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Bacterial Adherence to the Upper Respiratory Tract of Ferrets Infected with Influenza A Virus (42525)

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Abstract. A ferret model was used to study bacterial adherence in animals with influenza. Ferrets were inoculated intranasally with influenza A3/Hong Kong/1/68 virus. Antiviral serum antibodies were apparent by Day 5. On Days 3, 5, 7, 9, and 11, three virus-inoculated and two uninoculated controls were anesthetized, exsanguinated, and decapitated, and the lower jaw was removed. Each animal was inoculated intranasally with a 1-ml suspension containing 20 mg (dry wt) of either ^{3}H-labeled *Staphylococcus aureus* or ^{3}H-labeled group B *Streptococcus* type Ia and incubated for 45 min at ambient temperature. In animals challenged with staphylococci, 80% of the original inoculum remained free in suspension; of the remaining 20%, the distribution in the upper respiratory tracts of virus-infected and control animals was significantly different. Of the staphylococci remaining in the nasopharynx of control animals, 74% was present in mucinous plugs, 11% was bound to host cells present in washes of the nasal cavity, and 15% was released by protease treatment of the nasopharynx. Of the staphylococci remaining in the upper respiratory tract of virus-infected ferrets, 36% was recovered in plugs, 24% was bound to cells in nasal washes, and 40% was released by enzyme treatment. Overall, adherence-positive staphylococci represented 64% of recoverable bacteria in virus-infected ferrets versus 26% in controls. Adherence was increased twofold (Days 5 and 7) to threefold (Days 3, 9, and 11) in virus-infected ferrets compared to uninfected controls. In contrast, only 7% of the original streptococcal inoculum was recovered from virus-infected and uninfected control animals and virus infection did not enhance streptococcal adherence except for an approximately threefold increase that was seen on Day 11.

Influenza alone is rarely lethal, but it promotes secondary bacterial infections, particularly with *Staphylococcus aureus,* that are often fatal (1). It is evident from reports published during the past decade (2–8) that influenza virus infection significantly enhances the adherence of specific bacteria in *in vitro* model systems, suggesting a possible mechanism for the synergistic interaction between influenza virus and bacterial respiratory pathogens during epidemics of influenza. The study by Mulder and Hers (9) of 176 cases of *S. aureus* pneumonia and bronchitis in suspected influenza deaths inadvertently supports the view that this mechanism is also operational *in vivo.* They observed that staphylococcal infection follows the trail laid for it by the spread of virus along the respiratory tree and that staphylococcal infection apparently occurs only in virus-infected epithelium and not in normal epithelium immediately surrounding the infected area.

The phenomenon of virus-induced enhanced staphylococcal adherence has yet to be confirmed in an animal model system. For this purpose, we elected to study the phenomenon in the ferret (*Mustela putorius furo*) because influenza in ferrets resembles as closely as possible the well-documented clinical picture seen in humans (10). Considering the superiority and extensive use of the ferret in influenza research, it is difficult to understand why this animal has been used as a model for viral–bacterial synergism in only two studies published over 40 years ago. In 1935, Brightman (11) observed that group C streptococcal infections could not be produced in ferrets by intranasal inoculation alone. He also observed that ferrets inoculated with influenza virus alone had only a mild illness. However, when both the virus and the streptococci were given simultaneously, severe synergistic infection resulted. These results were confirmed in a subsequent study by Glover in 1941 (12). It is generally assumed that during epidemics of influenza, bacterial superinfection is endogenous; that is, the patient is the carrier of the infecting bacterium. However, Glover was able

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to show that group C streptococci could be acquired via aerosol by influenza A virus-infected ferrets but not by normal ferrets.

In ferrets, the pattern of infection after nasal instillation of human influenza viruses is similar to that in humans, mainly an infection of the upper respiratory tract with only rare lung involvement and viremia (10). Consequently, in the present study, we concentrated on determining what effect influenza virus infection of the upper respiratory tract had on the adherence and distribution of two exogenous bacteria—*S. aureus* and group B *Streptococcus* type Ia. In part, these two particular bacteria were selected because previous studies (2, 5, 7, 13–15) indicate that they adhere to virus-infected cells *in vitro* by different host cell receptors.

Materials and Methods. *Virus.* Influenza A/Hong Kong/1/68 virus (H3N2) was used to inoculate ferrets. The virus, kindly supplied by Vee E. Davison (Chief of Virology Section of the U.S. Air Force Epidemiology Division, Brooks Air Force Base, San Antonio, TX) was propagated in the allantoic cavities of embryonated chicken eggs. Subtype of the virus strain was confirmed by the hemagglutination inhibition (HI) test (16) using reference-typing antisera. Virus infectivity was titrated in Madin–Darby canine kidney cells, grown to confluency in 24-well Mark II tissue culture clusters (Costar, Cambridge, MA) as previously described (17), and the $TCID_{50}$ was calculated by the method of Reed and Muench (18).

Ferrets. Fifty 12-week-old unvaccinated female ferrets were obtained from Marshall Farms (North Rose, NY). Animals were observed for at least 10 days before being used for experiments. Preinoculation sera were obtained from each animal via the caudal artery (19). Thirty animals were anesthetized by intramuscular injection with 30 mg of Vetalar (Ketamine hydrochloride, Parke-Davis, Morris Plains, NJ) and 0.4 mg of Rompun (xylazine, Miles Laboratories, Inc., Shawnee, KS) and inoculated intranasally with 3×10^6 $TCID_{50}$ of virus contained in 1 ml of allantoic fluid. Five animals (three virus-inoculated and two controls) were anesthetized and then sacrificed by exsanguination via cardiac puncture on Days 3, 5, 7, 9, and 11. Blood specimens were allowed to clot and sera were collected for further testing. Each animal was decapitated, the lower jaw was removed, and the head was placed in a sterile petri dish. The anterior nares of each animal were swabbed, inoculated onto blood agar, and incubated at 37°C for 24 hr. Heads were immediately used in the radioassay system with one of the test bacteria.

Radiolabeling of bacteria. Two test strains of bacteria were used in the present study—*Staphylococcus aureus* and group B *Streptococcus* type Ia. The *S. aureus* strain (Ci-1) was isolated from a sputum specimen of a patient who died from postinfluenzal staphylococcal pneumonia; the phage type, phenotypic characteristics, and hydrophobicity of Ci-1 have been reported previously (20). In addition, the Ci-1 strain possessed a type 8 polysaccharide capsule based on serological typing kindly provided by W. W. Karakawa (Pennsylvania State University, University Park, PA). *S. aureus* was labeled with [methyl-^{3}H]thymidine (15 Ci/mmole; ICN Chemical and Radioisotope Div., Irvine, CA) as previously described (17) and freeze-dried for storage. Group B *Streptococcus* type Ia, prototype strain 090 (21), was also labeled with [methyl-^{3}H]thymidine as previously described (8) and stored frozen at −70°C in sterile deionized water (104.5 mg [dry wt] of streptococci per vial).

Radioassay. A 100-mg amount of the freeze-dried labeled staphylococci was suspended in 5 ml of Waymouth's MB 752/1 medium with L-glutamine (Flow Laboratories, Inc., McLean, VA) and 0.01% $NaHCO_3$; a 1-ml volume of the bacterial suspension was dispensed into each of five microcentrifuge tubes (West Coast Scientific, Inc., Emeryville, CA). The tubes were centrifuged in an Eppendorf Model Mark 5414 microcentrifuge (Brinkman Instruments, Inc., Westbury, NY) for 10 sec and drained, and each pellet (20 mg [dry wt]) was suspended in 1 ml of medium. One vial of frozen labeled streptococci was thawed, and equal volumes were dispensed into each of five microcentrifuge tubes. After centrifugation the pellets were washed once in medium, centrifuged, and drained, and each pellet (20 mg [dry wt]) was suspended in 1 ml of medium. A 1-ml volume of ^{3}H-labeled staphylococci or ^{3}H-labeled streptococci was used to inoculate the nasal cavity of each of the heads obtained from three virus-infected and two control ferrets; a tuberculin syringe, with an attached 25-gauge $\frac{5}{8}$-in. needle (Bec-

ton–Dickinson, Rutherford, NJ) inserted approximately 4 mm into the anterior nares, was used to slowly and gently dispense 500 μl of the bacterial suspension into each side. After inoculation, each head was gently rotated at frequent intervals during an incubation period of 45 min at ambient temperature.

At the end of the incubation period, the tracheal remnant was retracted to expose the distal portion of the nasopharynx and the presence of any bacterial "plug" formed during incubation. If a plug was present, it was carefully removed to a vial containing 1 ml of H_2O and dispersed with a Pasteur pipet. A 7-ml amount of ScintiVerse I scintillation cocktail (Fisher Scientific, Fair Lawn, NJ) was added to each vial, and radioactivity was measured in a Beckman LS 7800 liquid scintillation spectrometer. The cpm were converted to micrograms of bacteria by the following method: radioactivity of 5 μl of the test bacterial suspension, which contained 100 μg (dry wt) of labeled bacteria, was determined; cpm obtained were then divided by 100 to give counts per minute per microgram of bacteria (specific activity). The resultant factor (cpm) was used as the denominator in converting the cpm of each test specimen (numerator) to micrograms of labeled bacteria. In some ferrets, a 10-μl volume of the plug suspended in H_2O was used to make a smear that was subsequently stained with hematoxylin and eosin (H&E). Photomicrographs were made using Kodak EPY film.

The nasal cavity and nasopharynx were vigorously washed twice with 10-ml volumes of Waymouth's MB 752/1 medium (total of 20 ml) introduced through the anterior nares (5 ml per side per wash) and collected from the tracheal remnant into a tube. The tube contained host cells, free labeled bacteria, and labeled bacteria bound to host cells. Host cells, with and without bound labeled bacteria, were separated from unbound labeled bacteria by differential centrifugation at 1300 rpm for 3 min; the supernate was discarded and the cell pellet was repeatedly washed with medium until shown to be free of unbound bacteria by microscopic examination. The pellet was suspended in 1 ml of H_2O and processed for determination of radioactivity as described above. In addition, the total number of host cells present in the wash was determined by counting in a blood cell hemocytometer after staining with trypan blue vital dye and a smear was stained by H&E.

In order to remove labeled bacteria that remained bound in the nasal cavity after washing, a 2-ml volume of 0.125% protease (repurified type VI obtained from *Streptomyces griseus;* Sigma, St. Louis, MO) diluted in PBS was slowly added to the nasal cavity via the anterior nares (1 ml per side) and allowed to incubate for 45 min at ambient temperature. Again the nasal cavity was washed with medium (6-ml volume per side) and the released bacteria and host cells were collected. After centrifugation, the supernate was discarded and the pellet was suspended in 0.5 ml of H_2O and transferred to a scintillation vial. The tube was rinsed with 0.5 ml of H_2O which was also added to the vial. Scintillation cocktail was added as described above and the radioactivity of enzyme-released labeled bacteria was measured.

Serology. Each serum was pretreated with kaolin to remove nonspecific inhibitors and antibodies against the infecting Hong Kong strain of influenza A virus were titrated by HI (16).

Chemical determinations. The presence of C-reactive protein (CRP) was determined by the latex agglutination assay according to the instructions of the manufacturer (Difco Laboratories, Detroit, MI). Total serum protein and total albumin were determined by the clinical chemistry laboratory (Department of Pathology, Medical Center Hospital, San Antonio, TX) using the Sequential Multiple Analyzer Computer.

Results. Nasal inoculation of ferrets with the Hong Kong strain of virus caused a mild upper respiratory tract illness that was characterized by lethargy and sneezing. Symptoms lasted for only a few days after which time the animals appeared normal. Tests for the presence of CRP were negative in all of the serum specimens. Mean serum concentrations of albumin for control animals was 2.88 g/dl ± 0.32 ($SD\bar{x}$) versus 3.03 g/dl ± 0.24 ($SD\bar{x}$) for virus-inoculated animals; mean values for total serum proteins were 5.21 g/dl ± 0.29 ($SD\bar{x}$) for control animals and 5.15 g/dl ± 0.20 ($SD\bar{x}$) for virus-inoculated animals. Albumin concentrations represented $\bar{x}$54% of the total

serum proteins in control ferrets and $\bar{x}$59% in virus-inoculated ferrets. All serum values for the virus-inoculated ferrets were within 1 SD$\bar{x}$ of control values. Seroconversion was the only reliable indicator of virus infection. Serum antibody titers against the homologous infecting Hong Kong strain of influenza A virus were <10 (lowest dilution tested) in control uninoculated animals and remained <10 in animals 3 days postinoculation with virus. In virus-inoculated ferrets, the titers increased to 20 (Day 5), 80 (Day 7), 160 (Day 9), and 320 (Day 11).

Nasal specimens taken immediately after each animal was sacrificed were culture negative for both *S. aureus* and β-hemolytic streptococci. ^{3}H-labeled *S. aureus* and group B *Streptococcus* type Ia were injected into the nasal cavity of control and virus-inoculated ferrets; during the incubation period, plugs were formed in the nasopharynx as seen in Fig. 1. These plugs were comprised of numerous cocci bound to strands of mucinous material as seen in Fig. 2A. Following removal of the plug, labeled bacteria were washed from the nasal cavity; host cells, with and without bound bacteria (Fig. 2B), were separated from free unbound bacteria. The remaining adherence-positive labeled bacteria were harvested from the nasal cavity by enzyme treatment. Results are shown in Fig. 3. The adherence of ^{3}H-labeled *S. aureus* in virus-infected ferrets was significantly increased when compared to adherence in control ferrets. The enhanced adherence was not uniform across the five time periods tested. An approximately threefold increase was seen on Days 3, 9, and 11; adherence was increased approximately twofold on Days 5 and 7. There was no direct correlation between enhanced adherence and the appearance of specific antiviral serum antibodies. As also seen in Fig. 3, radioassay results

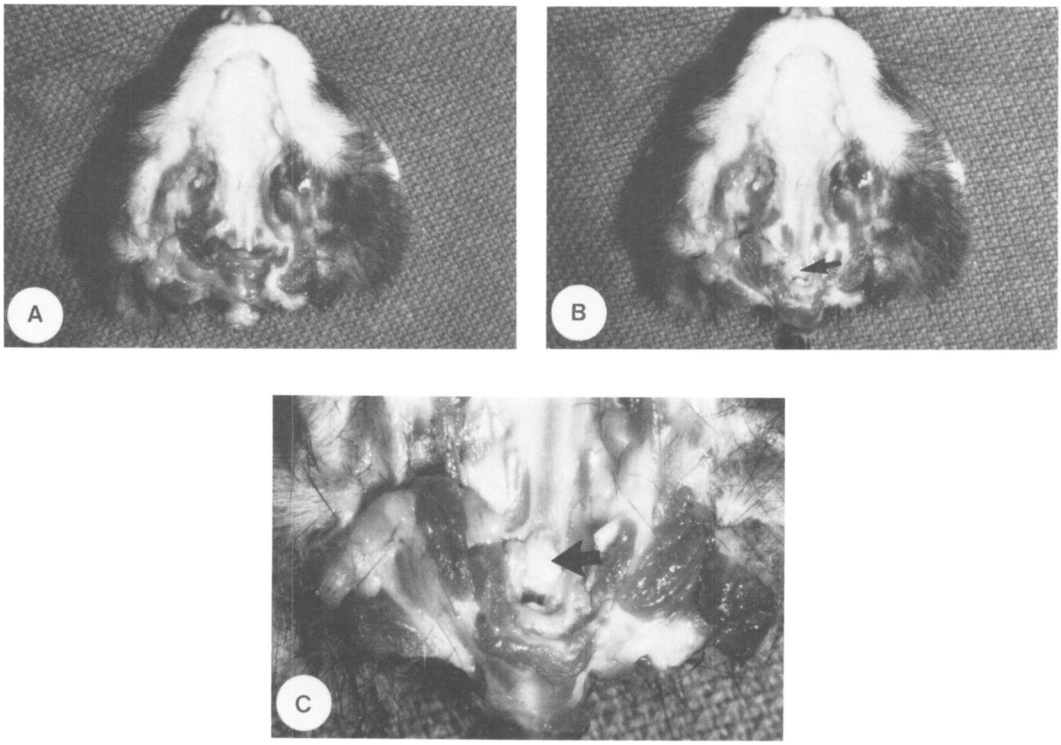

FIG. 1. Ventral view of a ferret head after removal of the lower jaw and inoculation of the anterior nares with a suspension of ^{3}H-labeled *Staphylococcus aureus* (A). During the incubation period, a mucinous plug (arrow) containing labeled bacteria formed in the nasopharynx and was exposed by retraction of the epiglottis (B and C).

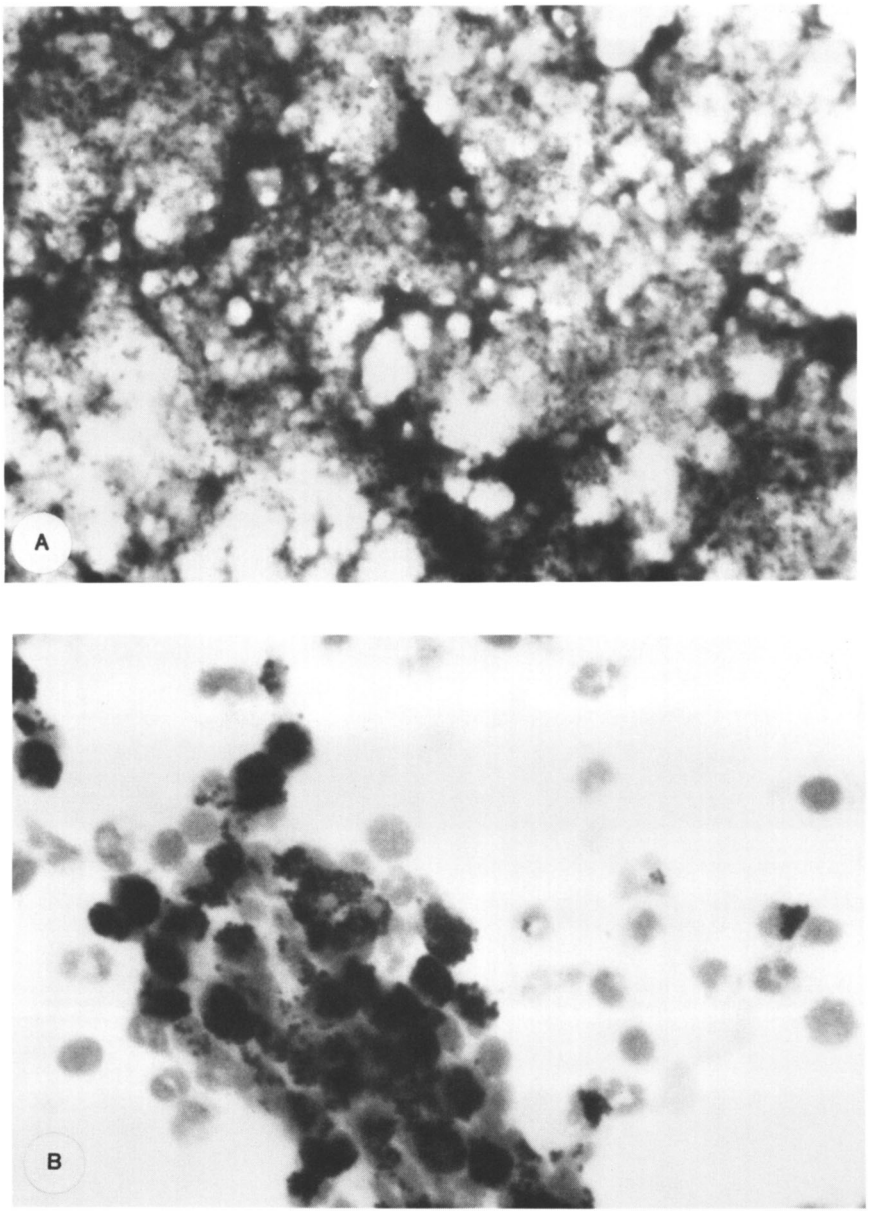

FIG. 2. Photomicrograph of a stained smear made from the plug harvested from the nasopharynx of a ferret inoculated with ^{3}H-labeled *Staphylococcus aureus* (A). The plug was comprised of a dense network of mucinous strands loaded with cocci and apparently free of host cells. Photomicrograph showing numerous staphylococci attached to host cells from the upper respiratory tract of the same animal (B). The host cells were collected in the nasal washes and separated by differential centrifugation from free unbound staphylococci. Also in (B), note the presence of segmented neutrophils with and without bound staphylococci. (both, H&E, ×500).

obtained with *S. aureus* were strikingly different from results obtained from animals receiving group B streptococci. While data from both sets of controls were similar, it is obvious that virus infection did not enhance streptococcal adherence except for an approximately

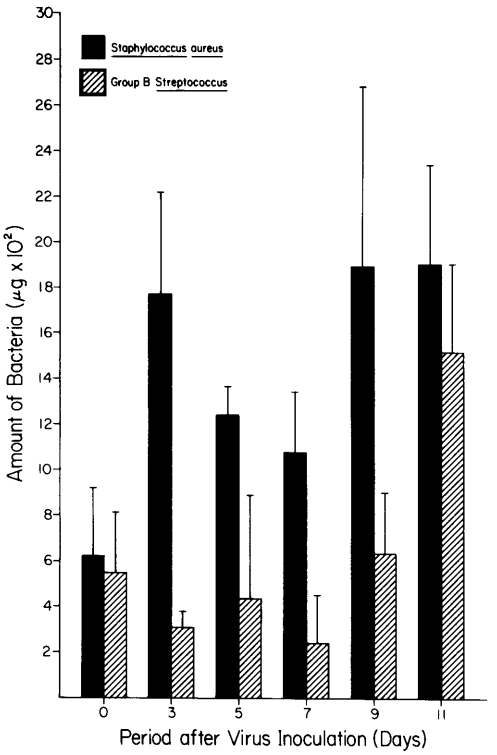

FIG. 3. Mean adherence of ^{3}H-labeled *Staphylococcus aureus* (closed bar) and ^{3}H-labeled group B *Streptococcus* type Ia (striped bar) to cells in the nasal cavity of ferrets either uninoculated (0 period) or postinoculation with influenza A/Hong Kong/1/68 virus. The bacteria were released only after enzyme treatment of the nasal cavity. Thin bars represent the standard deviation of results with triplicate testing (Days 3 through 11) and with 10 control animals in each test set at the 0 period.

threefold increase that was seen on Day 11. In fact, streptococcal adherence was decreased over twofold on Day 7.

The overall distribution of recoverable staphylococci and streptococci from the two groups of ferrets (uninfected and virus-infected) is shown in Fig. 4. By definition, recoverable bacteria included the ^{3}H-labeled bacteria that were (i) present in plugs, (ii) attached to host cells present in the nasal washes, and (iii) released by enzyme treatment. Each ferret represented in Fig. 4 was initially inoculated with 20 mg of radiolabeled bacteria. In ferrets receiving staphylococci, we recovered a mean of 20% (about 4 mg) of the original inoculum from each of the control and virus-infected animals which means that 80% of the staphylococci remained free in suspension and was removed during the nasal wash step. In contrast, only 7% (about 1.4 mg) of the original streptococcal inoculum was recovered. The overall distribution of ^{3}H-labeled *S. aureus* in control ferrets (Fig. 4A) shows that 74% (mean of 2.894 mg) of the recovered bacteria was present in plugs compared with 54% (mean of 0.908 mg) for the streptococci (Fig. 4B).

During the nasal wash step, 15–20 $\times$ 10^9 nasopharyngeal cells were released into the medium. Of the total recovered staphylococci, 24% was bound to host cells present in the nasal washes of virus-infected ferrets versus 11% in washes from the uninfected control group (Fig. 4A). Similar results were seen when enzyme treatment of the nasopharynx was used to release the remaining adherent bacteria. Of the total recoverable staphylococci, 40% was released in virus-infected ferrets versus only 15% in uninfected control ferrets (Fig. 4A). Results of determining the mean total amount of host cell-bound staphylococci (staphylococci bound to host cells in the nasal wash plus adherence-positive staphylococci released by enzyme treatment) indicated that 64% of the staphylococci recovered from virus-infected ferrets was host cell associated versus only 26% from uninfected control ferrets (Fig. 4A). As shown in Fig. 4B, 78% of the recoverable streptococci in virus-infected animals was bound to host cells versus 46% in control uninoculated animals. However, the mean values were not significantly different (0.936 mg versus 0.778 mg, respectively).

Discussion. In the present study, we used a ferret model system to determine the effect of influenza A virus infection on the adherence of *S. aureus* and group B streptococci. The tissue specificity for influenza A virus in ferrets is similar to that in humans. We focused on the upper respiratory tract (nasal cavity and nasopharynx) rather than the lower respiratory tract (larynx, trachea, and bronchial tree) because the nasal mucosa is the tissue most susceptible to virus infection (10) and the infection is primarily a rhinitis. In natural combined infections, the virus-infected nasal cavity is also the most likely area of the respiratory tract in which the early events leading to bacterial superinfection are initiated. *S. aureus* was selected as a test strain because of its predominant clinical association with influenza.

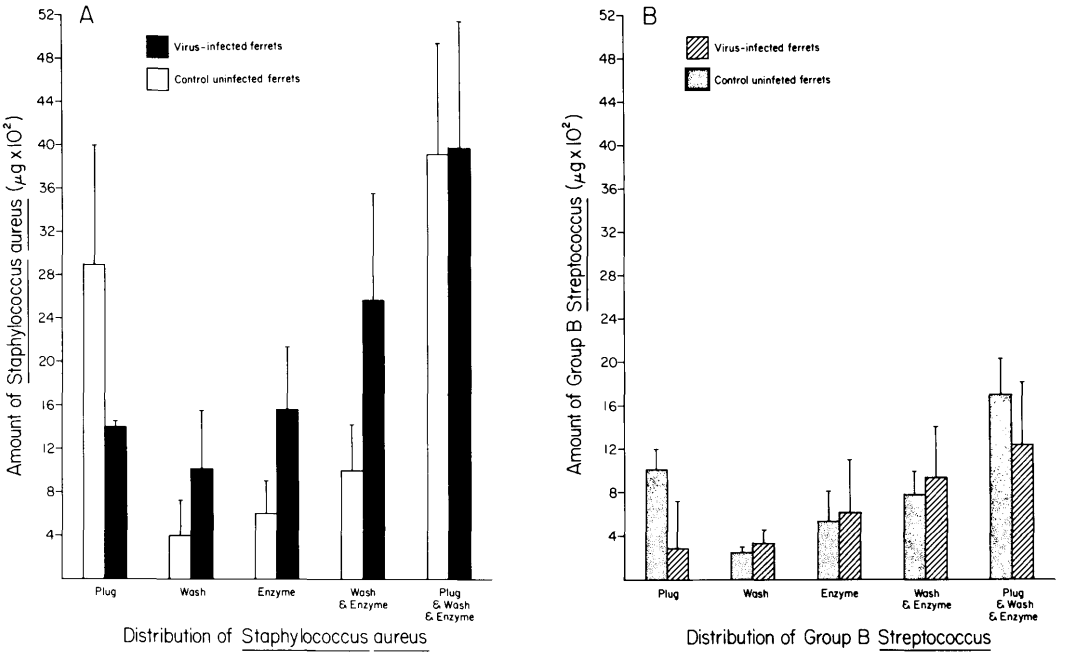

FIG. 4. Mean distribution of ^{3}H-labeled *Staphylococcus aureus* (A) and ^{3}H-labeled group B *Streptococcus* type Ia (B) recovered: in the mucinous plugs retrieved from the nasopharynx, attached to nasopharyngeal cells collected in the nasal washes, and released by enzyme treatment of the nasal cavity of control uninfected ferrets and ferrets infected with influenza A/Hong Kong/1/68 virus. Total of bound bacteria is the sum of bacteria adherent to host cells in the nasal washes plus adherent bacteria released only after enzyme treatment. Total recoverable bacteria is the sum of bacteria in the plug plus adherence-positive bacteria in the nasal washes and released by enzyme treatment. Thin bars represent the standard deviation of results from the two sets of control animals (10 animals per set) and the two sets of virus-infected animals (15 animals per set). For direct comparison, the scale is the same for both (A) and (B).

No association has yet been reported in the medical literature between influenza virus and group B streptococci; however, such an association has been demonstrated *in vivo* (23) and *in vitro* (2, 13, 14). In addition type Ia seems to preferentially localize in the respiratory tract of humans (22). In our model system, we used the Hong Kong strain of influenza A virus because clinical infection with this virus has been associated with staphylococcal superinfection (1).

Results from our study indicated that the distribution of staphylococci and group B streptococci in control uninfected animals differed appreciably in only one respect. The amount of staphylococci trapped and removed in mucinous plugs was 3-fold greater than the streptococci. The adherence to host cells was basically the same (Fig. 4). In fact, the amount of adherence-positive streptococci in virus-infected animals was also the same as in the two control groups. These results are in contrast with those of Jones and Menna (23) who showed that influenza A virus infection of mice effected a 120-fold increase in the adherence of type 1a group B streptococci to trachea compared to uninfected controls; the enhanced adherence in virus-infected animals was reduced by 90% in animals pretreated with specific antibody against the infecting virus. The difference in results between our study and that of Jones and Menna may reflect the fact that different animals models and different strains of influenza A virus were used and different areas of the respiratory tract were studied.

Of the four experimental groups studied, only one showed significantly enhanced adherence—the virus-infected ferrets challenged with *S. aureus* (Fig. 4). Adherence was in-

creased about threefold over that seen in control animals and in virus-infected animals challenged with group B streptococci. Once again, these results are in contrast to those reported by Nugent and Pesanti (24) in which control and influenza A virus-infected (Day 7) mice were exposed to aerosols of *S. aureus* and no significant difference in the number of staphylococci deposited on the proximal trachea was observed between the two groups. They did, however, report that the numbers of staphylococci remaining in virus-infected mice 3 to 6 hr after exposure were significantly higher compared with controls. It is interesting to note, in the present study, that the amount of recoverable staphylococci was also the same in both control and virus-infected ferrets; however, the distribution was significantly different. If trapping the staphylococci in mucinous strands, either specifically or nonspecifically, constituted a potential clearance mechanism, then the control ferrets were twice as efficient at clearing the organisms than virus-infected ferrets.

We also examined the adherence of staphylococci and streptococci in the upper respiratory tract of ferrets at various times after inoculation with virus. In these experiments, we concentrated on those organisms that were released from host cells only after enzyme treatment of the nasal cavity (Fig. 3). The ratio of staphylococcal adherence to streptococcal adherence was 5.7 (Day 3), 2.8 (Day 5), 4.5 (Day 7), 2.9 (Day 9), and 1.2 (Day 11, the only time we saw a significant increase in group B streptococcal adherence). From detailed descriptions of the nasal histology of influenza in ferrets by Francis and Stuart-Harris (25) and by Hotz and Bang (26) and of the distribution of viral antigen in the nasal mucosa by Liu (27), it is obvious that numerous changes occur at the level of the mucous membrane over time, ranging from local foci of virus infection, cell degeneration and destruction, and repair. Our data suggest that the changes that occur as a result of virus infection favor the specific adherence of *S. aureus* over group B streptococci. These two organisms have been shown to adhere to virus-infected cells, *in vitro,* by different mechanisms. *S. aureus* appears to have multiple adhesins on its surface capable of interacting with multiple receptors on the external surface of the plasma membrane of virus-infected cells (15, 17). In addition to these "virus-induced" receptors, virus-infected cells may also acquire receptors from the surrounding milieu. For example, *in vitro* studies indicate that virus-specific antibody (28) and fibrinogen (7, 8) act to enhance staphylococcal adherence by binding the cocci directly to the surface of influenza virus-infected cells. In the ferret model, antiviral serum antibodies increased substantially between Days 5 and 11. If these antibodies appeared in the upper respiratory tract of infected animals, they may have acted as "acquired" receptors and contributed to enhanced staphylococcal adherence. In contrast, sialic acid on the surface of group B *Streptococcus* type Ia acts as the adhesin that mediates binding to virus-specific hemagglutinin expressed on the surface of virus-infected cells (2, 13, 14). Adherence of these streptococci is actually blocked by the presence of virus-specific antibody (2) and is unaffected by the presence of fibrinogen (8). These studies suggest that virus infection may promote staphylococcal adherence simply because these organisms are equipped with more potential adhesins, unlike the group B streptococci in which sialic acid radicles present in nasal mucous might competitively inhibit the binding of these organisms to the hemagglutinin receptors present in virus-infected respiratory cells (29, 30).

In summary, our data demonstrate the potential usefulness of the ferret model system in elucidating the sequence of events resulting ultimately in postinfluenzal bacterial superinfection. In addition, the data suggest the importance of studying the mechanism(s) of respiratory viral–bacterial synergism in the cells and tissues of the upper respiratory tract, particularly when the object of a study is to determine the earliest steps in synergistic interactions between virus and bacteria.

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