INFECTION AND IMMUNITY, Apr, 1975, p. 742-747
Vol. 11, No. 4
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# Evidence for the Non-Protein Nature of the Receptor for the Enterotoxin of *Vibrio cholerae* on Murine Lymphoid Cells

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Received for publication 2 December 1974

Lymphoid cells from A/J and BALB/c strains of mice were iodinated with $^{125}I$ by the lactoperoxidase method and the plasma membranes were disrupted by freezing and thawing or with 0.5% Nonidet P-40, a nonionic detergent. Attempts to find choleragen reactive iodinated material in 0.5% Nonidet P-40 lysates were unsuccessful even when the cells were incubated with choleragen before lysis. Freezing and thawing the cells resulted in the release of iodinated choleragen reactive material. The interaction of choleragen with the iodinated material could be inhibited (at low choleragen concentrations) or enhanced (at high choleragen concentrations) by the addition of the ganglioside $G_{M1}$ to the immune precipitation system. The results are consistent with the hypothesis that the receptor for choleragen is a glycolipid and reduce, but do not totally eliminate, the likelihood that the receptor is glycoprotein in nature.

The enterotoxin of *Vibrio cholerae* has been shown to affect the metabolic activity of several cell types (reviewed in 6) including lymphoid cells (2, 8, 17; D. A. Hart and R. A. Finkelstein, J. Immunol., in press). The mechanism of action of the enterotoxin (choleragen) is believed to be the activation of membrane-bound adenylate cyclase after interaction with cell surface receptors. Several indirect lines of evidence indicate that the receptor may be the ganglioside $G_{M1}$ (GGnSLC) (3, 4, 9, 18). Staerk et al. (J. Staerk, H. I. Ronneberger, H. Wiegandt, and W. Ziegler, submitted for publication) have demonstrated that the specificity of choleragen is toward the oligosaccharide portion of the ganglioside molecule rather than toward the lipid portion. Previous evidence obtained with mouse lymphoid cells (8, 17), human lymphoid cells (Hart and Finkelstein, J. Immunol., in press), and with guinea pigs lymph node cells (Hart, unpublished observations) indicate that binding to the receptor alone does not lead to altered metabolic activity since the inactive derivative of choleragen, choleragenoid, binds to cells but does not have biological activity.

The present study was initiated to determine if choleragen and choleragenoid could possibly be interacting with cell surface glycoproteins that had the same configuration in the oligosaccharide portion of the molecule as the $G_{M1}$ glycolipid and also to investigate the possibility that choleragen was interacting with molecules on the cell surface in addition to the primary binding molecule.

Lactoperoxidase catalyzed iodination of cell surface proteins has been used to identify plasma membrane surface proteins in several cell systems and has been used with great success with lymphoid cells (12, 20). Most, if not all, cell surface proteins are labeled by this procedure. Thus, if the enterotoxin and enterotoxoid were interacting with an iodinatable surface glycoprotein receptor, it should be detectable by this method.

Treatment of iodinated lymphoid cells with the nonionic detergent Nonidet P-40 (NP-40) has been shown to result in the release of 95% of the iodinated trichloroacetic acid-precipitable proteins (19). Freezing and thawing cells results in the release or solubilization of smaller quantities of iodinated material (21) primarily as small membrane fragments, which are probably lipid-protein complexes (see chapter 3, ref. 22, for a review of membrane disruption). Both techniques were used in the present study to evaluate the binding of choleragen and choleragenoid to membrane components.

## MATERIALS AND METHODS

**Reagents.** Choleragen and choleragenoid were the generous gift of R. A. Finkelstein (University of Texas Health Science Center, Dallas, Texas) and were prepared as described (7). The nonionic detergent NP-40 was obtained from Shell Oil Co., New York. Bovine brain ganglioside $G_{M1}$ (95% purity) was prepared by J. Dain, Department of Biochemistry, University of Rhode Island, Kingston, R.I., and was obtained from R. A. Finkelstein. Trasylol, a protease inhibitor, was obtained from FBA Pharmaceuticals,

New York. Fluorescein isothiocynate, isomer I (10% on Celite) was obtained from Sigma Chemical Co., St. Louis, Mo. Sodium iodide ($^{125}$I), carrier free, 100 mCi/ml, was obtained from Amersham-Searle Corp., Chicago, Ill. Lactoperoxidase was obtained from Calbiochem, San Diego, Calif.

**Antiserum.** Rabbit anticholeragen serum was prepared by injection of a rabbit with 0.5 mg of choleragen in Freund complete adjuvant three times at 2-week intervals. Serum was obtained 7 to 10 days after the last injection. The antiserum showed only a single line of identity when tested by double diffusion in agar against choleragen and choleragenoid. The antiserum was absorbed extensively with murine lymphoid cells before use. An immunoglobulin G (IgG) fraction was obtained from the antiserum by sodium sulfate fractionation (11) followed by chromatography on diethylaminoethyl-cellulose in 0.0175 M phosphate buffer, pH 6.9 (10). The fluoresceinated derivative of anti-choleragen IgG was prepared as described by Rinderknecht (15).

Normal rabbit IgG was obtained from a pool of preimmune rabbit serum as described above.

Rabbit anti-A/J thymocyte serum was prepared by three injections of a rabbit with $10^8$ to $2 \times 10^8$ A/J thymocytes at 2-week intervals. Serum was obtained 10 days after the last injection and an IgG fraction was prepared as described above.

Goat anti-rabbit IgG Fc antiserum was obtained from a goat injected with four doses of 3 mg of twice recrystallized normal rabbit IgG Fc in Freund complete adjuvant. The fragments were obtained from papain digestion of normal rabbit IgG (14). The antiserum was absorbed extensively with murine lymphoid cells and decomplemented by heating to 56 C for 30 min before use.

**Lymphoid cells.** Murine thymocytes and splenocytes were obtained from strain A/J and strain BALB/c mice, 6 to 8 weeks of age, obtained from Jackson Laboratories, Bar Harbor, Me.

**Iodination of lymphoid cells.** Lactoperoxidase catalyzed iodination was carried out as described by Bauer et al. (1). Iodinated cells were washed three times with phosphate-buffered saline (PBS) and then resuspended at a concentration of $10^8$ cells per ml. Lysis in 0.5% NP-40 was at 37 C for 15 min and lysis by freezing and thawing was performed by seven cycles of freezing and thawing in an acetone-dry ice bath. Unsolubilized material was removed by centrifugation at $1,000 \times g$ for 15 min, unless otherwise indicated.

**Iodination of choleragenoid.** Choleragenoid was iodinated enzymatically with lactoperoxidase and sodium ($^{125}$I) iodide to a specific activity of 71,600 counts/min per $\mu$g of choleragenoid. Eighty-five to ninety percent of the labeled material retained activity as evidenced by binding to unlabeled splenocytes.

**Preincubation experiments.** Unlabeled cells were incubated at $10^8$ cells/ml of PBS and 50 $\mu$g of [$^{125}$I[choleragenoid was added with mixing. After 30 min at 22 C, the cells were obtained by centrifugation and the counts per minute was determined. The cells were resuspended in 1 ml of 0.5% NP-40 and incubated at 37 C for 15 min. The cell debris was pelleted by centrifugation, the supernatant fraction was removed, and the $^{125}$I counts per minute in the fraction was determined.

Unlabeled cells were incubated at $10^8$ cells/ml of PBS in the presence or absence of 50 $\mu$g of choleragen or choleragenoid for 30 min at 22 C. After centrifugation and washing, the cells were resuspended in 1 ml of PBS and fluoresceinated rabbit anti-choleragen IgG was added to the suspension. After incubation at 22 C for 30 min, the cells were washed three times with PBS. The cells were then observed with a Leitz Ortholux II with a Ploem illuminator (E. Leitz, Wetzlar, West Germany.)

Iodinated cells were resuspended at $10^8$ cells/ml of PBS and 50 $\mu$g of choleragen or choleragenoid was added rapidly. After 30 min at 22 C, the cells were obtained by centrifugation and lysed in 0.5% NP-40 as usual.

**Immune precipitations.** Immune precipitations were carried out in glass tubes (10 by 75 mm). Each tube contained 0.9 ml of 1 mg of bovine serum albumin plus 2 mg of NaI/ml of PBS; 0.05 or 0.1 ml of $^{125}$I sample; choleragen or choleragenoid where appropriate; 200 $\mu$g of normal rabbit IgG, rabbit anti-choleragen IgG, or rabbit anti-A/J thymocyte IgG; and 0.4 ml of goat anti-rabbit IgG Fc antisera. After several hours at 4 C, the precipitates were obtained by centrifugation and washed four to five times with 1 ml of 1 mg of bovine serum albumin plus 2 mg of NaI/ml of PBS until the counts per minute in the precipitate was not altered by an additional washing. All assays were performed in duplicate or triplicate and values agreed within 5%.

**Polyacrylamide electrophoresis.** Two percent sodium dodecyl sulfate/agarose/2.5% polyacrylamide electrophoresis was carried out as described previously (16). The gels were fractionated and counted as described (16).

## RESULTS

**Fluorescent antibody staining of cells treated with choleragen or choleragenoid.** When A/J and BALB/c unlabeled splenocytes or thymocytes were incubated with saturating concentrations of choleragen or choleragenoid (50 $\mu$g/ml), it was found with fluoresceinated anti-choleragen IgG that 95 to 100% of the cells stained with a diffuse ring pattern. No staining was observed in the absence of preincubation with toxin or toxoid. Thus, antibody can recognize cell-bound choleragen and choleragenoid.

**Interaction of lymphoid cells with [$^{125}$I]choleragenoid.** When A/J lymphoid cells were incubated with 50 $\mu$g of [$^{125}$I]-choleragenoid, it was found that thymocytes bound $10 \pm 1$ ng/$10^6$ cells and splenocytes bound $35 \pm 5$ ng/$10^6$ cells. Treatment of these cells with 0.5% NP-40 resulted in the solubilization of 80 to 90% of the bound choleragenoid. Greater than 90% of the released choleragenoid could be precipitated with anti-choleragen IgG.

**Immune precipitation of iodinated cell surface material treated with choleragen and**

**choleragenoid.** Treatment of samples of iodinated A/J splenocytes and thymocytes by the NP-40 and the freeze-thaw techniques followed by choleragen-anticholeragen immune precipitation yielded the results summarized in Table 1. Treatment of iodinated splenocytes with NP-40 results in the release of 2.4 times the trichloroacetic acid-precipitable counts per minute as obtained by the freeze-thaw treatment. In the case of thymocytes, 6 times as many trichloroacetic acid-precipitable counts per minute were obtained by NP-40 lysis as opposed to the freeze-thaw method. However, as shown in Table 1, whereas significantly more radioactivity was precipitable by choleragen or choleragenoid-specific immune precipitation in the freeze-thaw preparations of both splenocytes and thymocytes, this was not the case with the NP-40 lysates. About 50% of the radioactivity of the NP-40-lysed thymocyte preparation was precipitable with anti-thymocyte serum.

The differences in results between the NP-40 and freeze-thawed preparations raised the possibilities that the choleragen-choleragenoid receptor was degraded during the NP-40 lysis procedure or that NP-40 in some way interfered with the interaction of choleragen with its receptor. Addition of a protease inhibitor, trasylol, as suggested by Moroz and Hahn (13) did not alter the results shown in Table 1. That choleragen was capable of interaction with receptor in the presence of NP-40 was demonstrated by Ouchterlony double diffusion in agar analyses carried out with choleragen and bovine brain gangliosides (9) in plates that had 0.5% NP-40 incorporated into the agar. Precipitin lines were observed after 48 h at 22 C, indicating that choleragen could recognize the oligosaccharide configuration in the presence of 0.5% NP-40.

**Interaction of choleragen and choleragenoid with iodinated whole cells followed by NP-40 lysis.** When iodinated whole cells were subjected to preincubation with unlabeled toxin or toxoid followed by 0.5% NP-40 lysis and then immune precipitation with either normal rabbit IgG (control) or anti-choleragen IgG (specific), there was no increase in the counts per minute in the specific precipitate over that of the control precipitate (Table 2). Therefore, after the interaction of the toxin with its receptor no iodinatable material could be specifically precipitated after NP-40 dissociation of the cells. This experiment raised the possibility that the receptor is not an iodinatable surface protein.

**Characterization of freeze-thaw material reactive with choleragen.** In an attempt to recognize receptor substance, immune precipitates, obtained from freeze-thaw lysates of iodinated splenocytes or thymocytes, were solubilized in sodium dodecyl sulfate-urea and electrophoresed on agarose/2.5% acrylamide gels. The resulting pattern was very complex, indicating that the iodinated cell surface proteins in the membrane fragments precipitated by the choleragen-anticholeragen system were very heterogeneous. The same percentage of trichloroacetic acid-precipitable counts per minute could be precipitated with the choleragen-anticholeragen system from the supernatant material obtained after centrifugation at 10,000 $\times$ *g*.

TABLE 1. *Immune precipitation of iodinated cell surface material reactive with choleragen and choleragenoid*

| Expt | Trichloroacetic acid-precipitable counts/min (%)[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0.5% NP-40 lysis | | | | Freeze-thaw lysis | | | |
| | Control | Choleragen | | Choleragenoid (10 μg) | Control | Choleragen | | Choleragenoid (10 μg) |
| | | 5 μg | 10 μg | | | 5 μg | 10 μg | |
| Splenocytes[b] | | | | | | | | |
| Anti-choleragen IgG | 3.6 | | 4.3 | | 9.1 | 37.5 | 36.1 | 34.9 |
| Normal rabbit IgG | | | 3.2 | | | | 10.1 | |
| Thymocytes[c] | | | | | | | | |
| Anti-choleragen IgG | 8.9 | 8.3 | | 10.1 | 15.0 | 43.0 | 42.0 | |
| Anti A/J thymocyte IgG | 48.8 | | | | | | | |

[a] Triplicate aliquots of $10^7$ cell equivalents were utilized for each value. Immune precipitations were carried out as described in Materials and Methods. Standard error of the mean was less than 5%.

[b] The 0.5% NP-40 lysate contained $6.6 \times 10^5$ trichloroacetic acid counts/min per $10^7$ cell equivalents and the freeze-thaw lysate contained $2.77 \times 10^5$ trichloroacetic acid counts/min per $10^7$ cell equivalents.

[c] The 0.5% NP-40 lysate contained $9.3 \times 10^5$ trichloroacetic acid counts/min per $10^7$ cell equivalents and the freeze-thaw lysate contain $1.55 \times 10^5$ trichloroacetic acid counts/min per $10^7$ cell equivalents.

Electrophoresis of this material again showed a very heterogeneous pattern.

When immune precipitates of freeze-thaw lysates of iodinated thymocytes or splenocytes were treated with 0.5% NP-40 at 37 C, 75 to 85% of the precipitated counts per minute could be solubilized (Table 3). Equal percentages of the immune precipitated material could be solubilized from choleragen and choleragenoid immune precipitates. Determination of the presence of cell surface immunoglobulin by immune precipitation with anti-immunoglobulin reagents, in the material solubilized from immune precipitates of splenocyte lysates, indicated that 4 to 5% of the released counts per minute were accounted for by immunoglobulin. This percentage is similar to that found for NP-40 lysates of whole splenocytes (19).

TABLE 2. *Effect of preincubation with choleragen and choleragenoid on precipitation of iodinated cell surface material from NP-40 lysed BALB/c thymocytes*

| Expt | Trichloroacetic acid-precipitable $^{125}$I counts per minute (%)[a] | | |
|---|---|---|---|
| | Control | Choleragen (5 $\mu$g) | Choleragenoid (5 $\mu$g) |
| No preincubation | | | |
| Anti-choleragen IgG | 8.9 | 8.3 | 10.0 |
| Preincubated with choleragen | | | |
| Anti-choleragen IgG | 9.2 | | |
| Normal rabbit IgG | 7.1 | | |
| Preincubated with choleragenoid | | | |
| Anti-choleragen IgG | 8.8 | | |
| Normal rabbit IgG | 6.8 | | |

[a] Triplicate aliquots of $10^7$ cell equivalents containing $9.3 \times 10^5$ trichloroacetic acid-precipitable counts per minute were utilized for each value. Immune precipitations were carried out as described in Materials and Methods. Standard error of the mean was less than 5%.

**Effect of ganglioside $G_{M1}$.** Incubation of freeze-thaw lysates with the ganglioside $G_{M1}$ before immune precipitation with the choleragen plus anti-choleragen system resulted in an inhibition of precipitation of labeled material at low concentrations of choleragen (Fig. 1) but resulted in enhanced precipitation at higher concentrations (Fig. 1 and 2). It would appear that exogenously added ganglioside associated with membrane fragments not containing $G_{M1}$ or containing amounts insufficient to allow precipitation in the choleragen plus anti-choleragen system.

## DISCUSSION

Several lines of evidence, derived from a variety of indirect approaches, have led to the postulate that the receptor for choleragen is the ganglioside $G_{M1}$ (3, 9, 18; Staerk et al., submitted for publication). The toxin has been shown to be reactive to the oligosaccharide portion of the $G_{M1}$ molecule (Staerk et al., submitted for publication). However, the previous evidence does not exclude the possibility that the natural membrane receptor may be a glycoprotein with a similar oligosaccharide structure rather than a glycolipid. Results of the present study lend additional weight to the hypothesized glycolipid

TABLE 3. *Effect of 0.5% NP-40 on immune precipitated choleragen reactive material from freeze-thaw lysates of A/J lymphoid cells*

| Expt | $^{125}$I counts/min in immune precipitate[a] | | | Counts/min released by NP-40[d] |
|---|---|---|---|---|
| | Buffer[b] | 1st NP-40[c] | 2nd NP-40[c] | |
| Splenocytes | | | | |
| Anti-choleragen IgG | 41,200 | | | |
| Anti-choleragen IgG + 10 $\mu$g of choleragen | 238,300 | 65,100 | 36,400 | 85 |
| Thymocytes | | | | |
| Anti-choleragen IgG | 24,000 | | | |
| Anti-choleragen IgG + 10 $\mu$g of choleragen | 148,300 | 44,900 | 25,300 | 83 |

[a] All assays were performed in triplicate with $10^7$ cell equivalents per assay. Indicated values are the mean of the triplicate values. Deviation from the mean was less than 5%.

[b] Immune precipitates were washed five times. The indicated values are the fifth wash precipitates.

[c] The washed immune precipitates were resuspended in 1 ml of 0.5% NP-40 for 1 h at 37 C and then centrifuged. The indicated values are the counts per minute remaining in the precipitate.

[d] Determined from 100 [1 − (counts per minute remaining after NP-40 washes/counts per minute in buffer-washed precipitate) ].

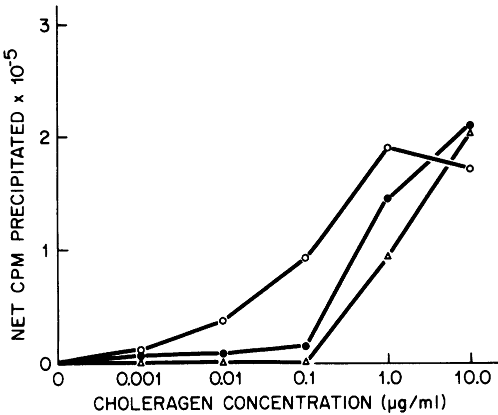


FIG. 1. *Effect of ganglioside $G_{M1}$ on the precipitation of iodinated membrane fragments by choleragen plus anti-choleragen. Iodinated BALB/c splenocytes were lysed by the freeze-thaw method. Triplicate aliquots of $10^7$ cell equivalents in 1 ml of standard assay solution were incubated with buffer (○), 1 µg of $G_{M1}$ (●), or 5 µg of $G_{M1}$ (Δ) for 30 min at 37 C. The indicated quantities of choleragen were then added and the immune precipitation was carried out as described in Materials and Methods. Control values (no choleragen) were subtracted from the experimental values. The variation between values at each point was less than 5%.*

nature of the receptor but still do not rigidly exclude the possibility of the receptor being a cell surface protein.

It has been confirmed both by specific immunofluorescence and by use of radiolabeled choleragenoid that binding to surface of splenocytes and thymocytes occurs and that cell-bound choleragen and choleragenoid can still be recognized by antibody. However, when iodinated cells were lysed, even after interaction with toxin, with NP-40, a method which is known to release greater than 90% of the iodinated protein (19), toxin reactive cell surface proteins were not detected by the immunoprecipitation system (Tables 1 and 2). The alternate method of cell disruption, freezing and thawing the cells, did result in the release of choleragen-reactive iodinated material. However, up to 85% of this material could be solubilized from immune precipitates by washing them with 0.5% NP-40 (Table 3). These experiments therefore provide rather compelling evidence for the non-protein nature of the choleragen receptor since if the receptor were a glycoprotein one would expect it to be iodinated by this lactoperoxidase procedure.

It is possible that the receptor was degraded during the lysis procedure with NP-40 by a protease not sensitive to the inhibitor, trasylol. This appears unlikely since the freeze-thaw extracts should have been exposed to the same intracellular proteases as the NP-40 extracts. In experiments in which choleragen was bound to the iodinated cells before lysis by NP-40, the complex was precipitated within 2 h of lysis so degradation should have been minimal. Also, 85% of the immune precipitated material from freeze-thaw lysates could be solubilized by treatment of the immune precipitates with NP-40, conditions where intracellular proteases would not have been present.

A second possibility is that the receptor is a glycoprotein that does not have any exposed tyrosine residues, in which case it would not have been iodinated by the lactoperoxidase method which catalyzes the incorporation of iodine into the tyrosine molecule.

Finally, the results obtained with the ganglioside $G_{M1}$ (Fig. 1 and 2) verify the interaction of choleragen with this ganglioside. At low concentrations of choleragen, $G_{M1}$ blocks interaction with cell surface components (Fig. 1). However, at higher concentrations of choleragen, the reaction with cell surface components is enhanced, as evidenced by increased immunoprecipitation of iodinated material (Fig. 1 and 2). These results indicate that $G_{M1}$ can associate with membrane fragments such that the molecules orient themselves so as to be recognized by the choleragen molecules. The association is

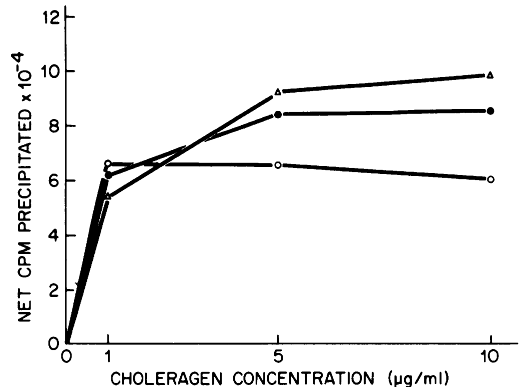


FIG. 2. *Enhancement by $G_{M1}$ of the precipitation of iodinated thymocyte membrane fragments. Iodinated BALB/c thymocytes were lysed by the freeze-thaw method. Triplicate aliquots of $10^7$ cell equivalents were incubated in 1 ml of the standard assay with buffer (○), 1 µg of $G_{M1}$ (●), or 5 µg of $G_{M1}$ (Δ) for 30 min at 37 C. Indicated quantities of choleragen were then added and the immune precipitations were carried out as described in Materials and Methods. Control values (no choleragen) were subtracted from the experimental values. Standard error of the mean was less than 5%.*

stable enough to allow precipitation in the choleragen plus anti-choleragen system. Relevant to this finding are the results of Cuatrecasas (3), who found that fat cells incubated with this ganglioside could bind greater quantities of choleragen than cells not incubated with the ganglioside.

The results obtained in this study are very similar to those found by Vitetta et al. (21) in their investigation of the Thy-1 antigen on murine thymocytes. They found that NP-40 lysis of iodinated thymocytes, followed by immune precipitation with anti-Thy-1, did not yield specifically precipitable iodinated cell surface protein. On the other hand, freeze-thaw lysis of iodinated cells allowed the precipitation of iodinated material. These authors postulated that the antigen may be a glycolipid. Support for this hypothesis came from the work of Esselman and Miller (5), who found that the ganglioside $G_{D1b}$ had Thy-1 activity. Thus, the freeze-thaw procedure apparently results in the release of lipid-protein complexes which in the present study could be separated by treatment with NP-40.

The results obtained in the present study, their similarity with those obtained with Thy-1 antigen (21), and the indirect evidence of other authors (4, 18; Staerk et al., submitted for publication) increase the likelihood that the receptor for choleragen on lymphoid cells is the ganglioside $G_{M1}$.

**ACKNOWLEDGMENTS**

I wish to thank Richard A. Finkelstein for many helpful discussions during the course of this study and his critical review of the manuscript.

This investigation was supported in part by Public Health Service grant 1-PO1-AI 11851-01 from the National Institute of Allergy and Infectious Diseases.

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