Enhancement of Random Migration and Chemotactic Response of Human Leukocytes by Ascorbic Acid

EDWARD J. GOETZL, STEPHEN I. WASSERMAN, IRMA GIGLI, and K. FRANK AUSTEN

From the Departments of Medicine and Dermatology, Harvard Medical School, and the Department of Medicine, Robert B. Brigham Hospital, Boston, Massachusetts 02120

ABSTRACT Incubation of human leukocytes with ascorbic acid at neutral pH and at concentrations 10–50 times that of normal blood levels augmented both the in vitro random migration and chemotaxis of the cells by 100–300% without influencing their phagocytic capacity. Enhancement of mobility by ascorbate was evident for isolated neutrophils, eosinophils, and mononuclear leukocytes and was independent of the specific chemotactic stimulus. Stimulation by ascorbate of the hexose monophosphate shunt of adherent neutrophils and augmentation by ascorbate of neutrophil mobility had comparable dose-response relationships, could be reversed by washing the cells, and were both suppressed by preincubation of the neutrophils with 6-aminonicotinamide, but not with the neutrophil-immobilizing factor. Glutathione, the proposed intermediate for ascorbate action, similarly stimulated hexose monophosphate shunt activity and enhanced migration. The enhancement in vitro of leukocyte mobility by ascorbate at concentrations found in some normal tissues, therefore, appears to be dependent upon stimulation of the leukocyte hexose monophosphate shunt.

INTRODUCTION

Adherent human neutrophils interacting with purified chemotactic factors demonstrated a two- to six-fold increase in the activity of their hexose monophosphate shunt (HMPS)[1] (1). Maneuvers which suppressed the chemotactic response of mobile leukocytes, such as treatment with diisopropyl fluorophosphate or with the neutrophil-immobilizing factor (NIF), did not prevent stimulation of the HMPS of adherent neutrophils. Although stimulation of the HMPS alone was not sufficient for chemotaxis, preincubation with 6-aminonicotinamide, which blocked the enhanced HMPS activity associated with the introduction of a chemotactic stimulus, partially suppressed the chemotactic response to diverse stimuli (2). To assess further the relationship of HMPS stimulation to a chemotactic response, ascorbic acid, which is known to increase leukocyte HMPS activity (1, 3), was examined for its effect on the random migration and chemotaxis of human leukocytes.

METHODS

Polystyrene disposable chemotactic chambers (Adaps, Inc., Dedham, Mass.) and acrylic radiochemotactic chambers (Neuro Probe, Inc., Bethesda, Md.) were assembled with 3-μm and 8-μm pore size micropore filters (Millipore Corp., Bedford, Mass.) as previously described (4, 5). Hanks's solution and Medium 199 with or without phenol red (Microbiological Associates, Inc., Bethesda, Md.), ovalbumin five-times recrystallized (Miles Laboratories, Inc., Miles Research Div., Kankakee, Ill.), dextran, Sephadex, and Ficoll (Pharmacia Fine Chemicals Inc., Piscataway, N. J.), sodium diatrizoate (Hypaque, Winthrop Laboratories, New York), two times recrystallized trypsin and soybean trypsin inhibitor (Worthington Biochemical Corp., Freehold, N. J.), sodium [^{51}Cr]chromate, [1-^{14}C]-glucose, and [6-^{14}C]glucose (Amersham-Searle Corp., Arlington Heights, Ill.), sodium lauryl sulfate (SLS), L-ascorbic acid (Fisher Scientific Co., Medford, Mass.), lactic acid dehydrogenase (LDH), L-lactic acid as 0.40 mg per ml solution, NAD (Sigma Chemical Co., St. Louis, Mo.), sodium metrizoate (Nyegaard and Co., Oslo, Norway), 6-aminonicotinamide (Mann Research Laboratories Inc., New York), iodoacetate (Eastman Kodak Co., Rochester, N. Y.), and plastic 35 × 10 mm Petri dishes (Falcon Plastics, Div.

Dr. Goetzl is an Investigator of the Howard Hughes Medical Institute. Dr. Wasserman is a postdoctoral trainee supported by training grant AI-00366 from the National Institutes of Health. Dr. Gigli is the recipient of a Research Career Development Award (AM-46409) from the National Institutes of Health.

Received for publication 16 July 1973 and in revised form 5 October 1973.

[1] *Abbreviations used in this paper:* AU, absorbancy units; ECF-A, eosinophil chemotactic factor of anaphylaxis; HMPS, hexose monophosphate shunt; hpf, high power fields; KRPG-ovalbumin, Krebs-Ringer phosphate glucose solution made 0.1 g per 100 ml in ovalbumin; LDH, lactic acid dehydrogenase; NIF, neutrophil-immobilizing factor; SLS, sodium lauryl sulfate.

of B-D Laboratories, Inc., Los Angeles, Calif.) were obtained from the manufacturers. Rice starch (Whittaker, Clark and Daniels, Inc., New York) was washed five times in distilled water, incubated with autologous fresh human serum for 1 h at 37°C, and washed again three times in saline before use. Ascorbic acid was made up as a 0.1 N stock solution and titrated to pH 7.4 in an ice bath by dropwise addition of 0.4 N NaOH.

NIF, derived from 1×10^7 human leukocytes which had either been incubated at 37°C in 1 ml of buffer alone (control for NIF) or engaged in phagocytosis of starch particles (PhNIF), was partially purified by heating and by gel filtration on Sephadex G-25 (4).

Gamma radiation from ^{51}Cr-containing micropore filters used in radiochemotactic chambers was measured with a dual-channel gamma well counter, and beta radiation from [^{14}C]glucose solutions was quantitated with Bray's fluid in a liquid scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.).

Collection and purification of human leukocytes. Mixed peripheral leukocytes from normal human subjects were collected and separated from erythrocytes by dextran sedimentation, and the contaminating erythrocytes were lysed with 0.84% NH_4Cl as previously described (1). Neutrophils and mononuclear leukocytes were further purified by centrifugation of mixed leukocytes on Ficoll-Hypaque cushions (6). Eosinophils from patients with hypereosinophilic syndromes were purified from mixed leukocytes in dextran-treated plasma by centrifugation on metrizoate cushions (7). All leukocytes were washed twice with Hanks's solution before their function was assessed.

Measurement of chemotaxis and random migration. Chemotaxis of human leukocytes was assayed by a previously detailed modification (4) of the Boyden micropore filter assay, employing 3-μm micropore filters for neutrophils, eosinophils, and mixed leukocytes, and 8-μm micropore filters for mononuclear leukocytes (8), and also by a radiochemotactic method which utilizes modified chambers that hold a layer of two micropore filters between ^{51}Cr-labeled leukocytes and the chemotactic stimulus (5). The medium for all cells was Hanks's balanced salt solution-0.5 g per 100 ml in ovalbumin (pH 7.4). The chemotactic factors were eosinophil chemotactic factor of anaphylaxis (ECF-A) purified from anaphylactic diffusates of human lung by Sephadex G-25 chromatography (9), human C5a generated by tryptic digestion of highly purified C5 (10), and human kallikrein generated from purified prekallikrein by activation with Hageman factor fragments (11). For the modified Boyden technique, initial cell suspensions contained $1.5–2.0 \times 10^6$ cells and the incubation time was 2½ h. The leukocyte counts were expressed as the mean of 10 high power fields (hpf), 5 from each of duplicate chambers, corrected for background counts in filters from chambers without a chemotactic stimulus. For the radiochemotactic procedure, the initial cell suspensions contained $5–6 \times 10^6$ cells and the incubation time was 4 h. The chemotactic response in duplicate chambers was calculated as net percent radioactivity = ([RS − RC]/[RT − RC]) × 100, where RS represents the counts per 4 min in the bottom filter of stimulated chambers, RC the counts per 4 min in the bottom filter of control chambers, and RT the counts per 4 min in the 0.5 ml of initial leukocyte suspension added to each chamber.

Random migration of leukocytes was assessed by eliminating the chemotactic stimulus and utilizing either the modified Boyden technique with a 4-h incubation or the [^{51}Cr]leukocyte assay with a 6-h incubation. In the Boyden technique, leukocytes were enumerated in 10 hpf from duplicate chambers, as per the assessment of chemotactic migration. In the [^{51}Cr]leukocyte assay, the response was expressed as the mean counts per 4 min in the bottom filters of duplicate chambers. For either assay, alterations of random migration by agents which act on the leukocytes were calculated from the random migration of pretreated leukocytes after correction for random migration of untreated cells. The effect of various agents on directed and random migration was assessed after preincubation of the cells with varying dilutions of the agents for 15 min at 37°C.

Erythrophagocytosis. Phagocytosis by human leukocytes of antibody-sensitized sheep erythrocytes (EA) or EA coated with the first four complement components (EAC-1423) was determined as described (12). The percent phagocytosis of the erythrocytes was calculated by subtracting the optical density (OD) at 414 nm of a 0.84% NH_4Cl lysate of uningested erythrocytes in each phagocytosis mixture from the OD_{414} of a lysate of the total initial erythrocytes and dividing this difference by the latter OD_{414}. The effect of ascorbate on this process was studied by preincubation for 20 min at 37°C of leukocytes or purified neutrophils with varying dilutions of ascorbate before the addition of the target erythrocytes; the net percent of erythrocyte ingestion attributed to ascorbate was calculated by subtracting the mean percent in its absence.

Aerobic glucose metabolic rates of adherent leukocytes. Mixed leukocytes or purified neutrophils were allowed to adhere to plastic Petri dishes by incubation in Hanks's solution without added protein as described (1). The adherent leukocytes were washed and covered with 1 ml of Krebs-Ringer phosphate glucose solution (2) made 0.1 g per 100 ml in ovalbumin (KRPG-ovalbumin). The activity of the HMPS of adherent leukocyte layers was determined by measuring the extent of conversion of [1-^{14}C]glucose to $^{14}CO_2$ after 80 min at 37°C (1, 2). The glycolytic activity of adherent leukocyte layers was assessed by measuring the lactate concentration in KRPG-ovalbumin after 3 h at 37°C with the LDH enzymatic assay (2). Both the cpm of $^{14}CO_2$ and the μg lactate per ml were standardized by dividing each value by the OD at 280 nm of a 3% SLS solution of the adherent leukocytes in the same dish. The resulting activities were expressed as cpm per 0.2 absorbancy units$_{280}$ (AU) for the HMPS and μg lactate/ml per 0.2 AU_{280} for the glycolytic pathway. The effect of various agents on these pathways, expressed as net cpm per 0.2 AU_{280} for the HMPS and net μg lactate/ml per 0.2 AU_{280} for glycolysis, was calculated by subtraction of the mean value for duplicate unchallenged dishes from the corresponding mean value for treated dishes. The effect of such agents under investigation were studied by preincubation of these agents with the leukocyte layers for 20 min at 37°C before the addition of chemotactic factors, cold glucose, and [1-^{14}C]glucose.

RESULTS

Enhancement of leukocyte mobility by ascorbate. Preincubation of human leukocytes with ascorbate resulted in an augmentation of their random migration (Fig. 1) and directed leukotaxis in response to the chemotactic stimuli, C5a (Fig. 1) and kallikrein. Ascorbate was less effective when present only on the stimulus side of the micropore filter opposite from the leukocytes, while its presence in both compartments of the chamber to elimi-

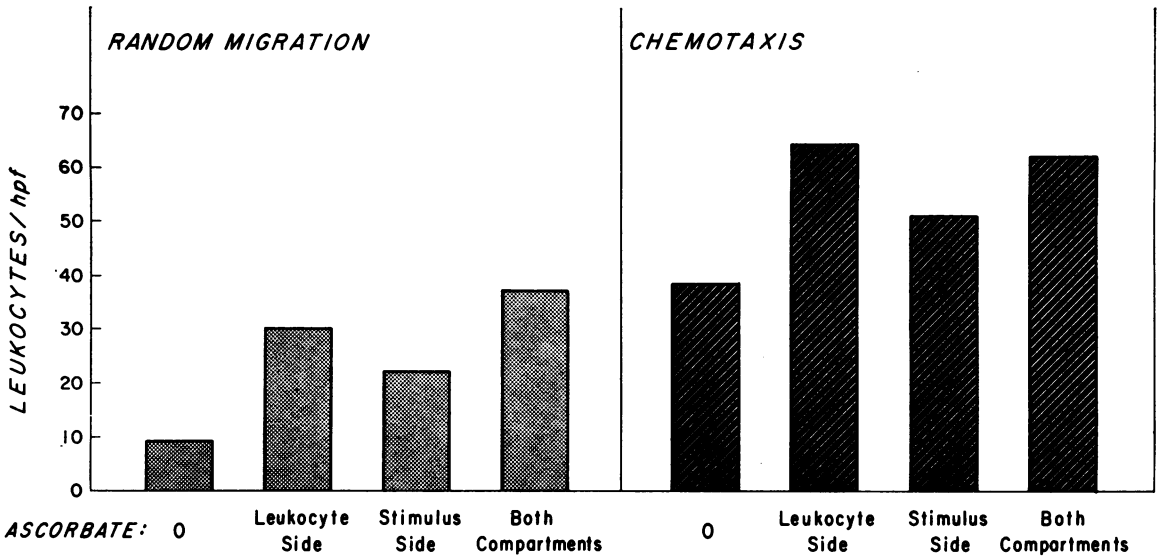

FIGURE 1 Enhancement by ascorbate of random migration and chemotactic response by mixed human leukocytes. The modified Boyden assay was used to assess random migration and the chemotactic response to C5a of mixed human leukocytes. C5a, produced by tryptic digestion of C5, was present at a final concentration equivalent to that derived from 10 μg of C5 per ml. The ascorbate concentration was 2.5×10^{-3} M. Cells were preincubated with ascorbate and introduced into the chambers without washing (leukocyte side), ascorbate was placed into the stimulus compartment alone (stimulus side), or these two maneuvers were combined (both sides).

nate a concentration gradient allowed for a maximum enhancement of random migration and chemotaxis. The random migration of purified human neutrophils, eosinophils, and mononuclear leukocytes, and the chemotactic response of these leukocytes to the selective stimuli, kallikrein, ECF-A, and C5a, respectively, were each enhanced by exposure of the leukocytes to ascorbate (Fig. 2). The capacity of ascorbate to enhance directed and random migration of neutrophils did not extend to an effect on erythrophagocytosis (Fig. 3).

Relationship of enhancement of leukocyte mobility by ascorbate to stimulation of HMPS activity. The activity of the HMPS of neutrophils was increased by the addition of ascorbate, with a dose-response relationship comparable to that found for the stimulation by ascorbate of random mobility (Fig. 3). The stimulation of both random migration and the activity of the HMPS of human leukocytes by two concentrations of ascorbate falling on the plateau of the dose-response curve was partially reversible with washing of the cells (Table I). The augmentation of the activity of the HMPS by ascorbate alone or in combination with C5a was greater than 90% reversible by washing the leukocytes. The enhanced random migration by ascorbate was also largely reversible upon washing of the leukocytes, while the enhancement of directed migration was only partially reversed by washing. The action of ascorbate was not unique, since glutathione which also stimulated HMPS activity enhanced random migration and chemotaxis of human leukocytes with similar dose-response patterns (Fig. 4). Methylene blue, the most potent agent in terms of HMPS stimulation, had no effect on migration, and therefore, HMPS stimulation was not alone sufficient to enhance migration.

Inhibition of the ascorbate effect on leukocyte mobility. Prior incubation of neutrophils with either 6-aminonicotinamide, which blocks HMPS activity by competing with $NADP^+$ (13), or low concentrations of the alkylating agent iodoacetate resulted in concomitant inhibition of enhanced HMPS activity and random migration (Table II). These doses did not affect base-line glycolytic activity, which was also not influenced by ascorbate. In contrast, NIF, which specifically inhibits both random and directed migration of these human neutrophils, had virtually no effect on the augmentation of random migration and HMPS activity by ascorbate.

DISCUSSION

Exposure of human peripheral leukocytes to ascorbic acid at neutral pH increased both the random migration of the leukocytes and their chemotactic responsiveness to diverse stimuli by 100–300%, without influencing their phagocytic capacity (Figs. 1, 3). The effect of ascorbate in enhancing mobility was not limited to a

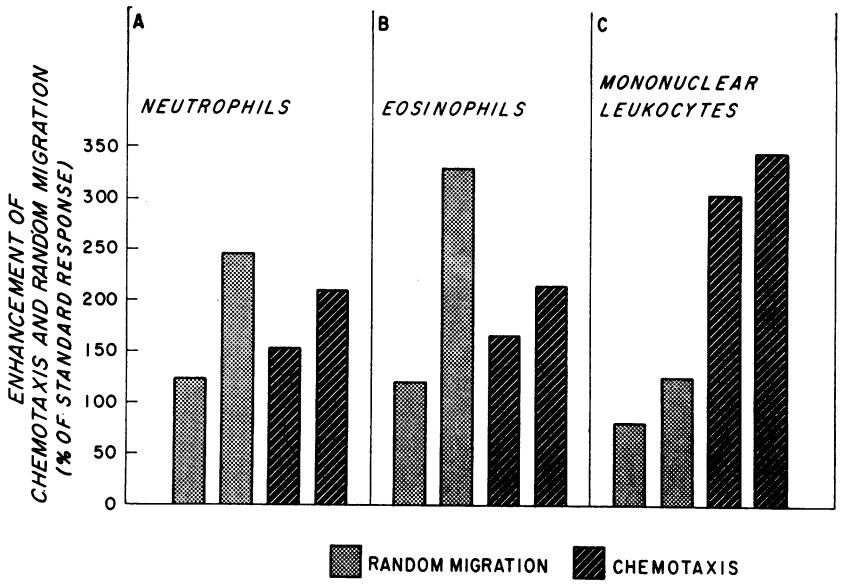

FIGURE 2 Enhancement by ascorbate of random migration and chemotaxis of purified human leukocytes. The net increase in the chemotactic response or the net increase in random migration with the modified Boyden method was expressed as a percent of these respective responses without ascorbate, termed the standard response. Leukocytes were preincubated for 15 min at 37°C with ascorbate at 2.5×10^{-3} M or 5.0×10^{-3} M; in each pair of bars the left-hand bar represents the effect of the lower concentration. Kallikrein was present at a concentration which generated 3 μg bradykinin per ml from 0.2 ml of heat-inactivated plasma, and C5a was employed at a final concentration equivalent to that derived from 10 μg C5 per ml. (A) Neutrophils. The random migration and chemotactic response to kallikrein of neutrophils in the absence of ascorbate were 10 and 39 neutrophils per hpf, respectively. (B) Eosinophils. The random migration and chemotactic response to ECF-A of eosinophils without ascorbate were 8 and 37 eosinophils per hpf, respectively. (C) Mononuclear leukocytes. The random migration and chemotactic response to C5a of mononuclear leukocytes without ascorbate were 12 and 33 leukocytes per hpf, respectively.

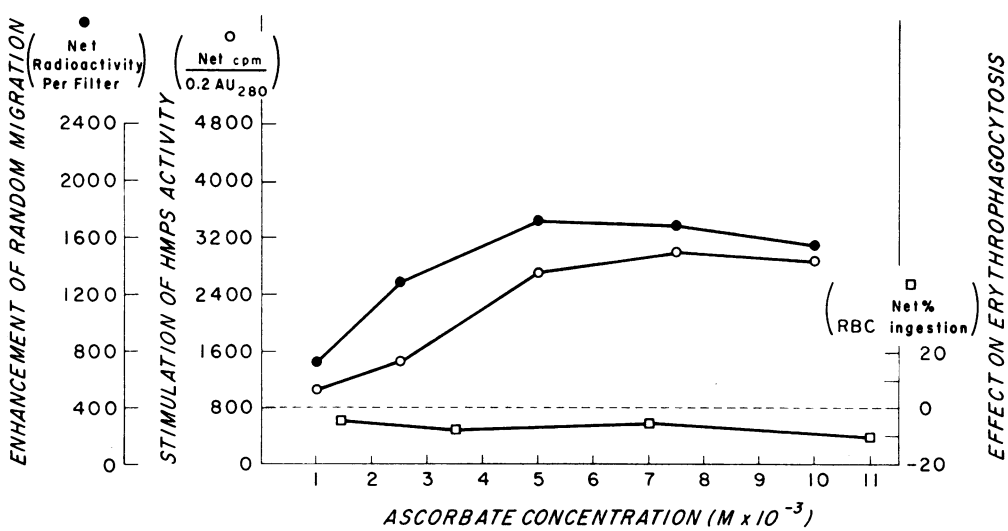

FIGURE 3 Dose-response of the effect of ascorbate on human neutrophil random migration, phagocytosis, and HMPS activity. The values for unstimulated purified neutrophils were: random migration, 1,609 counts per 4 min; phagocytosis, 35.1% EAC1423 ingestion per 5×10^6 neutrophils; and HMPS activity, 1,537 cpm per 0.2 AU_{280}. The data plotted represent increments or decrements above or below these base-line values.

TABLE I

*Reversibility of Ascorbate Effect**

Ascorbate concentration	Washing	Leukocyte migration‡	HMPS stimulation
M		*net % radioactivity*	*net CPM per 0.2 AU_{280}*
Ascorbate alone		Random	
5×10^{-3}	0	7.5	1,373
5×10^{-3}	+	1.8	78
1×10^{-2}	0	11.7	1,156
1×10^{-2}	+	2.5	25
Ascorbate and C5a		Directed	
0	0	2.1	496
0	+	—	168
5×10^{-3}	0	8.7	1,903
5×10^{-3}	+	4.1	204
1×10^{-2}	0	13.4	2,112
1×10^{-2}	+	7.0	187

* Mixed leukocyte layers or suspensions were preincubated with ascorbate at 2 different final concentrations of ascorbate for 15 min at 37°C, and then either studied directly or washed twice in Krebs-Ringer solution prior to study.

‡ Leukocyte migration refers to enhanced migration with ascorbate alone or chemotaxis with the C5a stimulus, both measured by the [^{51}Cr]leukocyte method.

§ C5a was produced by digestion of C5 with trypsin followed by dilution to a final concentration equivalent to that derived from 3 μg C5 per ml.

particular leukocyte type or chemotactic factor (Fig. 2), since stimulation was observed for isolated neutrophils, eosinophils, and mononuclear leukocytes in terms of their random mobility and their response to a chemotactic factor specifically appropriate to each, namely kallikrein,

TABLE II

Influence of Metabolic and Chemotactic Inhibitors on Ascorbate Effect‡*

Inhibitor	HMPS stimulation§	Glycolytic stimulation§	Enhanced random migration
	net CPM per 0.2 AU_{280}	*net μg lactate/ml per 0.2 AU_{280}*	*net % radioactivity*
0	992	1	12.0
6-aminonicotinamide (10^{-5} M)	46	0	−2.1
Iodoacetate (10^{-7} M)	−5	−2	1.5
Ph NIF 1/16‖	894	ND	10.9
Ph NIF 1/256‖	913	ND	10.4

* The final concentration of ascorbate was 2.5×10^{-3} M.

‡ Neutrophil suspensions or layers were preincubated with each inhibitor for 15 min at 37°C before being tested.

§ Baseline values without ascorbate were: HMPS activity, 1269 CPM per 0.2 AU_{280}; glycolysis, 26 μg lactate/ml per 0.2 AU_{280}.

‖ These concentrations of Ph NIF inhibited neutrophil random migration by 68% and 43%, and chemotaxis in response to kallikrein by 79% and 52%, respectively.

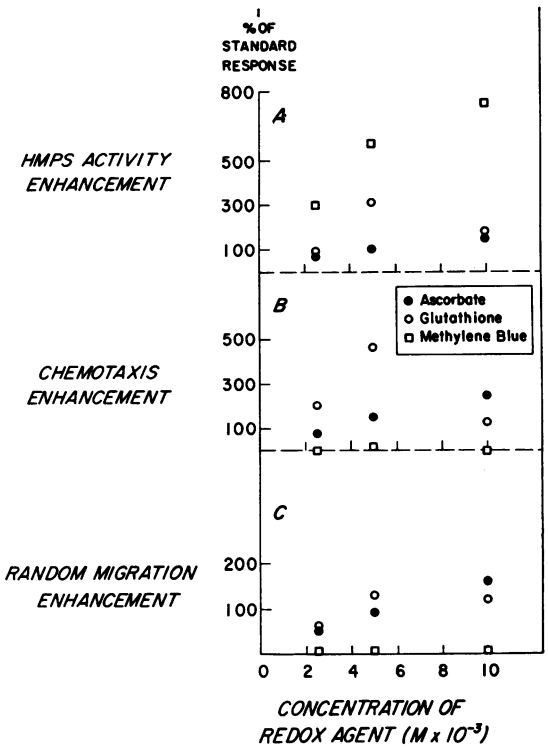

FIGURE 4 Dose-response of the effect of redox agents on mixed human-leukocyte random migration, chemotaxis, and HMPS activity. The values for unstimulated leukocytes were: random migration, 1,259 counts per 4 min; chemotaxis, 2,848 counts per 4 min; and HMPS activity, 504 cpm per 0.2 AU_{280}. The chemotactic stimulus was C5a, prepared as in Fig. 1, at a final concentration equivalent to that derived from 5 μg C5 per ml. The net increase in HMPS activity, random migration, and chemotaxis at each concentration of ascorbate was expressed as a percent of the response in the absence of ascorbate. (A) HMPS activity. (B) Chemotaxis. (C) Random migration.

ECF-A, and C5a, respectively. Although ascorbate exhibited this general effect on cell mobility, it did not enhance phagocytosis of sensitized erythrocytes previously interacted with the first four complement components (Fig. 3).

As ascorbate had been observed to increase the activity of the HMPS of human leukocytes (1, 3), the relationship between this stimulation and the enhanced mobility produced by ascorbate was examined in terms of dose-response relationship, reversibility, and the influence of metabolic inhibitors. The presumed mechanism of the ascorbate effect was the conversion of ascorbate to dehydroascorbate, which occurs rapidly and spontaneously at neutral pH conditions, followed by, presumably via the glutathione shuttle, the oxidation of NADPH by dehydroascorbate to $NADP^+$, the coenzyme in the rate-limiting initial step of the HMPS (3). The stimulation of HMPS activity and migration by glutathione (Fig. 4) supports its role as an intermediate

between dehydroascorbate and NADPH. Ascorbate is not itself a chemotactic factor, and its action in augmenting random and directed mobility is dependent upon its prior interaction with the leukocytes (Fig. 1). There is a dose-response relationship between the effect of ascorbate on HMPS activity and neutrophil mobility (Fig. 3), and both are reversed by washing (Table I). In addition, treatment of leukocytes with 6-aminonicotinamide or iodoacetate so as to inhibit stimulation of the HMPS by ascorbate resulted in complete blockage of enhancement by ascorbate of random migration (Table II) and chemotaxis. This inhibition of mobility by 6-aminonicotinamide was more profound than that previously demonstrated for suppression of chemotaxis in the absence of ascorbate (2). Thus, although the failure of methylene blue to enhance migration demonstrates that HMPS stimulation is not alone sufficient, it appears that ascorbate enhancement of random migration and chemotaxis is dependent upon stimulation of HMPS.

Previous work (1, 4) showed that irreversible inhibition of human neutrophil directed and random mobility by NIF was not lethal to the cells and was functionally specific, since neither neutrophil phagocytosis nor adherence to surfaces was altered by NIF. The concomitant finding that NIF did not suppress stimulation of the HMPS during phagocytosis, nor after introduction of a chemotactic factor, (1) has now been extended to show that NIF does not prevent ascorbate enhancement of HMPS activity (Table II). In addition, the failure of NIF to suppress ascorbate augmentation of mobility contrasts with previous studies (1, 4) in which this factor rapidly suppressed polymorphonuclear leukocyte mobility.

It was demonstrated more than two decades ago that deficiency of ascorbate resulted in impaired phagocytosis by leukocytes, a defect which could be reversed by the addition of exogenous ascorbate (14). However, while concentrations of ascorbate 10–50 times the usual plasma level (15) did not enhance the phagocytic capacity of human leukocytes (Fig. 3), these concentrations were effective in enhancing leukocyte mobility in vitro. Human leukocytes, as well as the choroid plexus, cerebral cortex, kidney, and eye, concentrate ascorbate to levels comparable to those which were associated with the augmentation of random and directed leukocyte mobility (16–20). It may well be that the high ascorbate levels in some tissues contribute to their ability to mount an inflammatory reaction in response to infection or other noxious stimuli.

ACKNOWLEDGMENTS

The expert technical assistance of Ms. Janet Woods is gratefully acknowledged.

This work was supported by grants AI-07722, AI-10356, and RR-05669 from the National Institutes of Health.

REFERENCES

1. Goetzl, E. J., I. Gigli, S. I. Wasserman, and K. F. Austen. 1973. A neutrophil immobilizing factor derived from human leukocytes. II. Specificity of action on polymorphonuclear leukocyte mobility. *J. Immunol.* **111**: 938.
2. Goetzl, E. J., and K. F. Austen. 1974. Stimulation of human neutrophil leukocyte aerobic glucose metabolism by purified chemotactic factors. *J. Clin. Invest.* **53**: 591.
3. DeChatelet, L. R., M. R. Cooper, and C. E. McCall. 1972. Stimulation of the hexose monophosphate shunt in human neutrophils by ascorbic acid: mechanism of action. *Antimicrob. Agents Chemother.* **1**: 12.
4. Goetzl, E. J., and K. F. Austen. 1972. A neutrophil-immobilizing factor derived from human leukocytes. I. Generation and partial characterization. *J. Exp. Med.* **136**: 1564.
5. Goetzl, E. J., and K. F. Austen. 1972. A method for assessing the *in vitro* chemotactic response of neutrophils utilizing ^{51}Cr-labeled human leukocytes. *Immunol. Commun.* **1**: 421.
6. Böyum, A. 1968. Isolation of leukocytes from human blood, further observations. *Scand. J. Clin. Lab. Invest.* **21** (Suppl. 97) : 31.
7. Day, R. P. 1970. Eosinophil cell separation from human peripheral blood. *Immunology.* **18**: 955.
8. Boyden, S. 1962. The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leukocytes. *J. Exp. Med.* **115**: 453.
9. Kay, A. B., and K. F. Austen. 1971. The IgE-mediated release of an eosinophil leukocyte chemotactic factor from human lung. *J. Immunol.* **107**: 899.
10. Ward, P. A., and L. J. Newman. 1969. A neutrophil chemotactic factor from human C5. *J. Immunol.* **102**: 93.
11. Kaplan, A. P., A. B. Kay, and K. F. Austen. 1972. A prealbumin activator of prekallikrein. III. Appearance of chemotactic activity for human neutrophils by the conversion of human prekallikrein. *J. Exp. Med.* **135**: 81.
12. Gigli, I., and R. A. Nelson. 1968. Complement dependent immune phagocytosis. I. Requirements for C1, C4, C2, C3. *Exp. Cell Res.* **51**: 45.
13. Ammon, H. P. T., and J. Steinke. 1972. Effect of 6-aminonicotinamide on insulin release and C-14 glucose oxidation by isolated pancreatic rat islets: difference between glucose, tolbutamide and aminophylline. *Endocrinology.* **91**: 33.
14. Numgester, W. J., and A. M. Ames. 1948. The relationship between ascorbic acid and phagocytic activity. *J. Infect. Dis.* **83**: 50.
15. Vilter, R. W. 1967. Pharmacology. *In* The Vitamins. W. H. Harris and R. S. Harris, editors. Academic Press Inc., New York. **1**: 487.
16. Cuttle, T. R. 1938. Observations on the relation of leukocytosis to ascorbic acid requirements. *Q. J. Med.* **7**: 575.
17. Loh, H. S., and C. W. M. Wilson. 1970. The origin of ascorbic acid stored in the leucocytes. *Br. J. Pharmacol.* **40**: 169.
18. Spector, R., and A. V. Lorenzo. 1973. Ascorbic acid homeostasis in the central nervous system. *Am. J. Physiol.* **225**: 757.
19. Becker, B. 1967. Ascorbate transport in guinea pig eyes. *Invest. Ophthalmol.* **6**: 410.
20. Martin, G. R. 1961. Studies of the tissue distribution of ascorbic acid. *Ann. N. Y. Acad. Sci.* **92**: 141,