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Influenza Type A Virus-Mediated Adherence of Type 1a Group B Streptococci to Mouse Tracheal Tissue In Vivo

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Influenza type A virus-mediated adherence of pathogenic bacteria to the mucosal surface of the respiratory tract may be one of several mechanisms whereby influenza predisposes to bacterial pneumonia. In the present study, we quantified the adherence of intranasally administered type 1a group B streptococci to the tracheal tissue of influenza type A/PR8/34 (HONI) virus-infected and mock-infected mice. Influenza type A/PR8/34 virus infection effected a 120-fold increase in the adherence of type 1a group B streptococci to tracheal tissue relative to that observed in mock-infected mice. Adherence of type 1a group B streptococci to the trachea of influenza type A/PR8/34 virus-infected mice was reduced by more than 90% by prior intranasal instillation of chicken antiserum to influenza type A/PR8/34 virus, whereas virtually no reduction in adherence was noted with normal chicken serum or rabbit antiserum to herpes simplex virus type 2. These findings suggest that adherence of type 1a group B streptococci to the tracheal tissue of influenza type A/PR8/34 virus-infected mice is effected by a viral component(s).

Secondary bacterial pneumonia is the most frequent complication of influenza in humans (2, 10, 15). Although several theories have been proposed (3, 6, 8, 9), the mechanism(s) whereby influenza predisposes to secondary bacterial pneumonia is unknown.

Since it is known that adherence to host tissue is an essential step in bacterial infection of mucous membranes (1, 7, 14), a plausible hypothesis of influenza-induced predisposition to bacterial infections would be enhanced bacterial adherence. Sanford et al. (12) tested this hypothesis by using an in vitro system consisting of influenza type A/NWS/33 (HONI) virus-infected Madin-Darby canine kidney cells and various species of bacteria known to cause respiratory tract disease in humans or to be commonly found in the upper respiratory tract. Of the 18 species of bacteria tested, only *Streptococcus sanguis* and group B streptococci (GBS) adhered to the cytoplasmic membranes of influenza type A virus-infected cells but not to uninfected control cells. Of the five serotypes of GBS tested, three adhered, types 1a (strain 090), 1c, and II.

Although the in vitro finding of Sanford et al. (12) of influenza type A virus-induced adherence of GBS may lack direct clinical relevancy because influenza has not been shown to predispose to secondary GBS pneumonia in humans, it is nevertheless inherently relevant, for Wennerstrom and Menna (Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, B155, p. 40) have documented a lethal synergism between influenza type A/PR8/34 (HONI) virus (Flu-A) and type 1a GBS (strain 090) in mice, and, as reported herein, influenza type A/PR8/34 virus infection effects the adherence of type 1a GBS (strain 090) to tracheal tissue in mice.

Flu-A was used in all experiments. Stock preparations of Flu-A consisted of infectious chicken embryo allantoic fluid. Viral infectivity was quantified by conventional in ovo assay. Titers were calculated by the Reed and Muench method of calculating 50% endpoints (11) and were expressed as 50% egg infectious doses (EID_{50}) per milliliter. When used in experiments, stock virus maintained at −70°C was thawed and diluted in phosphate-buffered saline (PBS), pH 7.2, in such a way that 20 μl contained 100 EID_{50}.

GBS type 1a (strain 090), originally obtained from R. K. Lancefield, Rockefeller University, was grown in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.). At 8 to 10 h post-seeding, the culture was harvested by centrifugation at 10,000 × *g* for 10 min at 4°C. The cell pellet was then washed once with PBS and suspended in a small volume of Todd-Hewitt broth containing 15% (vol/vol) glycerine. The bacterial suspension was then dispensed into 0.5-ml volumes and frozen at −70°C until needed. When used in

experiments, a sample of the GBS was thawed (37°C) and grown in Todd-Hewitt broth at 37°C. Approximately 8 h later, the GBS were harvested by centrifugation at 10,000 × *g* for 10 min at 4°C. The cell pellet was then washed twice in PBS, and the final cell pellet was suspended in a volume of PBS yielding a nephelometric reading of 30 with a Klett-Sumerson photometer equipped with a no. 42 blue filter, a reading corresponding to approximately 10^8 colony-forming units (CFU) of GBS per ml. The suspension of GBS was then further diluted with PBS in such a way that 10^6 or 10^7 CFU of GBS was contained in a volume of 30 μl.

Thirty male BALB/c mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.), weighing 18 to 22 g each, were anesthetized with methoxyflurane (Abbott Laboratories, North Chicago, Ill.) and inoculated intranasally (i.n.) with 100 EID_{50} of Flu-A in 20 μl of PBS, using a Hamilton 50-μl syringe outfitted with a 27-gauge, 0.05-inch (1.27-cm) needle and a Hamilton repeating dispenser. Immunofluorescence studies carried out in our laboratory have shown that an input dose of 100 EID_{50} results in Flu-A infection of the trachea; five of five BALB/c mice were assayed. As a control, 10 mice were mock infected with an equal volume of PBS. Two days later, the Flu-A-infected mice were anesthetized with methoxyflurane, and 10 mice were inoculated i.n. with 10^6 CFU of type 1a GBS in 30 μl of PBS. As a control to assess adherence of endogenous bacteria, 10 mice were inoculated i.n. with 30 μl of PBS. The remaining Flu-A-infected mice were inoculated i.n. with 30 μl of a 1:10 PBS dilution of heat-inactivated (HI) (30 min at 56°C) chicken antiserum directed against Flu-A antigens (Flow Laboratories, Inc., Rockville, Md.). This antiserum was prepared with egg-propagated Flu-A as immunogen and infectious allantoic fluid was exhaustively absorbed with normal BALB/c mouse lung homogenate before use. This antiserum failed to induce staining of normal mouse trachea by indirect immunofluorescence assay. As a means of assessing the attachment of GBS to mouse tracheae in the absence of Flu-A infection, the 10 previously mock infected mice were inoculated i.n. with 10^6 CFU of type 1a GBS as described above. Thirty minutes later, all of the mice, with the exception of those that received the Flu-A antiserum, were sacrificed by cervical dislocation, and their tracheae were ablated in toto. Immediately after excision, each trachea was lavaged with 1 ml of cold sterile PBS, using a 3-ml syringe equipped with a 18-gauge, 1-inch (2.54-cm) needle. Each trachea was then homogenized in 1 ml of PBS, using a Ten Broeck grinder. Individual homogenates and lavage fluids were then serially diluted in PBS, and the number of CFU of GBS were determined on Todd-Hewitt agar plates after a 48-h incubation period at 37°C. Thirty minutes after the instillation of the chicken antiserum to Flu-A, the remaining mice were inoculated with 10^6 CFU of GBS as described above. After an additional 30 min, the mice were sacrificed by cervical dislocation, and their tracheae were ablated in toto, lavaged, homogenized, and assayed for GBS along with the lavage fluids as described above. The mean numbers of CFU per trachea in the Flu-A-infected group were 28,400 in mice inoculated with GBS, 3,940 in control mice inoculated with PBS, and 5,800 in mice inoculated with anti-Flu-A and GBS. In mice mock infected with PBS and inoculated with GBS, the mean number of CFU per trachea was 203. Differences in mean numbers of CFU in individual mice in each group relative to those of Flu-A-infected, GBS-inoculated mice were analyzed by the rank sum test (4), with $P < 0.05$ considered significant ($P < 0.001$ for all of the above-described groups). Flu-A antiserum reduced the adherence of type 1a GBS by 92%, as determined by the following formula: 100% − {[(mean number of CFU of mice receiving Flu-A, anti-Flu-A, and GBS) − (mean number of CFU of mice receiving Flu-A and PBS)] ÷ [(mean number of CFU of mice receiving Flu-A and GBS) − (mean number of CFU of mice receiving Flu-A and PBS)]} = percent reduction in CFU. Taking into account that approximately 14% of the CFU detected in the tracheae of Flu-A-infected, GBS-inoculated mice represented endogenous bacterial flora by the formula [(CFU of Flu-A-infected, PBS-inoculated mice)/(CFU of Flu-A-infected, GBS-inoculated mice)] × 100, it can be seen that Flu-A infection effected a 120-fold increase in the adherence of GBS to tracheal tissue relative to the mock infected GBS-inoculated mice. Instillation of chicken antiserum to Flu-A antigens reduced the level of enhanced GBS adherence by 92%, suggesting that adherence was viral mediated. No significant differences were observed in the number of CFU recovered in the lavage fluids, irrespective of whether the mice were virus infected or mock infected (data not shown).

As a means of confirming that our CFU counts represented primarily GBS, colonies isolated from the tracheae of Flu-A-infected, GBS-inoculated mice were randomly sampled and identified with the API-20S system (Analytab Products, Plainview, N.Y.). Of 25 sampled colonies, 88% were GBS, a finding consistent with the observation that 14% of the CFU of Flu-A-infected mice represented endogenous flora. Thus, most of the bacteria that adhered to tracheal tissue were GBS.

To further document that enhanced adherence

TABLE 1. Effect of chicken antiserum to Flu-A, normal chicken serum, and rabbit antiserum to herpes simplex virus type 2 on the adherence of GBS to Flu-A-infected mouse trachea

Group[a]	Mean no. of CFU of bacteria per trachea (five mice)	% Reduction[b]	P[c]
Flu-A, PBS, GBS	154,000	—	—
Flu-A, anti-Flu-A, GBS	13,400	94	< 0.001
Flu-A, normal chicken serum, GBS	134,000	13	NS
Flu-A, anti-HSV-2, GBS	170,000	0	NS

[a] Twenty male BALB/c mice were inoculated i.n. with 100 EID_{50} of Flu-A; 48 h later, five mice received either PBS, a 1:10 PBS dilution of HI chicken antiserum to Flu-A, a 1:10 PBS dilution of HI normal chicken serum, or a 1:10 PBS dilution of HI rabbit antiserum to HSV-2 strain MS. See text for details of experimental protocol.

[b] Percent reduction in CFU was determined using the following formula: 100% − {[(mean CFU of test group) − (mean CFU of mice receiving Flu-A and PBS)] ÷ [(mean CFU of mice receiving Flu-A, PBS, and GBS) − (mean CFU of mice receiving Flu-A and PBS)] ×100} = percent reduction in CFU.

[c] Differences in CFU of individual mice in antiserum-treated and normal chicken serum-treated group relative to those of individual mice receiving Flu-A, PBS, and GBS were analyzed by the rank sum test (4), with $P \leq 0.05$ considered significant. NS, Not significant.

of type 1a GBS to tracheal tissue was owing to a Flu-A component(s) acting, presumably, as a receptor, the experiment was repeated with essentially the same experimental protocol. In this experiment, however, an input dose of 10^7 CFU, rather than 10^6 CFU, of type 1a GBS was employed, and two additional control groups were assayed; a Flu-A-infected group receiving HI normal chicken serum before i.n. instillation of GBS and a group receiving HI rabbit antiserum to herpes simplex virus type 2 (strain MS; a gift from M. Ito, Roswell Park Memorial Institute, Buffalo, N.Y.), before i.n. instillation of GBS. Mice receiving Flu-A alone or GBS alone were not assayed (Table 1). Instillation i.n. of antibody to Flu-A effected a 94% reduction in the adherence of type 1a GBS to the tracheae of Flu-A-infected mice, whereas normal chicken serum and rabbit anti-herpes simplex virus type 2 antiserum failed to significantly reduce the adherence of GBS; reduction was 13 and 0%, respectively.

These findings demonstrate that adherence of type 1a GBS strain 090 to tracheal tissue in vivo is significantly enhanced as a function of Flu-A infection and are consistent with the in vitro findings of Sanford et al. (12, 13) on the enhanced attachment of type 1a GBS strain 090 to influenza type A virus-infected Madin-Darby canine kidney cells relative to mock infected cells. In addition, the findings reported herein suggest that adherence of GBS to tracheal tissue is mediated by a cell surface-associated Flu-A component(s), for adherence was significantly reduced by prior instillation of homologous antiviral antiserum but not heterologous antiviral antiserum. This observation is consistent with the finding of Sanford and co-workers (13) that adherence of GBS to influenza type A/NWS/33 (HONI) virus-infected Madin-Darby canine kidney cells is significantly reduced by incubation with homologous antiviral antibody, but not heterologous antiviral antibody (antibody to herpes simplex virus type 2) before the addition of GBS.

Although the nature of the influenza type A virus receptor(s) involved in the attachment of GBS is unknown, studies by Sanford and Ramsay (Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, B153, p. 40) in which type 1c GBS and influenza A/NWS/33 (HONI) virus-infected Madin-Darby canine kidney cells were used suggest that it is the galactose side-chains of the viral hemagglutinin. Using the same virus-host cell system and type 1a GBS strain 090, Sanford and colleagues (13) have shown that bacterial adherence is negated by prior treatment of the bacteria with *Vibrio cholerae*-derived, receptor-destroying enzyme (neuraminidase). This finding suggests that the sialic acid group on the type-specific polysaccharide antigen (5) is acting as the adhesin in this system.

Studies are currently being carried out in our laboratory with mouse tracheal organ cultures to identify the Flu-A receptor and the type 1a GBS adhesin involved in adherence.

This study is the first, to our knowledge, to show that adherence of GBS to tracheal tissue in vivo is potentiated by Flu-A infection and that adherence is probably due to a viral component(s). These findings have important implications, for infections with influenza type A virus could effect the attachment of endogenously or exogenously acquired pathogenic bacteria to respiratory tract epithelia, leading to colonization and, in some cases, to secondary bacterial pneumonia as a function of a descending infection.

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