, SM FT, H_2O lysis in 0.2 ml quantities were

added to glass tubes and incubated for 15 minutes at 37°C, 5% CO_2.

c. Controls

A PBS (negative) control (0.2 ml) and an fMLP (positive) control (0.190 ml PBS plus 0.010 ml 1.2 x 10^{-8} M fMLP) were performed with each experiment.

d. Reaction

The neutrophil solution was spun for 1.5 minutes at 500 x g (International Clinical Centrifuge) to pellet the neutrophils. The PBS supernate was discarded, and the reagent media was added to the neutrophils. The test tube was hand-shaken to resuspend the neutrophils, and the solution was incubated for 5 minutes at 37°C, 5% CO_2, after which a 2X volume of buffered 2% glutaraldehyde was added to fix the cells. The suspensions were maintained at 4°C until analyzed.

J. Assessment of Neutrophil Shape Change

Fixed cells were examined under phase contrast microscopy with a 100X objective and were categorized according to shape (Figure 1):

1) spherical--spherical cells without obvious surface ruffles.
2) ruffled--spherical cells with ruffles around the edges.
3) bipolar--elongated cells with ruffles or lamellipodia at one pole.
4) uropod--elongated cells with ruffles or lamellipodia at one pole and having a tail, or uropod, at the other pole.

For the purposes of the present analysis, "spherical" and "ruffled" shapes were grouped together as spherical forms, and "bipolar" and "uropod"

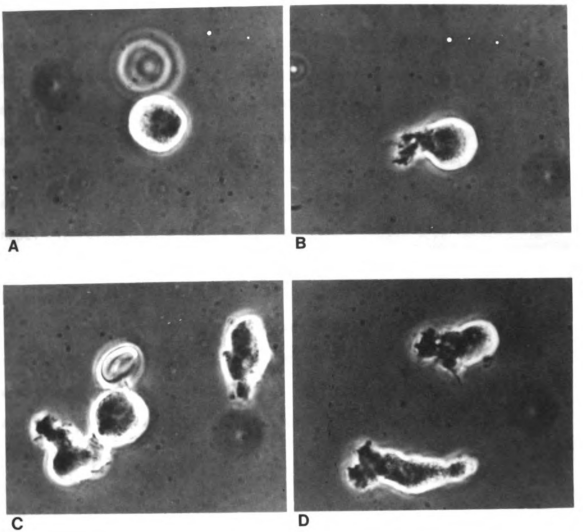

FIGURE 1 CHANGE IN SHAPE (x100) OF PMNs

PMNs WERE SUSPENDED IN PBS (2.3×10^6 CELLS/ML) AND STIMULATED WITH FMLP (6×10^{-10}M) AT 37°C FOR 5 MIN. AND THEN FIXED WITH GLUTARALDEHYDE. PHASE CONTRAST LIGHT MICROSCOPY WAS USED, AND PHOTOGRAPHS WERE MADE WITH POLAROID TYPE 665 POSITIVE/NEGATIVE FILM: A) SPHERICAL; B) RUFFLED; C) COMPARISON OF RUFFLED (LEFT), SPHERICAL (CENTER), AND BIPOLAR (RIGHT): D) COMPARISON OF BIPOLAR (TOP) AND UROPOD (BOTTOM).

shapes were classified together as bipolar forms. The shift to a bipolar form from a spherical form is an identifiable indicator of a stimulated neutrophil. For a given chemotactic factor, the change is rapid, occurring within minutes, and the glutaraldehyde-fixed shapes are discernible with light microscopy. An fMLP dose of 6×10^{-10} M stimulated approximately 50-60% of isolated neutrophils to undergo the transistion in shape. Therefore, this dose (ED_{50}) was used as a positive control with various agents in order to detect noticeable stimulation or inhibition by the agents (i.e., a shift up or down from the 50% level).

One hundred cells were counted per test, and shapes were recorded in terms of percentages. The data are expressed as mean ± standard deviation; n represents the number of separate experiments. Each experiment was done in duplicate. Student's t-test was used as a measure of significance.

RESULTS

A. Investigation of Media Controls

Preliminary experiments involved an exploration of appropriate media controls. Spent media (SM), defined as growth-supporting media from noninfected HeLa cell cultures, exposed for a time equal to the viral infection period (2 days), served as a measure for testing the effects of cellular metabolic products. SM caused an effect which appeared dose- and concentration-dependent.

Fresh media (FM), media used for growth of cell cultures, but not yet exposed to the cells, provided a test for the effects of media alone. The media stimulated neutrophils, seemingly in a dose- and concentration-dependent fashion, analogous to SM.

Fresh media incubated (FMI), which is fresh media incubated for the same time length as SM or virally infected media (VIM), was investigated as a test for the effects of media incubation upon neutrophil response. The incubated media showed stimulatory effects, but the neutrophil response was not significantly different from that produced by SM or FM (data not shown).

B. Experimental Conditions

To insure optimal PMN response and maximum virus activity, experimental conditions were adjusted to account for variables of time, pH, and

temperature. Preliminary investigations using control media preparations suggested that an interaction time of five (5) minutes was appropriate. Maximal bipolar formation occurred between two (2) and ten (10) minutes incubation. After ten minutes, in control media, the cells reverted to spherical form with increasing frequency over time.

To maintain an adequate buffering system all experimental incubations were performed in a 5% carbon dioxide incubator. A pH of 6.5-7.4, the necessary range for normal PMN function, was maintained. Furthermore, all reagents were adjusted to the same range.

All media samples and the PMNs were incubated separately for fifteen (15) minutes at 37°C to provide a uniform temperature (37°C) of interaction. Other experimenters showed that PMNs perform better at 37°C than at other temperatures (20), and that Coxsackie viruses exert a more pronounced influence on PMNs at 37°C than at other temperatures (87).

C. Large Volume Effects

In initial experiments comparing VIM with control media, a reaction volume of 1 ml, which included PBS as a solvent, was used. As depicted in Table 1, undiluted VIM, which contained approximately $10^{6.5}$ virus particles per 0.2 ml, stimulated significantly the neutrophils, in comparison to other media. Furthermore, there was a 26% increase in bipolar formation by VIM undiluted over the negative PBS control.

When the VIM was diluted 1:10 and 1:100 (not shown) in PBS, thereby reducing the virus to PMN ratio from 10:1 to 1:1 and 1:10 respectively, the PMN response did not differ from controls.

Table 1. Large Volume Solution Effects on PMN Shape

Medium	Dilution	V/PMN	N	$\bar{X}$ % Spherical ± S.D.
FM	1:1	-	5	97.70 ± 1.86
	1:10	-	5	99.80 ± 0.45
SM	1:1	-	6	99.42 ± 0.66
	1:10	-	6	99.75 ± 0.27
VIM	1:1	10:1	6	74.0 ± 5.25 ($P < 0.0002$)
	1:10	1:1	6	97.67 ± 1.60
PBS	-	-	6	100 ± 0

The noninfected media preparations did not cause neutrophil shape change, at any dilution, compared to PBS control. Earlier experiments, however, demonstrated bipolar shape formation when 0.7 ml media was used in the reaction mixture rather than 0.2 ml (data not shown).

D. Small Volume Effects

To increase the probability of interaction between virus particles and neutrophils, the reaction volume was decreased, by eliminating PBS. As shown in Table 2, the undiluted VIM (Coxsackie virus titer $10^{6.5}$ per 0.2 ml) caused bipolar shape formation. The shift in morphological shape was significant as compared with the PBS control and with other media preparations.

VIM, at a 10-fold dilution, resulting in a virus to PMN ratio of 1:1 in the reaction mixture, still produced a significant PMN shape change

Table 2. Small Volume Solution Effects on PMN Shape

Medium	Dilution	V/PMN	N	$\bar{X}$ % Spherical ± S.D.	
FM	1:1	-	5	81.00 ± 8.49	
SM	1:1	-	6	82.17 ± 14.13	
VIM	1:1	10:1	6	31.08 ± 25.05	(P< 0.02)
FM	1:10	-	5	99.20 ± .45	
SM	1:10	-	6	97.50 ± 1.30	
VIM	1.:10	1:1	6	70.83 ± 8.92	(P< 0.005)
FM	1:100	-	5	99.80 ± .45	
SM	1:100	-	6	99.25 ± .88	
VIM	1:100	1:10	6	94.92 ° 4.71	(NS> 0.05)
PBS	-	-	6	99.67 ± .82	

response, compared to PBS and to other media. The effect, however, was definitely less pronounced than that produced by media containing 10 times more viral particles than PMNs. When diluted further, 100-fold or 1000-fold (not shown), the shape change-producing effect disappeared.

Fresh media and spent media preparations when undiluted, caused neutrophils to polarize. The change was significant, in both instances, with respect to the PBS control, but less marked than the bipolar formation attributable to the VIM. At higher dilutions, 10-, 100-, 1000-fold (not shown), no neutrophil shape change response, compared to PBS control, was detectable.

Several paramount questions arose from these studies. Is PMN shape change caused by the virus itself; and, if so, can the viruses be concentrated so as to amplify the response? Secondly, is the effect on PMN

shape due to a component in the media, possibly liberated from HeLa cells as a result of viral action (indirect viral influence)? Thirdly, if the virus affects PMNs indirectly, is the action on the HeLa cells specific, or could nonspecific destruction of the cells generate the same inducer of PMN shape change?

E. Ultracentrifugation

Ultracentrifugation of the viral media was performed in an attempt to answer the first two questions. As shown in Table 3, most of the activity for PMNs resided in the supernatant. The supernatant induced approximately the same amount of polarity as did the uncentrifuged preparation, and produced a significantly greater neutrophil response than did the PBS control ($P < 0.0002$) or the virus samples ($P < 0.05$).

Table 3. Effects of Viral Media Constituents on PMN Shape

Medium	V/PMN	N	$\bar{X}$ % Spherical ± S.D.
Cell Debris	---	3	95.33 ± 3.51
Supernatant	---	3	18.50 ± 2.00 ($P < 0.05$)
Virus (ucfg*)	50:1	3	67.83 ± 13.28
Virus (ucfg, diluted 1:5)	10:1	3	86.67 ± 10.15
VIM (non ucfg)	10:1	3	26.17 ± 5.80
PBS	---	3	97.00 ± 1.00

*ucfg = ultracentrifuged
stock virus solution (VIM) titer was $10^{6.5}$/0.2 ml

The viral pellet sample, containing about 15 x 10^6 particles, produced a shape change response. Additional studies likely would confirm the seemingly nonsignificant effect produced by the pellet. In comparison with the undiluted VIM or with the separated supernatant, however, the response was minimal. Furthermore, when the pellet was diluted so that the number of viral particles equalled the number in the noncentrifuged sample, 3 x 10^6 particles, neutrophil polarization was practically nonexistent.

In a concomitant study, the effects of viral media constituents upon inhibition (or potentiation) of fMLP--induced neutrophil shape change were assessed. As noted in Table 4, none of the media components affected markedly the fMLP response. The supernatant plus fMLP, however, demonstrated a greater polarization response than did other fMLP-media preparations.

Table 4. Effects of Viral Media Constituents on Inhibition of PMN Shape Change

Medium	V/PMN	N	$\bar{X}$ % Spherical ± S.D.
Cell Debris + fMLP	---	2	31.75 ± 5.30
Supernatant + fMLP	---	2	13.75 ± 6.72
Virus (ucfg) + fMLP	50:1	2	17.50 ± 2.12
Virus (ucfg, diluted 1:5) + fMLP	10:1	2	31.25 ± 8.84
VIM (non ucfg) + fMLP	10:1	2	19.50 ± 4.95
fMLP (6 x 10^{-10} M)	---	2	29.50 ± 16.26

Verification of separation of media constituents by ultracentrifugation was ascertained through titering the supernatant, using the method of Reed and Muench (86), for the presence of virus. In each of the three experiments, greater than 90% reduction in viral titer was determined.

F. Nonspecific HeLa Cell Injury

Since most of the activity towards PMNs resided in the ultracentrifuged supernatant of virally infected media, viral-specific effect was investigated by disrupting HeLa cells with alternate methods. The supernatant from preparations of frozen-thawed HeLa cells produced minor stimulation of PMNs (Table 5), compared to the PBS control. Nonetheless, the neutrophil activity produced from this media was not as marked as the activity induced by VIM or viral media supernatant (Tables 2 and 3). For cell maintenance, the HeLa cells were suspended in fresh media, during freeze thawing.

Table 5. Effects of Frozen-Thawed Media on PMN Shape

Medium	N	$\bar{X}$ % Spherical ± S.D.
HeLa cells FT	7	77.86 ± 15.89
SM	7	88.14 ± 5.27
SM FT	7	94.00 ± 5.35
PBS	7	99.29 ± 1.11

Spent media was frozen and thawed to ascertain whether the process of freeze-thawing would affect neutrophil shape. No stimulation resulted from frozen-thawed spent media supernatant, as compared to control. Indeed, the freeze-thawing process may depress a neutrophil response, as suggested by SM and SMFT data.

As another type of nonspecific cell injury, HeLa cells were hypotonically lysed with water, and the supernatant was tested for shape change effect. Table 6 illustrates that the media from the lysed cells caused no PMN polarization.

Table 6. Effects of Hypotonically Lysed HeLa Media on PMN Shape

Medium	N	$\bar{X}$ % Spherical ± S.D.
Water	7	99.29 ± .81
PBS	7	99.57 ± .53

DISCUSSION

The data indicates that Coxsackie B5 virus infected media stimulates PMNs. Upon isolation, neutrophils are spherical. When exposed to the chemotactic factor fMLP (6×10^{-10} M), 50-60% adopt bipolar forms within five minutes. The rapid change in shape is a component of the chemotactic process and may be an important indicator of that phenomenon. Smith *et al.* (20) and Smith and Hollers (42) used shape change as a measure of neutrophil responsiveness to several chemotactic factors with consistent results. PMN response varies little from day to day and between donors.

The level of PMN stimulation produced by VIM was 75% of that achieved with fMLP at 2×10^{-9} M, the dose of optimal responsiveness for the peptide. Williams *et al.* demonstrated the presence of fMLP receptors on neutrophils (15). PMN membrane receptors for viruses, including the Coxsackie virus group, however, are not defined. Indeed, adsorption studies by Gomez *et al.* showed that Coxsackie B3 binds to mononuclear phagocytes and not to granulocytes (88). Preliminary work in this laboratory using Coxsackie B5 virus indicates minimal adsorption by PMNs (Dennis L. Murray, personal communication). Regardless, the virus probably does not compete for the same binding sites as fMLP, as no inhibition of fMLP-induced shape change was found using either VIM or a centrifuged viral pellet.

VIM stimulation of PMNs is apparently dose and concentration dependent and occurs rapidly. In both large and small volume experiments (Tables 1 and 2), the stimulating effect of VIM decreased with increased dilution. Furthermore, the more concentrated media solution, i.e., small volume, elicited a more pronounced PMN response. Apparently, decreasing the reaction volume creates more interaction between media components, including virus, and PMNs. Studies with other PMN chemotactic factors support the dose and concentration dependence phenomena, as well as the acute response, illustrated by VIM (8,13).

Previous experimenters using viral preparations described neutrophil chemotactic depression. Most researchers used influenza virus (57, 61, 63, 64), and only one group purified the virus (61). None of the investigators, however, discussed possible media influences.

Analysis of enterovirus effect upon PMN chemotaxis is recent. In an ECHO virus investigation, Bültmann et al. incubated virus solution with PMNs for one hour before exposing the mixture to a gradient of chemotactic factor in the Boyden Chamber assay (75). The inhibition they visualized may be attributable to the virus, but components in the solution also could be responsible. No spent media controls were performed.

In a later study, Bültmann and Gruler used a Zigmond orientation chamber and a statistical mathematical model to describe selective chemotactic disturbance by ECHO virus (89). They demonstrated that the degree of PMN orientation declines with both increasing virus dose and with increasing time of virus-PMN interaction (89). Their analysis appears sophisticated, but, again, they used a virus suspension, rather than purified virus, and they did not discuss spent media controls.

The above concerns regarding viral suspension-PMN interactions point to the necessity for designing and performing careful media controls. Spent media and fresh media, in the present Coxsackie B5 virus studies, caused PMNs to change shape, albeit minimally. Ostensibly, the spent media contains some constituents, possibly liberated from the HeLa cells, which stimulate neutrophils, to some extent. Moreover, breakdown of media over time may generate certain factors which influence PMN shape.

PMNs are sensitive to many substances, including bacteria (10) and C5a (9). Bacterial contaminants in the media could trigger a PMN response. Moreover, HeLa cell debris could be chemotaxigenic, i.e., induce the cleavage of C5 to C5a upon interaction with supporting serum in the media. Both of these possibilities were unlikely to this study because first, bacterial contamination likely would be evident in the culture flask and would elicit significant PMN shape alteration; and second, the serum used for cell growth was 2% heat inactivated fetal calf serum, presumably devoid of any complement components.

Undoubtedly there are many unknown components within the media solution. One substance could activate neutrophils; whereas, another substance might mitigate a granulocyte response. Depending on the circumstances, one factor could prevail over another. There was no evidence for depressed PMN activity within the constraints of this study. Boyden Chamber analysis or longer incubation period tests may reveal different information.

A decreased chemotactic response over time could result from chemotactic deactivation, a phenomenon first categorized by Ward and Becker, whereby exposure to PMNs to a high dose of a chemotactic factor, which

could happen within minutes, makes the cells unresponsive to the same factor at a future time (25). In the present study, VIM stimulated PMNs within the five minute time frame, but at a later time, e.g., 45 minutes, VIM might fail to elicit a PMN response. This is a possibility to test.

The next step was to separate the viral particles from the surrounding milieu by ultracentrifugation. Testing each fraction independently showed most of the stimulating activity to be in the supernatant. Lack of any effect by the viral pellet suggests that Coxsackie B5 virus neither stimulates nor inhibits stimulation of PMNs. The absence of neutrophil stimulation by the virus is in excellent agreement with all previous investigations. To date, there is no experimental evidence for direct viral chemotactic stimulation. Lack of demonstrable inhibition of shape change by Coxsackie B5 virus is not disconcerting, though, as the studies mentioned above did not employ isolated virus pellets.

The presence of chemotactic activity in the supernatant is not, however, unprecedented. Ward et al., ultracentrifuged Newcastle Disease Virus--and Mump Virus--infected culture media and discovered chemotactic activity solely in the virus-free supernatant (84). As with Coxsackie B5 virus, the viral pellets did not activate neutrophils. A nonsedimenting material in the virally infected fluid (and not in uninfected media) was responsible for activation in their study and, likewise, in the present work. They did not attempt to elucidate the nature of that material (Peter Ward, personal communication).

Presumably, Coxsackie B5 virus is inducing HeLa cells to release products, which then stimulate PMNs. Identification of the specific agent might be achieved via more sophisticated ultracentrifugation and

affinity chromatography. Interval experiments where VIM is removed at certain time points and then tested with PMNs would indicate at which point maximal stimulation occurs. Preliminary investigation in this laboratory indicates that the greatest neutrophil response occurs with VIM of 36 to 48 hours incubation, a time point correlative with highest virus titer. There may be a causal linkage between virus proliferation and exudation of cellular constituents.

Another interesting question concerns the possible chemotaxigenity of HeLa cell lysates. Upon incubation with serum, could the lysates generate a chemotactic factor? Brier et al. (83) and Snyderman et al. (85) described factors released from culture cells which, upon interaction with serum or with complement alone, could generate the chemotaxin C5a. Ward et al. also demonstrated the same phenomenon using small volumes (as such, nonchemotactic) of a virus-free supernatant (84).

Nonspecific injury to the HeLa cells did not generate shape change-inducing activity. Although supernatant from frozen-thawed cells stimulated neutrophils, most of the activity might be due to FM, which was used as supporting media. Similarly, Ward et al. did not show chemotactic activity from supernatants of uninfected frozen-thawed culture media (84). As an additional test, hypotonic lysis, which visibly destroyed cellular integrity, did not cause the release of any substance that affected neutrophil shape.

The seeming conclusion is that viral action upon HeLa cells is specific. In a typical viral lytic infective mechanism, a virus adsorbs to the cell, enters, replicates using host machinery, and finally destroys the cell with simultaneous release of cellular products. Blackman and

Bubel showed that poliovirus, an enterovirus, induces release of cellular proteins from HEp2 cells as a result of infection (82). Presumably, Coxsackie B5 virus interacts with HeLa cells similarly, via specific receptors. A substance which activates neutrophils is among the cellular exudates.

Future investigation of virus-specific generation of neutrophil activators probably should focus on the virus. For instance, a synthetic chemical might duplicate viral action. Alternatively, one could inactivate the virus by ultraviolet irradiation, by heating, or with immune serum; infect HeLa cells with the inactivated specimen; and assay the media for PMN effect. Perhaps viral replication is not required to cause cellular leakage.

Further Coxsackie B5 studies might include other aspects of the PMN response. One could examine each of the myriad facets of chemotaxis, individually or in series, including: directional motility using the Boyden chamber, the Nelson under agarose assay, or the Zigmond orientation chamber; random motility (chemokinesis); adherence; oxidative metabolism; and lysosomal enzyme release.

The influence of Coxsackie B5 virus upon other essential neutrophil functions such as opsonization, phagocytosis and bactericidal (or viricidal) capacity could be clinically useful information. Some work has been done. Kańtoch et al. performed extensive investigations of Coxsackie A4 virus (supernatants of centrifuged suspensions derived from inoculated mice), influence upon horse and guinea pig leukocytes and consistently found inhibition of bacterial phagocytosis (87,90,91). Murray and Chakrabarty demonstrated that Coxsackie B5 virus

in media solution did not influence PMN bactericidal activity (92).

In summary, the data provide no evidence for direct Coxsackie B5 viral influence upon PMN morphology, though ostensibly the virus caused the release of cell culture constitutents, which caused PMN polarization. Whether Coxsackie B5 virus could engineer in vivo a similar phenomenon is unknown. Further work to elucidate the mechanisms involved is appropriate.

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