The Requirement for Membrane Sialic Acid in the Stimulation of Phagocytosis by the Natural Tetrapeptide, Tuftsin*

(Received for publication, July 11, 1972)

ANDREAS CONSTANTOPoulos AND VICTOR A. NAJJAR‡
From the Division of Protein Chemistry, Department of Molecular Biology and Microbiology, Tufts University
School of Medicine, Boston, Massachusetts 02111

SUMMARY

The presence of sialic acid on the polymorphonuclear (PMN) phagocyte is necessary for maximal stimulation of its phagocytic activity by the tetrapeptide, tuftsin, Thr-Lys-Pro-Arg. Treatment of polymorphonuclear cells with bacterial neuraminidases, purified by affinity chromatography, completely abolishes stimulation of phagocytic activity by free tuftsin or by tuftsin bound to the carrier leukokinin molecule. These enzymes cleave sialic acid at both 2-3' and 2-6' glycosidic bonds. By contrast, treatment of cells with influenza virus neuraminidase, which cleaves predominantly the 2-3' linkage, reduces the tuftsin effect by approximately 50%. Enzyme-treated cells remain viable and capable of phagocytosis. Cells treated with any of the neuraminidases and cells of the untreated controls show comparable levels of phagocytosis in the absence of tuftsin. While membrane sialic acid is necessary for stimulation of phagocytosis, it may function only as a binding site for the three positively charged residues of the tetrapeptide. Neuraminidases from Vibrio cholerae or Clostridium perfringens, in excess amounts, released in 2 hours a maximum of about 135 million molecules of sialic acid per polymorphonuclear cell. Under similar conditions, treatment with influenza virus released approximately 30 million molecules per cell.

It was shown earlier that a particular cytophilic γ-globulin, termed leukokinin, binds specifically to blood polymorphonuclear leukocytes and stimulates their phagocytic activity 2- to 3-fold. It was later found that the whole molecule was not necessary for this biological effect. All of the stimulatory activity resided in a small peptide termed tuftsin. This peptide is cleaved from the parent molecule by a membrane enzyme, leukokininase, leaving behind an inactive leukokinin molecule. The peptide then acts on the cell in some manner to stimulate phagocytosis, pinocytosis, and cell motility. The phagocytic activity of lung and peritoneal macrophages is also stimulated to the same degree by similar concentrations of tuftsin. A preliminary account of the findings that led to the discovery of tuftsin, its structure, and its chemical synthesis has already appeared.

Tuftsin is a highly basic tetrapeptide, L-threonyl-L-lysyl-L-prolyl-L-arginine, molecular weight 500. It might be expected that a basic peptide with no hydrophobic residues would interact with the cell membrane in a strictly electrostatic manner, perhaps with the negatively charged sialic acid groups. This paper presents evidence that membrane sialic acid is required for stimulation of phagocytosis by tuftsin and its parent molecule, leukokinin. The data also lend support to the existence of sialic acid in both 2-6’ and 2-3’ α-keto-linkages to carbohydrate residues on the membrane of PMN cells of man, dog, and rabbit.

MATERIALS AND METHODS

Preparations rich in (PMN) neutrophils were obtained fromuffy coats of human and dog blood and rabbit peritoneal exudates. Tuftsin was synthesized in our laboratory with a basic peptide with no hydrophobic residues would interact with a negatively charged sialic acid group. The virus, A2/Aichi/2/68 (800 chick cell agglutination units per ml), was a gift of Dr. Allen F. Woodhour (Merck Institute for Therapeutic Research). Other reagents used were sodium periodate, sodium arsenite, thiobarbituric acid, sialic acid, and bovine submaxillary mucin (Schwarz-Mann, Orangeburg, N. Y.). The neuraminidase reaction was carried out as described in the Worthington catalog with the addition of 2 mM calcium chloride and 2 mM EDTA. Bovine submaxillary mucin was used as the substrate. One unit of activity is equivalent to the release of 1 μ mole of sialic acid per min at 37°C. Protein was measured by the method of Lowry et al. (12) and sialic acid by the thiobarbituric acid method of Warren (13).

* This work was supported by Grant AI-09116, National Institutes of Health, United States Public Health Service and Grant GB 31535 X, National Science Foundation.
† American Cancer Society Professor of Molecular Biology (MA Div.).
‡ The abbreviation used is: PMN, polymorphonuclear.
PMN cells (approximately 3 × 10⁷) were incubated with or without neuraminidase in the indicated amounts for 30 min at 37° in 1 ml of Krebs-Ringer phosphate medium, pH 7.4, modified to contain 0.1 mM CaCl₂, 0.2% glucose and bovine serum albumin, 3 mg per ml. They were then washed three times each with 5 ml of the same medium. Phagocytosis was assayed in a total volume of 0.3 ml of the medium containing 2 × 10⁶ PMN cells and 4 × 10⁸ Staphylococcus aureus (2, 14, 15). Incubation was carried out at 37° for 30 min with gentle mixing in a vertical rotor at 8 rpm. Stained smears of samples of incubated cells were evaluated by two observers who counted a total of 400 cells. The phagocytic index is the percentage of cells counted that contained bacteria. Phagocytic stimulation in the presence of tuftsin, leukokinin, or serum equals the phagocytic index obtained minus the basal phagocytic index of cells treated identically except for the omission of tuftsin, leukokinin, or serum.

RESULTS

Abolition of Tuftsin Effect after Incubation of Phagocytic Cells with Bacterial Neuraminidases—As shown in Table I, incubation of human or canine PMN cells with 1.2 milliunits of C. perfringens or V. cholerae neuraminidase for 30 min abolished their ability to respond to tuftsin, γ-globulin, or serum by an increase in phagocytic index. It should be noted that the autologous serum had been incubated at 56° for 30 min to inactivate complement. Thus, the stimulation of phagocytosis by serum was presumably due only to the leukokinin present in it (1-3).

Incubation of PMN cells with increasing amounts of the bacterial neuraminidases between 0.12 and 1.2 milliunits produces a progressive diminution in the subsequent response of the cells to tuftsin. The activities of both enzymes are approximately the same given the limitation of the sensitivity of the method. Enough sialic acid is presumably removed with 0.8 to 1.2 milliunits to render the PMN cell completely unresponsive to phagocytic stimulation by either tuftsin or leukokinin (Fig. 1). However, the basal phagocytic index of 18 ± 2 of unstimulated cells remains within normal limits, a consistent finding shown by both human and canine PMN cells. This is in agreement with recent observations reported by Noseworthy et al. (16).

TABLE I

| PMN cells | Stimulating agent | Phagocytic index |
|-----------|------------------|------------------|
| Human     | None             | 17-19            |
|           | Tuftsin          | 40-54            |
|           | γ-Globulin       | 42-48            |
|           | Serum            | 42-48            |
| Canine    | None             | 17-20            |
|           | Tuftsin          | 41-49            |
|           | γ-Globulin       | 47-51            |
|           | Serum            | 42-48            |
|           | None             | 16-19            |
|           | Tuftsin          | 40-49            |
|           | γ-Globulin       | 41-50            |
|           | Serum            | 46-52            |

* Treated with V. cholerae neuraminidase. All other cells were treated with C. perfringens.

Fig. 1 (left). Stimulation of phagocytosis by tuftsin after incubation of PMN cells with bacterial neuraminidases. The procedures used are described in Table I. Human (●), canine (×), or rabbit (■) PMN cells were incubated with C. perfringens neuraminidase and human (○) or canine (△) cells with the V. cholerae enzyme. Phagocytosis was assayed with or without 1 m mole of tuftsin. Each point represents the difference between the phagocytic index of a sample of cells incubated with and another incubated without tuftsin.

Fig. 2 (center). Stimulation of phagocytosis by tuftsin after incubation of PMN cells with viral neuraminidase. Human (○) or canine (●) PMN cells were incubated with the indicated amount of neuraminidase and phagocytosis was assayed and plotted as in Fig. 1.

Fig. 3 (right). Effect of tuftsin on the liberation of sialic acid from mucin by C. perfringens neuraminidase. Mucin, 2 mg, and neuraminidase, 12 milliunits, were incubated at 37° in 0.1 M sodium acetate buffer, pH 5.5, containing 2 mM CaCl₂ and 0.2 mM EDTA (total volume 1 ml) with (●) or without (○) 7 m moles of tuftsin. An aliquot of 0.2 ml was taken at the times indicated for sialic acid assay. These are represented by the points on the curves.
Experiments were carried out as described in the legend for Table 1 with influenza virus (32 milliunits of neuraminidase) replacing the bacterial enzymes where indicated. The ranges of values from five replicate experiments are shown.

### Table I

#### Effect of desialation of polymorphonuclear cells with viral neuraminidases on stimulation of phagocytosis by tuftsin, γ-globulin, and serum

PMN cells were incubated with or without neuraminidase (1 milliunit of C. perfringens or 2 milliunits of V. cholerae enzyme) and 15 mg of mucin or 4.2 μmoles of tuftsin (1.4 μmoles added at 0, 10, and 20 min) as indicated. One of each pair was used for assay of phagocytosis with 1 nmole of tuftsin, the other without tuftsin.

| Source of neuraminidase | Number of rabbit peritoneal PMN leukocytes | Incubation time | Sialic acid released |
|-------------------------|-------------------------------------------|-----------------|---------------------|
| V. cholerae             | 1.9 x 10⁶                                | 60 min          | 134                 |
| V. cholerae             | 1.9 x 10⁶                                | 120 min         | 137                 |
| C. perfringens          | 2.5 x 10⁶                                | 120 min         | 132                 |
| Influenza virus         | 2.5 x 10⁶                                | 120 min         | 29                  |

#### Table II

**Release of sialic acid from polymorphonuclear cells by incubation with neuraminidases**

Peritoneal exudates were harvested from rabbits 14 hours after the intraperitoneal injection of 400 to 500 ml of 0.15% glycogen in 0.15 M NaCl. After washing with Krebs-Ringer phosphate medium, PMN cells were incubated for 60 or 120 min with 12 milliunits of each of the bacterial enzymes or 60 milliunits of the viral enzyme in a total volume of 1 ml. The values represent the average of two such identical experiments.

| Source of neuraminidase | Number of rabbit peritoneal PMN leukocytes | Incubation time | Sialic acid released |
|-------------------------|-------------------------------------------|-----------------|---------------------|
| V. cholerae             | 1.9 x 10⁶                                | 60 min          | 134                 |
| V. cholerae             | 1.9 x 10⁶                                | 120 min         | 137                 |
| C. perfringens          | 2.5 x 10⁶                                | 120 min         | 132                 |
| Influenza virus         | 2.5 x 10⁶                                | 120 min         | 29                  |

PMN cells from peritoneal exudates were used for this study (7). The protective effect of tuftsin could, however, be due to a detrimental effect on the enzyme itself rather than due to its proposed binding to membrane sialic acid. The results depicted in Fig. 3 do not support this proposition. Here C. perfringens neuraminidase activity, with bovine mucin as substrate, was measured in the absence and presence of 7 μmoles (3.5 mg) of tuftsin. In the presence of this large excess of tuftsin, only a slight effect on the initial rate was discernible, which may well be due to tuftsin substrate interaction rather than an effect on the enzyme.

### Table III

**Protective action of bovine submaxillary mucin and tuftsin on neuraminidase effect on polymorphonuclear leukocytes**

Duplicate samples of canine PMN cells were incubated with or without neuraminidase (1 milliunit of C. perfringens or 2 milliunits of V. cholerae enzyme) and 15 mg of mucin or 4.2 μmoles of tuftsin (1.4 μmoles added at 0, 10, and 20 min) as indicated. One of each pair was used for assay of phagocytosis with 1 nmole of tuftsin, the other without tuftsin.

| PMN cells incubated with | Phagocytic index | Phagocytic index |
|-------------------------|------------------|------------------|
| Neuraminidase           | Without tuftsin  | With tuftsin     |
| None                    | None             | 19               | 43               |
| C. perfringens          | None             | 18               | 41               |
| C. perfringens          | Mucin            | 18               | 42               |
| None                    | None             | 22               | 46               |
| V. cholerae             | None             | 18               | 42               |
| V. cholerae             | Mucin            | 17               | 44               |
| None                    | None             | 17               | 46               |
| C. perfringens          | None             | 19               | 49               |
| C. perfringens          | Tuftsin          | 20               | 48               |

globulin or by serum was also reduced by about 50% after incubation of cells with the viral neuraminidase (Table II).

As shown in Table III, when mucin, a substrate for the bacterial neuraminidase, was present during incubation of cells with the enzyme, the PMN cells responded to tuftsin with an increase in the phagocytic index equal to that observed with control cells and, as usual, tuftsin was without effect on the cells incubated with the enzyme alone. The addition of tuftsin in high concentration also protected the cells from the effect of C. perfringens and, as usual, tuftsin was without effect on the cells incubated with 1 milliunit of neuraminidase the enzyme (Table III). The protective effect of tuftsin may well result from its ability to bind to membrane sialic acid residues and thereby make them inaccessible to the enzyme.

The protective effect of tuftsin could, however, be due to a detrimental effect on the enzyme itself rather than due to its proposed binding to membrane sialic acid. The results depicted in Fig. 3 do not support this proposition. Here C. perfringens neuraminidase activity, with bovine mucin as substrate, was measured in the absence and presence of 7 μmoles (3.5 mg) of tuftsin. In the presence of this large excess of tuftsin, only a slight effect on the initial rate was discernible, which may well be due to tuftsin substrate interaction rather than an effect on the enzyme.

### Table IV

**Presence of Sialic Acid in 2-3' and 2-6' α-Keto-linkage on PMN Cell Membrane**

As it was not practical to prepare PMN from blood in quantities sufficient to measure the amount of sialic acid released by incubation with neuraminidase, rabbit PMN cells from peritoneal exudates were used for this study (7). They are in all respects similar to blood PMN cells in their response to tuftsin and in their content of leukokinase in the cell membrane (4, 5, 14, 15). The effect of bacterial neuraminidase on the rabbit cells is similar to that of the enzyme on the human and canine cells (see Fig. 1). Of the amount of sialic acid released by the bacterial enzymes, about 20% was liberated in 2 hours by the influenza virus. Most of this is presumably present in 2-3' linkage. If it is assumed that all of the sialic acid on the membrane was equally accessible to the neuraminidases, and that the action of the viral enzyme went to completion, it may be concluded that about 80% of the sialic acid existed in 2-6' linkage. Thus, the number of molecules of sialic acid in 2-3' linkage was calculated to be about 30 x 10⁶ per cell. These must be considered only as approximate values since it has been assumed that all the sialic acid was removed by V. cholerae and C. perfringens and this was derived only from the outer cell membrane. This is not an invalid assumption since it has recently been shown that these enzymes do not penetrate the PMN cell membrane (19). Furthermore, in these experiments, the removal of membrane sialic acid per se, or the presence of an unsuspected contaminating enzyme might conceivably have damaged the cell to expose internal sialic acid. This possibility, however, was ruled out since all cells remained alive and actively motile. Their phagocytic potential was essentially unimpaired since phagocytosis by enzyme-treated PMN cells was identical with that of normal cells. When desialated cells were allowed to phagocytize a high multiplicity of staphylococci, 100 bacteria per PMN cell, fully 100% of the cells had engulfed great numbers of bacteria.
Sialic acid has been shown to play a significant role in cell physiology. It is apparently concerned with cell adhesion (20, 21), and in an elegant study it was shown to play a dual role in the metabolism of circulating glycoproteins (22). Its presence on liver cell membranes is essential for the binding and transport of serum glycoproteins into the liver cell. In order for binding to occur, however, the glycoprotein must first be desialylated (22).

The results in this communication indicate that membrane sialic acid, whether of the 2-3' or 2-6' linkage, can act as a receptor site for free tuftsin as well as for that covalently present in the parent molecule, leukokinin. On the reasonable assumption that 2-6' bonds not cleaved by the viral enzyme remain on the membrane surface, the data indicate that sufficient receptor site for free tuftsin as well as for that covalently present in the parent molecule, leukokinin. On the reasonable assumption that sialic acid, whether of the 2-3' or 2-6' linkage, can act as a receptor site for tuftsin. The limitation of viral enzyme activity is expressed in the phagocytic reaction by a residual stimulatory response of the PMN cell that was resistant to the action of the enzyme.

Inasmuch as tuftsin plays a significant role in body defense (14, 15), the importance of membrane sialic acid as a receptor site for tuftsin adds another dimension to the role of sialic acid in a variety of physiological functions. Protection of sialic acid in the membrane, but not that in mucin from the action of neuraminidase, indicates that sialic acid is a part of, but not necessarily all of, the tuftsin receptor site. The binding of tuftsin may include hydrophobic interactions involving the methylene carbons of lysine and arginine as well as ionic effects. The observation that the effect of neuraminidase on the ability of the PMN cell to be stimulated by tuftsin can be prevented by mucin lends support to the view that this effect is a consequence of the removal of membrane sialic acid rather than an effect of contaminants in the enzyme preparation.

The simple act of phagocytosis, which is not subjected to outside stimulation by tuftsin, leukokinin, or serum, and which is observed in both desialylated and normal PMN controls, is independent of the presence of sialic acid. Recently, Noseworthy et al. (16), who studied phagocytosis in the absence of serum stimulation, concluded that treatment of monolayers of PMN cells with "purified" neuraminidase does not affect this process. Our results are in agreement with their findings in that normal and desialylated PMN cells exhibited comparable phagocytic indices in the absence of tuftsin, leukokinin, or serum. Our main concern is that sialic acid is, however, absolutely required for stimulation of phagocytosis by these materials. It may well be that sialic acid is not the ultimate receptor for tuftsin. It may simply bind the tetrapeptide in order to provide a high local concentration in the vicinity of the ultimate protein receptor which then mobilizes the cell membrane for more effective phagocytosis, pinocytosis, and cell mobility (3, 6). This remains to be determined. When membrane proteins are successfully solubilized, the postulated protein component of the receptor might be identified by binding studies with the tetrapeptide.

REFERENCES

1. Fidalgo, B. V., and Najjar, V. A. (1967) Biochemistry 6, 3396-3399
2. Najjar, V. A., Fidalgo, B. V., and Stitt, E. (1968) Biochemistry 7, 2376-2379
3. Fidalgo, B. V., and Najjar, V. A. (1967) Proc. Nat. Acad. Sci. U. S. A. 57, 957-964
4. Najjar, V. A., and Nishioka, K. (1970) Nature 218, 672-673
5. Nishioka, K., Constantopoulos, A., Sato, F. S., and Najjar, V. A. (1972) Biochem. Biophys. Res. Commun. 47, 172-179
6. Nishioka, K. (1971) Ph.D. dissertation, Vanderbilt University
7. Constantopoulos, A., and Najjar, V. A. (1972) Cytohes 6, 97-100
8. Merrifield, R. B. (1964) Biochemistry 3, 1385-1390
9. Najjar, V. A., and Merrifield, R. B. (1965) Biochemistry 4, 3765-3770
10. Cuatrecasas, P. (1970) J. Biol. Chem. 245, 3059-3065
11. Cuatrecasas, P., and Illiano, G. (1971) Biochem. Biophys. Res. Commun. 44, 178-184
12. Lown, O. H., Rosebrugh, N. J., Fabr, A. L., and Randall, E. J. (1981) J. Biol. Chem. 256, 265-270
13. Warren, L. (1959) J. Biol. Chem. 234, 1971-1975
14. Constantopoulos, A., Najjar, V. A., and Smith, J. W. (1972) J. Pediat. 80, 564-572
15. Najjar, V. A., and Constantopoulos, A. (1972) J. Reticuloendothel. Soc. 12, 197-215
16. Noseworthy, J., Korchak, H., and Karnovsky, M. L. (1972) J. Cell. Physiol. 97, 91-96
17. Dreyfus, R. (1967) Biochem. Biophys. Res. Commun. 26, 631-638
18. Rapelson, M. E., Jr., Gold, S., and Prienb, E. (1966) Methods Enzymol. 6, 677-680
19. DePierre, J., and Karnovsky, M. L. (1972) in Inflammation (Lapow, I. H., and Ward, P. A., eds) p. 90, Academic Press, New York
20. Weiss, L. (1965) J. Cell Biol. 26, 735-739
21. Roseman, S. (1970) Chem. Phys. Lipids 5, 270-297
22. Price, W. E., Jr., and Ashwell, G. (1971) J. Biol. Chem. 246, 4825-4833
The Requirement for Membrane Sialic Acid in the Stimulation of Phagocytosis by the Natural Tetrapeptide, Tuftsin
Andreas Constantopoulos and Victor A. Najjar

*J. Biol. Chem.* 1973, 248:3819-3822.

Access the most updated version of this article at [http://www.jbc.org/content/248/11/3819](http://www.jbc.org/content/248/11/3819)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/248/11/3819.full.html#ref-list-1](http://www.jbc.org/content/248/11/3819.full.html#ref-list-1)