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EFFECTS OF AMMONIA ON HUMAN NEUTROPHIL N-FORMYL CHEMOTACTIC PEPTIDE RECEPTOR-LIGAND INTERACTION AND CYTOSKELETAL ASSOCIATION

Maddalena Coppi and Richard Niederman

Department of Cell Biology
Forsyth Dental Research Center
140 The Fenway
Boston, MA 02115

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Ammonia is a bacterial metabolite which is commonly used to alter cytoplasmic and lysosomal pH of eukaryotic cells. Here we examine its effect on external N-formyl peptide receptors of human neutrophils. Ammonia does not affect the number of N-formyl peptide receptors on the cell surface, nor the association of the ligand-receptor complex with the cytoskeleton. However, ammonia causes a marked decrease in the affinity of the chemotactic peptide receptor for its ligand. The Kd of untreated cell for the chemotactic peptide was 0.65 ± 0.06 nM, whereas that of ammonia treated cells was 1.02 ± 0.10 nM (Mean ± SEM, N = 6). These results suggest that ammonia can affect external as well as internal cellular components. Since ammonia is used to alter lysosomal and cytoplasmic pH, and is a metabolite of common bacterial pathogens, these results bear directly on its use in cell biology and on its potential as a virulence factor.

Neutrophils are crucial to defense against bacterial infections.Activation is initiated by the binding of chemotactic substances to specific receptors on the cell surface (1). One important class of chemotactic substances are the N-formyl peptides which are derived from bacterial proteins (2,3). The binding of an N-formyl peptide to its receptor results in the generation of a variety of intracellular signals. These signals, many of which are generated through the interaction of the chemotactic peptide receptor with a pertussis toxin sensitive G protein, trigger a variety of cellular responses including adhesion, chemotaxis, degranulation, and superoxide anion production (4,5,6).

The chemotactic peptide receptor-ligand interaction is critical for appropriate control of neutrophil function. Therefore, the ability of bacteria to interfere with this

ABBREVIATIONS:

Hanks balanced salt solution (HBSS); Fluoresceinated chemotactic peptide, N-formyl-Nle-Leu-Phe-Nle-Tyr-Lys-fluorescein (FLPEP); Dimethylsulfoxide (DMSO); N-[2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid] (HEPES); Bovine serum albumin (BSA).

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complex formation and/or its internalization can be crucial to the establishment of an infection. For example, the bacterial metabolite ammonia has been found to inhibit neutrophil function (including: chemotaxis, phagocytosis, degranulation, and oxidative dependent and independent killing mechanisms)(7,8,9). These effects have been purported to be due to the effect of ammonia on lysosomal pH. In fact, we have found that ammonia reduces the level of cytoskeletal actin, as well as raise the cytoplasmic pH (10). Here we test the hypothesis that ammonia can have an effect on the extracellular receptor-ligand interaction. In particular, we examined the effect of ammonia on the number, affinity, and cytoskeletal association of the N-formyl chemotactic peptide receptor for N-formyl-Nle-Leu-Phe-Nle-Tyr-Lys (11,12,13).

MATERIALS AND METHODS

Preparation of human neutrophils. Neutrophils were prepared essentially by the method of Boyum (14), and red blood cells removed by two hypotonic lyses. The final cell concentration was adjusted to 2 x 106 in a 1x Hanks balanced salt solution (HBSS; Gibco Laboratories, Grand Island, N.J.) supplemented with 10 mM HEPES and 0.1% RIA grade BSA (Sigma Chemical Co., St Louis, MO), and cell viability determined by trypan blue exclusion (>95% viable). These and all subsequent solutions were adjusted to pH 7.4 and sterile filtered prior to cell isolation and treatment.

Chemotactic Peptide. FLPEP (N-formyl-Nle-Leu-Phe-Nle-Tyr-Lys- fluorescein) was obtained as a dry powder from Molecular Probes (Eugene, Oregon). Stock solutions of 10^{-4} M were prepared in 50 % DMSO and 50% ethanol, and fresh dilutions made in HBSS-HEPES supplemented with 0.1% BSA at a pH of 7.4. Nonfluorescent chemotactic peptide was obtained from Sigma Chemical Co. and prepared exactly as the fluorescent peptide.

Equilibrium Binding Studies. Binding experiments were carried out essentially as described by Sklar and Finney (12). Isolated cells were suspended in HBSS-HEPES + 0.1% BSA at pH 7.4, ammonium chloride in 1x HBSS-HEPES, pH 7.4 (or an equivalent volume of HBSS-HEPES) was then added to a final concentration of 30 mM. Cells were incubated for five minutes at 25°C, and then cooled to 4°C for 15 min in icewater. The cells were neither washed not warmed after exposure to ammonia. All subsequent steps were carried out at 4°C. Aliquots of cells were exposed to 0-20 nN FLPEP in the presence or absence of 1 x 10^{-6} M nonfluorescent FLPEP for one hour. Specific binding was calculated by subtracting nonspecific binding from specific + nonspecific binding. These results and all others are reported as mean ± SEM.

Flow Cytometry. Neutrophil were identified by means of forward and right angle light scatter in a sheath buffer of isotonic saline containing 10 mM HEPES, pH 7.4, and fluorescence (Ex: 488 nm, Em: 510 nm) measured on a Coulter EPICS V flow cytometer equipped with the Coulter MDADS data acquisition system. Mean linear fluorescence for 5,000 cells was determined using a Low Level Quantitative Microbead Standards (Flow Cytometry Standards Corporation, Research Triangle Park, NC) to calibrate the machine, and generate a standard curves.

Measurement of the Receptor-Cytoskeletal Association. Ammonia treated and untreated cells were labeled with 20 nM FLPEP for one hour (as described above), washed twice in 4°C HBSS-HEPES, and their membranes stripped by Triton X-100 solubilization essentially as described Jesaitis *et al.* (15). Two alterations were made: the addition of 10 mM EGTA to the extraction buffers, and a second repetition of the extraction procedure. Cells were analyzed on the flow cytometer as described above. As a positive control, some cells were exposed to the fluorescent peptide for 5 min at 25°C, cooled, and analyzed as just described.

RESULTS

Effect of Ammonia Treatment on FLPEP Receptor Binding Characteristics.

We first examined the effect of 30 mM ammonia on N-formyl peptide binding to its receptor. This concentration was chosen because of its common use by others (7,8,9), and because pathogenic bacteria generate similar levels *in vitro* (16,17) and *in situ* (18). Fig. 1 displays typical specific and nonspecific binding curves for ammonia treated and untreated cells. The nonspecific and specific binding curves appear to be similar, suggesting that: (1) ammonia treatment does not affect either nonspecific binding, or FLPEP fluorescence; and (2) the number of receptors displayed on the cell surface are similar. However, it is important to note, as seen in Fig. 1c, that at concentrations below 2 nM, the FLPEP binding by ammonia treated cells is lower than that of untreated cells. This implies that ammonia treatment decreases the affinity of cell surface receptors.

To more accurately determine the effect of ammonia treatment on receptor number and receptor-ligand affinity, Scatchard analysis (19) was performed (Fig. 2). For the displayed experiment, the Scatchard plot indicates a slight increase in receptor number (from approximately 23,000 to 24,000) following ammonia treatment. In experiments with other donors, we observed similar small, but insignificant increases in receptor number when comparing untreated and ammonia treated cells (25,060 $\pm$ 2335 and 26624 $\pm$ 2774 receptors, N=6). For the displayed experiment, the Kd for untreated cells was 0.5 nM. In contrast, the ammonia treated cells displayed a receptor Kd of 0.74 nM. For all six repetitions of this experiment we found that ammonia caused a significant change in the affinity of the chemotactic peptide receptor. The mean Kd for untreated and treated cells was 0.65 $\pm$ 0.06 nM and 1.02 $\pm$ 0.10, respectively. On average, ammonia treatment increased the Kd of the receptor by 58 $\pm$ 12 %.

Effect of Ammonia Treatment on Receptor-Cytoskeletal Association.

By decreasing the cytoskeletal actin (10), it is possible that ammonia can alter receptor-cytoskeletal association and internalization, and thus affect the receptor Kd (15,20,21). To control for this we examined receptor-cytoskeletal association before and after ammonia treatment. Fig. 3 displays the results of these experiments. These results are the average of four experiments performed on four different donors. As expected, total binding (which reflects the total number of cell surface receptors) was very similar for treated and untreated cells (67 $\pm$ 6, and 64 $\pm$ 4 channel numbers, respectively). Following Triton extraction, cytoskeletally associated receptor binding was substantially reduced to 20% of initial levels for both ammonia treated and untreated cells (14 $\pm$ 1, and 12 $\pm$ 1 channel numbers, respectively).

Previous reports indicate that neutrophil activation results chemotactic receptor modulation, and increased receptor-cytoskeletal association (15,20,21). Therefore, as a positive control, we also examined the receptor number and cytoskeletal association

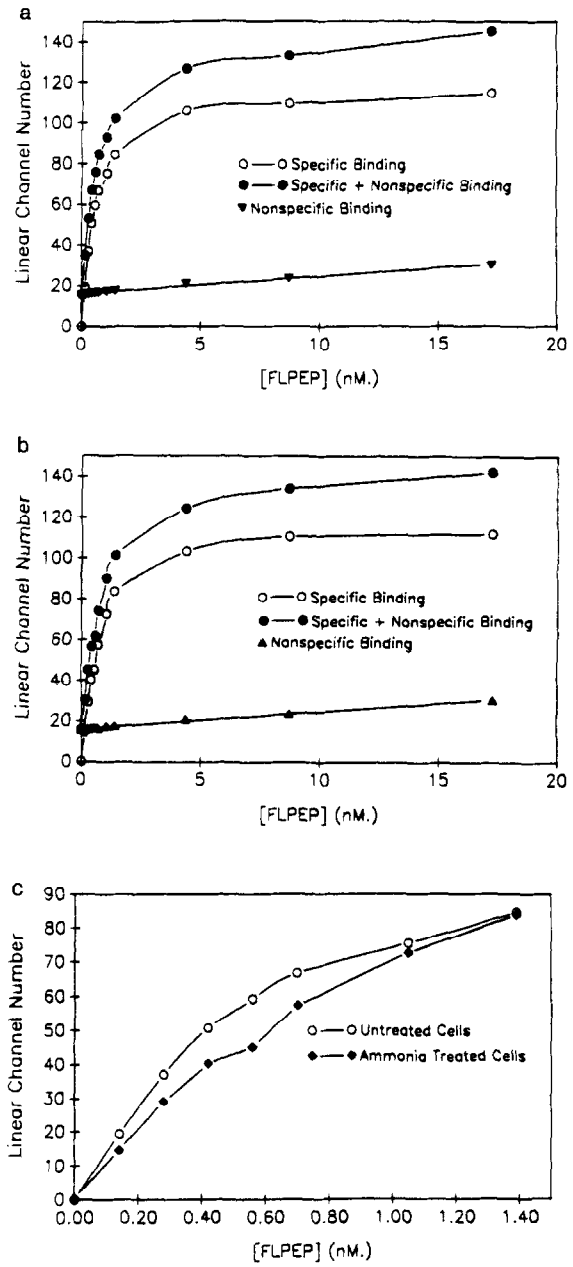

Figure 1. Equilibrium binding of FLPEP by human neutrophils. Typical experiment. **1a.** Untreated human neutrophils. **1b.** Ammonia treated human neutrophils. **1c.** Specific binding of FLPEP. The graphic indicates that at concentrations of FLPEP below 2 nM, specific binding is reduced in ammonia treated cells.

following neutrophil activation (Fig. 3). As expected, total FLPEP binding and cytoskeletally associated binding (110 ± 4 channel numbers, 75 ± 4 channel numbers, respectively) were much higher than either ammonia treated or untreated cells. Furthermore, receptor-cytoskeletal association increased to 69 ± 5%.

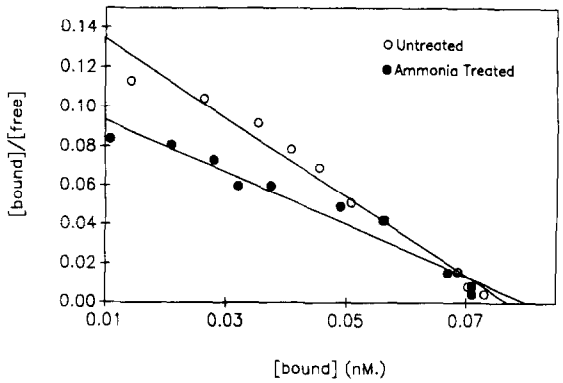

Figure 2. Scatchard analysis for untreated and ammonia treated cells. Typical experiment. The plot indicates that untreated and ammonia treated cells have a similar x-intercept, but differing slopes, thus indicating a similar number of FLPEP receptors, but with a different Kd.

DISCUSSION

The results reported here indicate that untreated and ammonia treated neutrophils display a similar number of N-formyl peptide receptors, and a similar number of cytoskeletally-associated receptors. However, ammonia increases the Kd of the receptors by a factor of approximately two. Since receptor occupancy is crucial in determining functional activity (1,6,22), these results suggest that ammonia can decrease cell function by decreasing the cell's sensitivity to N-formyl peptides. This finding is in contrast to other investigators (7,8,9) who linked ammonia's inhibitory action to its lysosomotropic properties. To date, then, the results suggest that the effects of ammonia are pleiotropic and affect both cytoplasmic and extracellular components of the neutrophil.

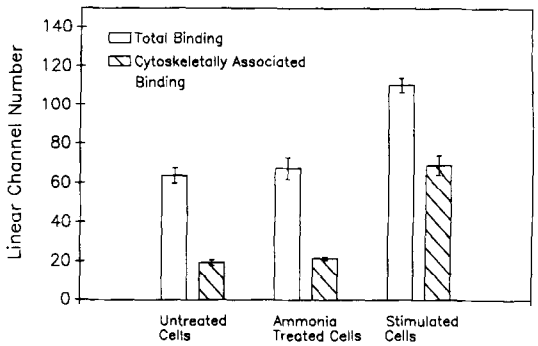

Figure 3. Effect of ammonia on receptor-cytoskeletal association. Total binding is the total specific fluorescence per neutrophil. Cytoskeletally associated binding is the total specific fluorescence per neutrophil after Triton X-100 extraction. Stimulated cells were exposed to FLPEP for 5 min at 25°C followed by cooling and equilibration. The results indicate that untreated and ammonia treated cells exhibit a similar number of cell surface, and cytoskeletally associated receptors. (Mean ± SEM, N=4).

Others have reported that exposure of permeabilized neutrophils to substances which interact with G proteins (guanine nucleotides and pertussis toxin), raises the N-formyl peptide receptor Kd without altering the number of receptors (23,24,25). We have obtained a similar result by ammonia treatment. Because ammonia can affect external cellular components, and also penetrate host cell membranes and alter cytoplasmic pH, there are several possible mechanisms by which ammonia could alter N-formyl peptide receptor affinity: ammonia may directly alter the N-formyl peptide receptor; ammonia may alter the receptor G-protein interaction, thus affecting the Kd; or ammonia may alter cytoplasmic constituents or pH, which in some undetermined way affects the Kd. Conversely, it is interesting that ammonia treatment had no effect on the association of the ligand-receptor complex with the cytoskeleton. Therefore, it is improbable that the noted changes in receptor affinity are due to alterations in receptor-cytoskeletal association.

The importance of these findings lie in two areas. First, pathogenic bacteria depend on their ability to both alter the environment and interact with host cells (26,27). One mechanism to accomplish this task is to generate copious amounts of ammonia (16,17,18), thus altering the bacterial environment (28), and based on the studies reported here, alter the host cell's ability to bind N-formyl peptides. Second, ammonia has been used for approximately 75 years to experimentally alter cytoplasmic pH (29) and thereby alter cell function (30). The results reported here suggest that ammonia, in addition to altering cytoplasmic cellular components, can also alter extracellular components of the cell.

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REFERENCES

1. Omann, G.M., Allen, R.A., Bokoch, G.M., Painter, R.G., Traynor, A.E., and Sklar, L.A. (1987) *Physiol. Rev.* **67**, 285-322.
2. Becker, E.L. (1979) *J. Reticuloendothel. Soc.* **26**, 701-709.
3. Marasco, W.A., Phan, S.H., Krutzsch, H., Showell, H.J., Feltner, D.E., Nairn, N., Becker, E.L., and Ward, P.A. (1984) *J. Biol. Chem.* **259**, 5430-5439.
4. Rotrosen and Gallin, (1987) *Ann. Rev. Immunol.* **5**, 127-150.
5. Boxer and Smolen (1988) *Hematol. Oncol. Clin. N. Am.* **2**, 101-134.
6. Allen, R.D., Traymor, A.E., Omann, G.M., Jesaitis, A.J. (1988) *Hematol. Oncol. Clin. N. Am.* **2**, 33-59.
7. Styrt, B. and Klempner, M.S. (1988) *J. Med. Microbiol.* **25**, 101-107.
8. Styrt, B. and Klempner, M.S. (1986) *Blood* **67**, 334-342.
9. Styrt, B. and Klempner, M.S. (1983) *J. Clin. Invest.* **72**, 1793-1800.
10. Kraus, E. and Niederman, R. (1989) *Cytometry*. In press.
11. Niedel, J., Wilkinson, S., and Cuatrecasas, P. (1979) *J. Biol. Chem.* **254**, 10700-100706.
12. Sklar, L.A., and Finney, D.A. (1982) *Cytometry* **3**, 161-165.
13. Sklar, L.A., Finney, D.A., Oades, Z.G., Jesaitis, A.J., Painter, R.G., and Cochrane, C.G. (1984) *J. Biol. Chem.* **259**, 5661-5669.

14. Boyum, A. (1968) *Scand. J. Clin. Lab. Invest.* **97**, 77-89.
15. Jesaitis, A.J., Naemura, J.R., Sklar, L.A., Cochrane, C.G., and Painter R.G. (1984) *J. Cell. Biol.* **98**, 1378-1387.
16. Holdeman, L., Cato, E.P., and Moore, W.E.C. (1977) *Anaerobe Laboratory Manual.* Virginia Polytechnic Institute, Blacksburg, VA.
17. van Steenbergen, T.J.M., van der Mispel, L.M.S., de Graff, J. (1986) *J. Dent. Res.* **65**, 909-912.
18. Margolis, H.C., Duckworth, J.H., and Moreno, E.C. (1988) *J. Dent. Res.* **67**, 1468-1475.
19. Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660-672.
20. Zigmond, S.H., Sullivan, S.J., and Lauffenburger, D.A. (1982) *J. Cell Biol.* **92**, 34-43.
21. Sklar, L.A., Finney, D.A., Oades, Z.G., Jesaitia, A.J., Painter, R.G., and Cochrane, C.G. (1984) *J. Biol. Chem.* **259**, 5661-5669.
22. Omann, G.M., and Sklar, L.A. (1988) *J. Cell Biol.* **107**, 951-958.
23. Koo, C., Lefkowitz, R.J., and Snyderman, R. (1983) *J. Clin. Invest.* **72**, 748-753.
24. Sklar, L.A., Bokoch, G.M., Button, D., and Smolen, J.E. (1987) *J. Biol. Chem.* **262**, 135-139.
25. Feltner, D.E., and Marasco, W.A. (1989) *J. Immunol.* **142**, 3963-3970.
26. Miller, J.F., Mekalanos, J.J., and Falkow, S. (1989) *Science.* **243**, 916-922.
27. Finlay, B.B., and Falkow, S. (1989) *Microbiol. Rev.* **53**, 210-230.
28. Margolis, H.C., Duckworth, J.H., and Moreno, E.C. (1988) *J. Dent. Res.* **67**, 1476-1482.
29. Busa, W.S. (1986) *Ann. Rev. Physiol.* **48**, 389-402.
30. Haussinger, D. (1988) *pH Homeostasis.* Academic Press, N.Y., N.Y.