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Volume Changes Induced in Rabbit Polymorphonuclear Leukocytes by Chemotactic Factor and Cytochalasin B

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Incubation of rabbit neutrophils with various chemotactic factors causes an expansion of their volume. The expansion shows a high correlation with the chemotactic responsiveness of these cells, requires metabolic energy, and is independent of the presence of divalent cations. Cytochalasin B causes a decrease in the volume of the neutrophil. This decrease also requires metabolic energy and is independent of divalent cations. In the presence of cytochalasin B, the chemotactic factor, instead of acting to expand cell volume, induces a further contraction of the cell; this decrease requires Ca^{2-} in the external medium. (Am J Pathol 81:1-14, 1975)

STIMULATED BY THE BELIEF that chemotaxis, lysosomal enzyme secretion, and phagocytosis of the neutrophil involve activation of the contractile mechanisms of the cell, we have shown that ATP induces a microscopically observable contraction of glycerol-treated rabbit neutrophils [1] similar to that found with other "contractile cell models." [2] A decrease in the volume of the neutrophil is also demonstrable, which is clearly a reflection of the microscopically observed contraction.[1] Subsequent quantitative studies of the ATP-induced volume decrease revealed that it possessed many of the characteristics of actomyosin interactions.[3] Moreover, Marsh [4] and Fay and Delise [5] have clearly demonstrated a decrease or increase in cell volume associated with the corresponding contraction or relaxation of skeletal or smooth muscle cells.

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The mechanisms are essentially unknown whereby chemotactic agents such as C5a, or a factor derived from *Escherichia coli* culture filtrates [6] induce chemotaxis or lysosomal enzyme secretion.[7] The findings cited above suggested that measurement of the changes in cell volume induced by chemotactic agents might provide insight into the mechanisms of their action. As will be shown in this report, chemotactic agents acting on rabbit peritoneal neutrophils in suspension induce an expansion of neutrophil cell volume which, under certain circumstances, correlates with the chemotactic responsiveness of the cell. Cytochalasin B in concentrations sufficient to inhibit neutrophil movement alters the response to the chemotactic agents from an expansion to a contraction. Based on these and other observations, we postulate that chemotactic agents activate cell movement by inducing a volume expansion and contraction of the neutrophil, and that the inhibition of cell movement by cytochalasin B stems from its inhibition of the mechanisms of cell expansion while leaving the mechanisms of volume contraction intact or even stimulated.

Materials and Methods

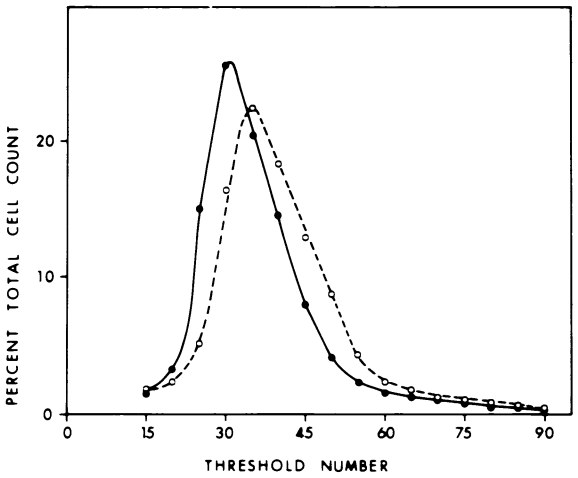
The rabbit peritoneal neutrophils and C5a were obtained as described previously [8] and the bacterial chemotactic factor (bacterial factor) as described by Schiffman *et al.*[6] The cytochalasin B was purchased from ICI Research Laboratory, Alderley Park, England. It was dissolved in dimethyl sulfoxide at a concentration of 4 mg/ml and stored at 4 C until used. The EDTA was obtained from J.T. Baker Co., Phillispsburg, N.J.; the iodoacetic acid and deoxyglucose were obtained from Sigma Chemical Co., St. Louis, Mo.

The mean cell volume of a given cell suspension was measured directly as the mean cell threshold using a Model ZBI Coulter electronic particle counter with a mean cell volume computer. The procedure is described in detail by Koza *et al.*[9] The final concentration of the cells was 4 to 6 $\times$ 10^4/ml. The volume changes of the leukocytes are expressed as the percent of the control cell volume measured at a comparable time interval. All experiments were carried out at room temperature except for the preincubation of the cells with glycolytic inhibitors (iodoacetic acid or deoxyglucose) which was done at 31 C. Cells isolated from the peritoneal exudate were preequilibrated in Hanks' buffer for 30 minutes at room temperature before each experiment. The spontaneous change in cell volume was usually, but not always, insignificant, and therefore a control measurement of mean cell volume in the absence of either cytochalasin B or bacterial factor was always carried out in each individual experiment. In studying the effect of bacterial factor in the presence of cytochalasin B, cells were preincubated with the cytochalasin B for 15 minutes at room temperature, and the control mean cell volume in such experiments was therefore observed in the presence of cytochalasin B. For the distribution curve of Text-figure 1, the model ZBI Coulter Counter was appropriately adjusted and the distribution was obtained according to the method of Hsu and Becker.[1]

Results

Chemotactic Factor-Induced Volume Expansion

Text-figure 1 shows the volume distribution curve of neutrophils before and after the addition of a 1:5000 dilution of bacterial factor. The entire



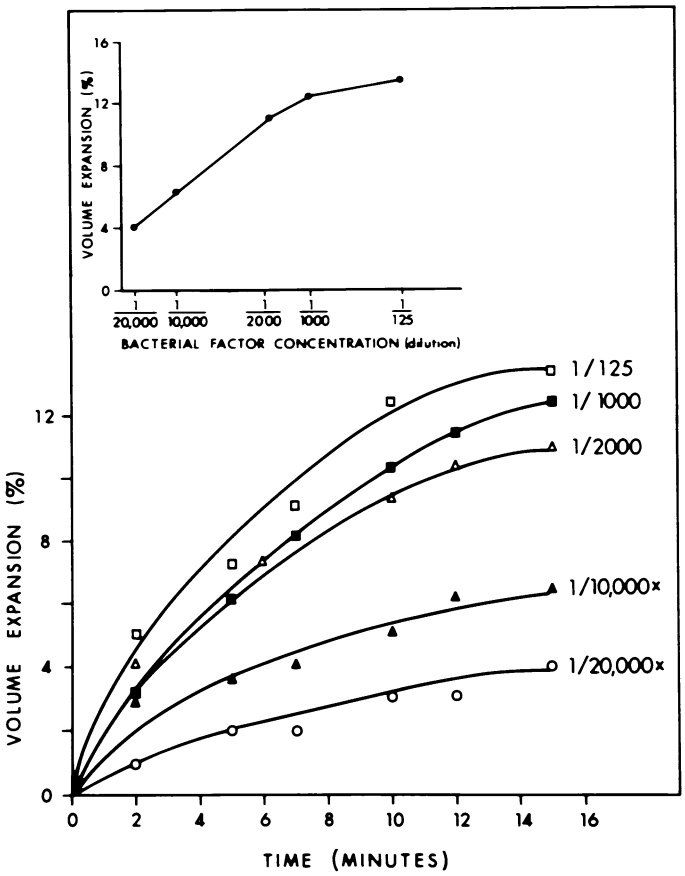
TEXT-FIGURE 1—Effect of bacterial factor on the volume distribution of neutrophils [Control cells (no bacterial factor added), *solid line*; at 20 minutes after the addition of bacterial factor in 1 : 500 dilution, *dotted line*].

distribution curve is seen to shift to the right, indicating that most of the cells responded to bacterial factor with an expansion of their volume. In this experiment, cells treated with bacterial factor increased their mean cell volume 10.5% over that of the control cells.

Text-figure 2 shows the time course of the expansion of a cell preparation following its reaction with dilutions of bacterial factor ranging from 1 : 20,000 to 1 : 125. Depending on the cell preparation and the concentration of chemotactic factor, the time required for cells to achieve maximal volume expansion in response to the bacterial factor ranged from 10 to 20 minutes (Text-figure 2).

The inset of Text-figure 2 shows the dose-response curve of bacterial factor-induced volume expansion observed 15 minutes after the addition of bacterial factor. In this instance, the effect of bacterial factor became constant at concentrations above a dilution of 1 : 1000. In other cell preparations, the volume expansion reached an optimum at a 1 : 1000 to 1 : 2000 dilution, higher concentrations being less effective. One of these convex dose-response curves is shown in Text-figure 3. This type of dose-response curves is also frequently encountered in chemotaxis for reasons which are not clear.[8]

Although partially purified bacterial factor was used in most of these studies, we have also demonstrated, in experiments not shown here, that a highly purified bacterial factor,[6] in concentrations inducing significant chemotactic activity, expanded the cell volume of rabbit neutrophils. Furthermore, the highly purified material also induced a volume contraction in the presence of cytochalasin B (see below). Moreover, the volume-expanding effect is not restricted to the bacterial chemotactic factors. As



TEXT-FIGURE 2—Time course and dose-response curve (*inset*) of bacterial-factor-induced volume expansion

shown in Text-figure 3, C5a (a chemotactic agent derived from the fifth component of human complement) was also able to expand the cell volume at the same concentrations that resulted in chemotactic activity. The time course of the volume expansion caused by C5a is similar to that induced by the bacterial factor. These observations make it highly unlikely that the volume response elicited by the partially purified bacterial material was due to the impurities present.

Correlation Between the Ability of the Cell to Expand its Volume and Its Chemotactic Activity in Response to Bacterial Factor

Neutrophils were obtained in the usual way from 16 rabbits and their chemotactic responsiveness measured over a range of dilutions of bacterial factor from 1:5000 to 1:62.5. At the same time, the ability of the same cells to expand their volume was measured at a 1:250 and 1:100 dilution of the same chemotactic factor. Because of technical difficulties, measure-

ments of the volume expansion of the leukocytes over a wider range of concentrations were not possible. The coefficients of correlation were calculated relating the chemotactic responsiveness of each dilution and the volume expansion obtained at each of the two dilutions studied. The results can be seen in Table 1.

A steady increase in the degree of correlation between chemotactic responsiveness and volume expansion was found as the chemotactic response was measured at higher and higher levels of chemotactic factor. There was a statistically highly significant correlation between the chemotactic response induced at the two highest concentrations of chemotactic factor and the volume expansion induced by either of the two dilutions of factor employed for that purpose. Chemotactic activity obtained at the lower concentrations of bacterial factor did not correlate significantly with the induced volume expansion.

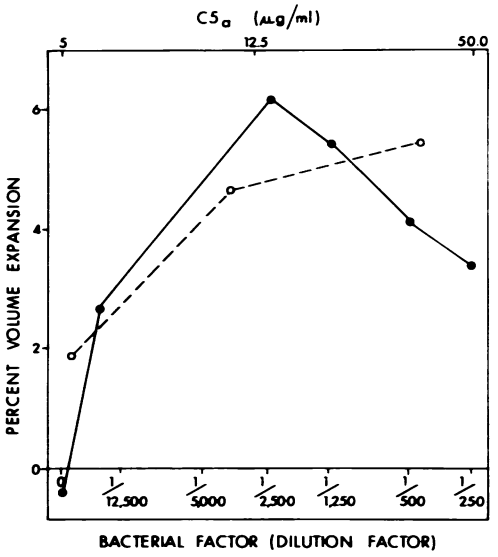
Lack of Requirement of Extracellular Divalent Cation for Volume Expansion

Ca^{2+} and Mg^{2+} are both required for an optimal chemotactic response of rabbit [10] and human [11] neutrophils, and EDTA strongly inhibits chemotaxis. We therefore compared the volume expansion induced by a 1:250 dilution of bacterial factor in the presence and absence of 5 mM EDTA. In general, as exemplified in Text-figure 4, EDTA significantly retards the rate but not the extent of volume expansion. In a very few instances, however, EDTA not only decreased the speed of the volume response but also significantly reduced its final extent. The underlying nature of this cellular variability is unknown. In the presence of 0.25% dimethylsulfoxide, EDTA consistently enhanced the cell expansion induced by bacterial factor (see Text-figure 8). Thus, in general, the extent of the volume expansion induced by chemotactic factor does not depend on the presence of divalent cations in the medium.

Table 1—Correlation of Chemotactic Responsiveness With Cell Expansion

Dilutions for testing chemotaxis	Correlation coefficient (r)	
	Dilutions for testing expansion	
	1:100	1:250
1:5000	0.0211	−0.0256*
1:2500	0.1442	−0.1114
1:1000	0.1287	0.09181
1:500	0.3346	0.2670
1:250	0.4417	0.3959
1:125	0.7734†	0.6049†
1:62.5	0.7104†	0.6808†

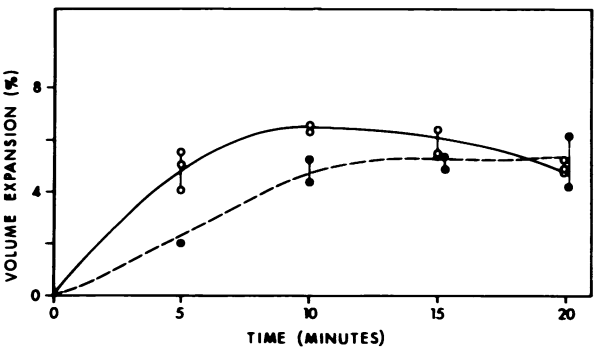
* N = 14; for the remainder, N = 16.
† Statistically significant, $P < 0.05$.



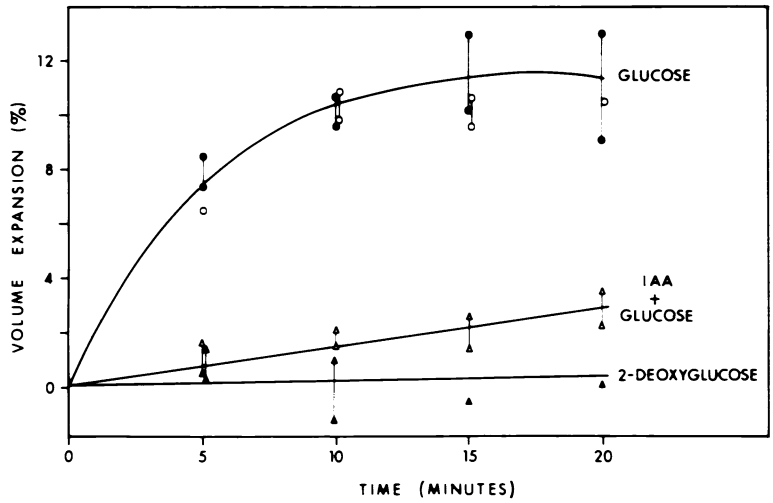
TEXT-FIGURE 3—Dose-response curves of volume expansion induced by bacterial factor (*solid line*) and C5a (*dotted line*)

Energy Requirement for Bacterial-Factor-Induced Volume Expansion

Cells incubated with 5 mM deoxyglucose in the absence of glucose or with 0.1 mM iodoacetate in the presence of 5 mM glucose for 30 minutes at 37 C lost their ability to expand in response to the bacterial chemotactic factor, as shown in Text-figure 5. In two studies not shown here, the inhibiting effect of deoxyglucose was partially or completely prevented by the inclusion of 5 mM glucose in the preincubation period. Treating cells with deoxyglucose either in the presence or absence of glucose caused no significant changes in cell volume. Incubating cells with iodoacetate, however, caused to 5 to 7% decrease in cell volume compared to the control. These findings exclude the possibility that the cells failed to respond



TEXT-FIGURE 4—Time course of bacterial-factor-induced volume expansion in the presence and absence of EDTA. The bacterial factor was used in a dilution of 1:2000. (Hanks' buffer containing 1.7 mM Ca^{2+} and 0.7 mM Mg^{2+}, *solid line;* 5 mM EDTA in Hanks' buffer containing no Ca^{2+} or Mg^{2+}, *dotted line*).



TEXT-FIGURE 5—Effect of deoxyglucose and iodoacetate on volume expansion induced by bacterial factor. Cells were preincubated with 5 mM deoxyglucose (*solid triangles*), 0.1 mM iodoacetate + 5 mM glucose (*open triangles*), or 5 mM glucose (*solid circles*) at 37 C for 30 minutes. Volume expansion induced by bacterial factor at 37 C in the absence of these reagents is indicated by the *open circles*.

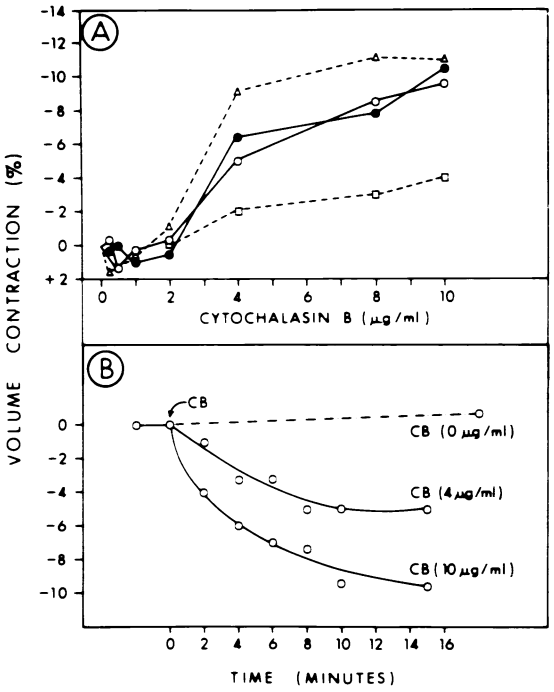
to bacterial factor because their volume had already been expanded by the glycolytic inhibitors. These observations therefore suggest that depletion of cellular ATP or other high energy intermediates may reduce the volume response elicited by bacterial factor.

Effect of Cytochalasin B on Cell Volume

Cytochalasin B acting on the cell in the absence of chemotactic factors induces a decrease in cell volume. Text-figure 6 shows the time course and dose-response curve of the cytochalasin-B-induced contraction of cell volume. The minimal concentration of cytochalasin B required for inducing a volume decrease is 4 μg/ml. The time required to reach the minimal volume ranged from 5 to 15 minutes; once attained, the cell volume remained stable for at least an hour.

In results not reported here, no demonstrable effect of EDTA on the decrease of volume induced by cytochalasin B was ever observed.

Similar to the bacterial-factor-induced expansion, the volume decrease induced by cytochalasin B is also significantly inhibited by either deoxyglucose or iodoacetate, as shown in Text-figure 7. Because of the variability encountered, the results of three experiments employing cells from three different rabbits are shown. The effect of deoxyglucose can be partially prevented by 5 mM glucose. Interestingly, in one of the cell samples tested, the presence of 5 mM glucose in equal concentration with



TEXT-FIGURE 6—The concentration dependence (**A**) and time course (**B**) of the volume decrease induced by cytochalasin B. In **A**, cytochalasin B concentrations at 4 minutes (*open squares*), 10 minutes (*solid circles*), 16 minutes (*open triangles*), and 18 minutes (*open circles*) are shown.

deoxyglucose caused an enhancement of the contraction induced by cytochalasin B compared to the control.

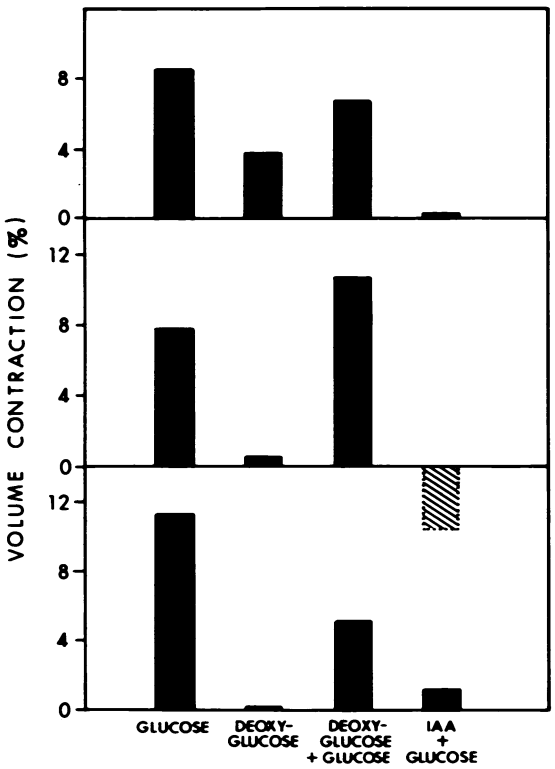
Bacterial-Factor-Induced Cell Volume Contraction in the Presence of Cytochalasin B

Because of the known inhibitory effect of cytochalasin B on chemotaxis it was of interest to see if cytochalasin B affected the activity of the bacterial chemotactic factor in causing volume expansion. Since cytochalasin B at concentrations above 4 μg/ml caused a decrease in volume by itself, the cells were first equilibrated with 4 μg/ml of cytochalasin B for 15 minutes. Within 2 minutes after the addition of chemotactic factor to these cells a rapid contraction of their volume occurred which was completely abolished (Text-figure 8) or greatly inhibited by 5 mM EDTA. This is completely unlike the lack of effect of EDTA on the expansion induced when the chemotactic factor reacts with leukocytes in the absence of cytochalasin B. As shown in the same experiment (Text-figure 8), EDTA in the presence of 0.25% dimethylsulfoxide decreases slightly the initial rate of the volume response to bacterial factor (see Text-figure 4); in this experiment, but not in others, it caused a significant increase in the maximal volume expansion achieved. Table 2 shows that

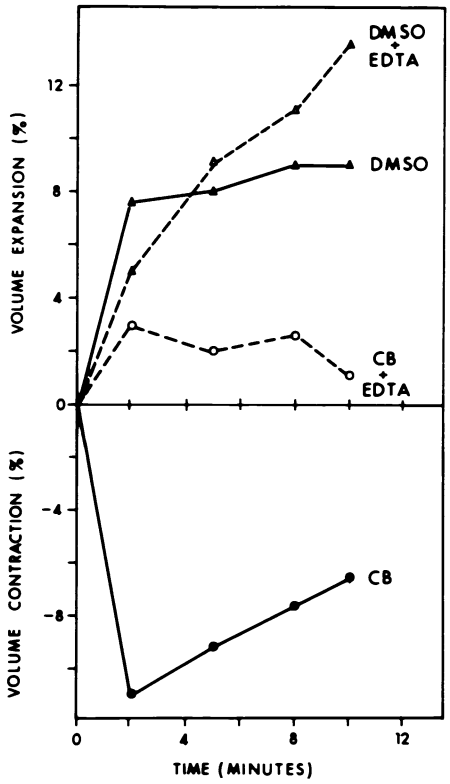
EDTA in the presence of Mg^{2+} causes a significant inhibition of the bacterial factor-induced contraction of the cell volume, suggesting that Ca^{2+} but not Mg^{2+} plays an important role in this process. EDTA itself has no inhibitory effect, since in the presence of excess Ca^{2+} the extent of volume decrease did not significantly differ from the control.

Discussion

In these studies we have demonstrated the ability of chemotactic factors, such as bacterial chemotactic factor and C5a, to induce a significant expansion of the volume of rabbit peritoneal neutrophils. The volume expansion seems closely related to the chemotactic response in the following respects: a) The volume expansion is an active process requiring intact glycolysis. b) Cytochalasin B, an agent inhibiting chemotaxis as well as spontaneous motility, completely abolishes the volume expansion at concentrations comparable to those required to inhibit chemotaxis. and c) A statistically significant correlation between the neutrophil's chemotactic activity and the volume expansibility is found (Table 1) when the



TEXT-FIGURE 7—Effect of 5 mM deoxyglucose and 0.1 mM iodoacetate on volume contraction induced by 8 μg/ml cytochalasin B; the results for cells from 3 different rabbits are shown. Experimental conditions were the same as those described in Text-figure 5. (Volume expansion is indicated by the hatched column)



TEXT-FIGURE 8—Time course of volume changes induced by bacterial factor (1 : 1000 dilution) in the presence and absence of 4 μg/ml cytochalasin B (CB) and the effect of EDTA on the volume responses. [Bacterial factor + CB, *solid circles*; bacterial factor + CB + 5 mM EDTA, *open circles*; bacterial factor + 0.25% dimethyl sulfoxide (DMSO), *solid triangles*; bacterial factor + DMSO + EDTA, *open triangles*].

chemotactic activity is measured at sufficiently high concentrations of chemotactic factor.

The 1 : 250 dilution of chemotactic factor was the concentration of chemotactic factor in this study which most frequently gave a maximum chemotactic response; the correlation coefficient r which relates the maximal chemotactic response to the chemotactic response elicited by the 1 : 250 concentration was 0.93. The chemotactic reactivities measured at the 1 : 125 and the 1 : 625 dilutions of chemotactic factor which correlated in a statistically significant manner with the volume expansion were therefore in the inhibitory region of the dose-response curve. The significance of this is not readily apparent. Nevertheless, the high degree of correlation obtained suggests that the volume expansion represents a primary response of the cells to the chemotactic factor, possibly related to the intrinsic mechanisms of locomotion of these cells.

A striking apparent discrepancy between chemotaxis and the volume expansion induced by chemotactic factors is the requirement of Ca^{2+} and Mg^{2+} in the external medium for optimal chemotactic activity [10,11] and the

Table 2—Requirement for Ca^{2+} in the Contraction of Neutrophils Induced by Bacterial Factor in the Presence of Cytochalasin B

Additions to Hanks' buffer*	Cell volume† (mean ± SE)
1.7 mM Ca^{2+}; 0.7 mM Mg^{2+}	7.5 ± 0.6
2.0 mM EDTA; 4 mM Mg^{2+}	2.1 ± 0.2
2.0 mM Mg^{2+}	3.2 ± 0.3
2.0 mM EDTA; 4 mM Ca^{2+}	7.0 ± 0.2

* In all instances, cells were suspended in Hanks' buffer without Ca^{2+} or Mg^{2+} but with the additions listed.

† Mean cell volume = percent change from control.

general lack of any need for divalent cations in the medium to obtain maximal expansion of cell volume (Text-figures 4 and 8). One interpretation is that the requirement for external divalent cations comes relatively late in the chemotactic sequence, and that the volume expansion does not involve these putatively later divalent-cation-dependent steps. The significance of the decrease in the rate at which the maximum expansion is obtained when EDTA is present is unclear.

The volume expansion of the neutrophils must be due to an increased influx of electrolytes and/or water. The requirement of metabolic energy for the volume expansion suggests that an alteration in membrane permeability due to a simple and direct interaction of the chemotactic agent with the cell membrane cannot be wholly responsible for the volume change. Energy-requiring processes, such as ion pump activities, and/or a mechanicochemical event, such as contractile or relaxation processes, are other possibilities to be considered. The nature of the ion pump in rabbit peritoneal neutrophils unfortunately remains unclear. Phagocytic stimuli inhibit the ouabain-sensitive influx of K^+ into human neutrophils.[12] By itself, this inhibition would be expected to decrease the volume of the neutrophils. However, concentrations of ouabain as high as 10 mM in the absence of K^+ failed to stimulate or inhibit the volume expansion induced by either high or low concentrations of bacterial factor. Furthermore, ouabain even at this high concentration failed to influence the cell volume significantly.[13]

There is no evidence for or against a mechanicochemical mechanism playing a role in the volume expansion the neutrophil-induced chemotactic factors. As described in the Introduction, volume changes in striated and smooth muscle cells are caused by actomyosin interactions. In nonmuscle cells such as erythrocyte ghosts,[14] isolated kidney tubule, or Ehrlich ascites tumor cells,[15] the possibility has been considered that contractile proteins are involved in regulating cell water content. Whether

such contractile proteins are involved in the volume expansion of neutrophils studied here remains to be investigated.

Cytochalasin B itself at concentrations of 4 μg/ml or greater activates an energy-dependent contraction of neutrophil volume (Text-figure 6), an effect similar to that of cytochalasin D on HeLa cells.[16] The contraction is apparently not due to the lysosomal enzyme secretion from rabbit peritoneal neutrophils induced by cytochalasin B.[9]

In the presence of greater than 0.16 μg/ml of cytochalasin B, the bacterial chemotactic factor induces a rapid contraction followed by a gradual reexpansion of cell volume (Text-figure 8). At 0.2 μg/ml, cytochalasin B neither inhibits nor enhances the volume expansion caused by concentrations of bacterial factor inducing maximal chemotactic response. In two experiments where a dilute concentration of bacterial factor was employed, a significant enhancement of volume expansion was found in the presence of 0.2 μg/ml of cytochalasin B,[18] but in other experiments, this concentration was without effect. These low concentrations of cytochalasin B induce an enhancement of chemotactic activity which also is quite variable from cell to cell.[17] Thus, the concentrations of cytochalasin B required to convert bacterial-factor-induced volume expansion to contraction parallel those causing a significant inhibition of chemotactic activity.[17]

The mechanism of the volume contraction induced by chemotactic factor in the presence of cytochalasin B is unknown, although it appears to be neither the cause nor the result of the lysosomal enzyme secretion induced under these circumstances.[9] The following findings suggest that at least part of the process, or processes, involved in the contraction induced by bacterial chemotactic factor in the presence of cytochalasin B appears to be different from those involved in the contraction of cytochalasin B alone: a) Bacterial-factor-induced contraction in the presence of cytochalasin B specifically requires extracellular Ca^{2+}. b) The contraction caused by cytochalasin B alone is slow when compared to that caused by the bacterial factor. c) The contraction caused by bacterial factor is followed by a gradual reexpansion of cell volume, whereas the contraction caused by cytochalasin B produced a reduced cell volume which is stable for a prolonged period of time.

In the earlier part of the Discussion we presented evidence which suggests that expansion of the volume is part of the motile process of the neutrophil induced by chemotactic factors. This conclusion necessarily implies that a contractile process exists to compensate for the volume increment, otherwise, one would expect a constant gain in cell volume during cell movement. Bacterial chemotactic factor can cause a contraction of

cell volume, but so far this has been demonstrable only at concentrations of cytochalasin B which reduce or prevent cell movement. The expansion induced by chemotactic factors was demonstrated with cells in suspension; neutrophils move only on surfaces. This suggests that in the absence of cytochalasin B, the proper surface and, particularly, the adherence of the cell to that surface might provide the conditions whereby the chemotactic factor could cause a contraction along the portion of the cell adhering to the surface. On this hypothesis, the part of the cell in contact not with the surface but with the bulk medium (the lamellapodia, perhaps) would then be capable of expanding in response to the chemotactic factor. Some support can be adduced for such speculation. In the absence of cytochalasin B, lysosomal enzyme release from neutrophils is caused by chemotactic factors only when the neutrophils adhere to the proper surface;[7] neutrophils in suspension do not secrete lysosomal enzymes in response to chemotactic factors or do so very poorly except in the presence of cytochalasin B.[7,18] Thus, in this instance, cytochalasin B can substitute for a surface and vice-versa.

These studies deal only with one gross aspect of cellular events: the volume changes elicited by agents which elicit and inhibit cell movement. Despite a lack of insight into the molecular nature of the processes involved, the fact that the volume responses of the neutrophil can be isolated and studied represents a great reduction in the complexity characterizing the movement of the whole cell.

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