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Lethal Synergism Induced in Mice by Influenza Type A Virus and Type Ia Group B Streptococci

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Intranasal inoculation of CD-1 or BALB/c mice with low doses of influenza A/PR8/34 (HON1) virus followed 48 h later by intranasal inoculation of low doses of type Ia group B streptococci effected a lethal synergism. At a constant input dose of virus, a direct relationship between input dose of bacteria and percent mortality was observed; the converse was also true. An inverse relationship between input dose of group B streptococci, but not input dose of virus, and mean time to death was observed in CD-1 but not in BALB/c mice. The kinetics of influenza A/PR8/34 virus and group B streptococcal replication in singly and dually infected BALB/c mice was determined by assaying samples from the lungs, liver, spleen, and blood for viable group B streptococci and infectious influenza A/PR8/34 virus. No significant difference in virus replication in the lung was observed between singly and dually infected mice. Extrapulmonary dissemination of virus was not observed. Concurrent virus infection effected a 10,000- to 100,000-fold increase in the levels of type Ia group B streptococci in the lung. Potentiation of group B streptococcal infection of the lung was not associated with bacteremia or infection of the liver or spleen, a finding contrary to previous observations of fulminant septicemia after intranasal inoculation of mice with input doses of group B streptococci less than one-tenth of the pulmonary levels observed in the present study.

Bacterial pneumonia as a complication of influenza is a well-documented phenomenon (16), but its epidemiological basis is presently unknown. The putative predisposing factors include diminution of phagocytic cell activity (1, 2, 9, 13) and virus-induced adherence of bacteria to respiratory mucosa (6). In vitro studies by Sanford and co-workers (14, 15) documented that influenza type A virus-infected cells possess a virus gene-coded cell surface receptor(s) that mediates the attachment of types Ia, Ic, and II group B streptococci (GBS). This in vitro finding was confirmed in part by Jones and Menna (6) in vivo using influenza type A virus-infected mouse tracheal tissue and type 1a GBS.

Wennerstrom (17) documented that type Ia GBS strain 090 inoculated intranasally (i.n.) into adult mice induces a lethal infection. Of interest was the finding (17) that this lethal infection was associated with an initial low-grade pulmonary infection and escape of GBS from the lung, followed by fulminant septicemia accompanied by levels of GBS in the lung in excess of those observed in the blood.

When mice are inoculated i.n. with lethal doses of influenza A/PR8/34 (HON1) virus (influenza A), a fulminant pneumonia characterized by marked inflammation, lung consolidation, and extensive pulmonary viral replication occurs (8). In contrast to the pathogenesis of type Ia GBS-induced pneumonia in mice, influenza virus-induced pneumonia generally is not associated with infection of extrapulmonary tissues.

These studies were carried out to ascertain the effect of concurrent infection with low input doses of influenza A and type Ia GBS in mice and to characterize the effect by defining the kinetics of replication of each agent in singly and dually infected mice.

MATERIALS AND METHODS

Virus. Influenza was used in all experiments. Stock preparations of influenza A consisted of infectious chicken embryo allantoic fluid. Viral infectivity was quantified by a conventional in ovo assay. The Reed and Muench method was used to calculate 50% endpoints (12), and virus titers are expressed as 50% egg infectious doses (EID_{50}) per milliliter. Stock virus was frozen at −80°C until needed, at which time it was rapidly thawed at 37°C and diluted appropriately in phosphate-buffered saline (PBS), pH 7.2.

Bacteria. Type Ia GBS strain 090, obtained from R. K. Lancefield, Rockefeller University, New York, was serially passaged in BALB/c mice as an infectious

spleen homogenate by the method of Lancefield et al. (7, 18). Before each experiment, a sample of the stock GBS spleen homogenate frozen at −80°C, was thawed and cultivated in Todd-Hewitt broth (DFCO Laboratories, Detroit, Mich.) at 37°C. After 12 h, the GBS were harvested by centrifugation at 10,000 × *g* for 10 min at 5°C. The harvested GBS were suspended in PBS to a concentration of 10^8 CFU, determined nephelometrically (18), and then further diluted in PBS to obtain the appropriate concentrations for the mouse inoculations. In each experiment, CFU of GBS were enumerated to confirm the calculated input doses (17).

Inoculation protocol. Adult male and female CD-1 outbred mice (20 to 22 g) and adult male (18 to 20 g) BALB/c mice (Charles River Breeding Laboratories, Wilmington, Mass.) were used in these studies. Groups of CD-1 mice were anesthetized with methoxyflurane (Abbott Laboratories, North Chicago, Ill.) and inoculated i.n. with 10^2, 10^3, or 10^4 EID_{50} of influenza A, using a standard inoculum volume of 20 μl, PBS as diluent, and a Hamilton 250-μl syringe outfitted with a 27-gauge, 0.5-in. (ca. 1.27-cm) needle and a Hamilton repeating dispenser. In studies with BALB/c mice, influenza A was administered i.n. at input doses of 10^1, 10^2, or 10^3 EID_{50}. After 48 h, the mice were anesthetized with methoxyflurane and inoculated i.n. with type Ia GBS, using an inoculum volume of 30 μl, PBS as diluent, and input doses of 10^1, 10^2, 10^4, or 10^6 CFU for CD-1 mice and 10^1, 10^2, or 10^3 CFU for BALB/c mice. Controls consisted of CD-1 and BALB/c mice inoculated singly with a dose of either agent or with an equal volume of diluent. The mice were observed for 10 days, and the mean time to death and cumulative percent mortality were determined.

Kinetics of infection. BALB/c mice were inoculated i.n. with 10^2 EID_{50} of influenza A or an equal volume of PBS followed 48 h later by i.n. inoculation of 10^2 CFU of GBS or an equal volume of PBS. Three experimental groups resulted; a combined-infection group, mice infected with influenza A only, and mice infected with GBS only. Five mice per group, including the mock-infected mice which were not assayed, were used to assess survival. On days 1, 3, 5, and 7 after GBS inoculation, mice from each group were sacrificed, and their livers, spleens, and lungs were ablated and frozen at −80°C until assayed for infectious influenza A and GBS. In addition, mice undergoing combined and GBS-only infection were serially bled via the retroorbital plexus on days 1, 3, 5, and 7 after GBS inoculation for quantitation of GBS.

Quantitation of influenza A. Infectious influenza A was quantified by quantal assay on confluent monolayers of Madin-Darby canine kidney cells. Tissue homogenates (10%, wt/vol) were prepared with Ten Broeck tissue grinders and Eagle minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 2% (vol/vol) heat-inactivated (56°C for 30 min) newborn calf serum (GIBCO). The tissue homogenates were then clarified by low-speed centrifugation and filtered through 0.45-μm filters (Millex; Millipore Corp., Bedford, Mass.), and serial 10-fold dilutions (10^{-3} to 10^{-7}) were prepared in Eagle minimal esential medium supplemented with 2% (vol/vol) heat-inactivated newborn calf serum and gentamicin (50 μg/ml). Replicates of four confluent monolayers of Madin-Darby canine kidney cells, grown in 16- by 125-mm tubes, were inoculated per dilution of tissue homogenate using 1 ml of homogenate per culture. After 48 h of incubation at 37°C in an atmosphere of 5% CO_2− 95% air, virus infection was assessed with a hemadsorption assay, using a 0.5% (vol/vol) suspension of chicken erythrocytes prepared in Eagle minimal essential medium supplemented with gentamicin (50 μg/ml). Virus titers were then calculated, using the REED and Muench (12) method of calculating 50% endpoints, and expressed as 50% tissue culture infectious doses per milliliter. In each assay virus- and mock-infected controls were assayed in parrallel.

The specificity of the hemadsorption reactions was randomly confirmed with identically inoculated and incubated cover slip cultures of Madin-Darby canine kidney cells and the indirect immunofluorescence assay procedure for detecting intracellular influenza A antigens after acetone fixation. All (100%) of the hemadsorption-positive reactions were also positive by the immunofluorescence assay. Negative hemadsorption reactions were not further assayed.

Quantitation of GBS. Viable GBS were quantified in 10% (wt/vol) tissue homogenates prepared as described above and in samples of whole blood. Serial 10-fold dilutions of the homogenates and of whole blood were prepared in PBS, and 0.1 ml of each dilution was inoculated onto each of three blood agar plates (BBL Microbiology Systems, Cockeysville, Md.). The plates were then incubated at 37°C for 48 h, at which time the GBS were enumerated.

Immunofluorescence assays. The indirect immunofluorescence assay procedure was used to detect influenza A antigens and GBS group antigen in frozen sections of mouse tissue. Tissues were quick-frozen in a Quick Freeze chamber (American Optical Corp., Buffalo, N.Y.) and stored at −80°C. Approximately 4-μm-thick tissue sections were prepared with a Cryo-Cut Cryo-Stat microtome (American Optical Corp). The sections were air-dried and then fixed in acetone for 8 to 10 min at 4°C. The acetone-fixed tissue sections were again air-dried and then stored at −20°C until assayed. Tissue sections were gently washed in PBS for 5 min and then treated with either a 1:10 dilution in PBS of heat-inactivated (56°C for 30 min) chicken antiserum directed against influenza A antigens (Flow Laboratories, McLean, Va.) or a 1:10 dilution in PBS of heat-inactivated rabbit antiserum directed against GBS group antigen (Burroughs Wellcome Co., Greenville, N.C.) for 30 min at 37°C in a humidified chamber. Before use, both antisera were exhaustively absorbed with a 10% (wt/vol) homogenate of normal BALB/c mouse lungs. As controls in each assay, a 1:10 dilution of preimmune chicken or rabbit serum and a conjugate control (tissue sections treated with PBS) were assayed in parallel. After incubation, the tissue sections were washed for 15 min in PBS to remove unreacted antiserum. Fluorescein isothiocyanate-conjugated rabbit immunoglobulin G (IgG) directed against chicken IgG (Cappel Laboratories, Cochranville, Pa.) or fluorescein isothiocyanate-conjugated goat IgG directed against rabbit IgG (Cappel Laboratories) diluted 1:10 in PBS was then added to the tissue sections, and the sections were incubated at 37°C in a humidified chamber for 30 min. The tissue sections were then washed with PBS for 15 min and counterstained with Evans blue dye for 5 min. They were then rinsed in PBS for 3 min and mounted

TABLE 1. Mortality of CD-1 mice

Virus dose (EID_{50})	% Mortality (no. dead/no. inoculated) at GBS dose (CFU):				
	PBS[a]	10^1	10^2	10^4	10^6
PBS[a]	ND[b]	0 (0/4)	15 (2/13)	21 (3/14)	60 (6/10)
10^2	0 (0/7)	ND	18 (2/11)	80 (4/5)	100 (12/12)
10^3	0 (0/9)	60 (3/5)	69 (11/16)	94 (17/18)	90 (9/10)
10^4	21 (3/14)	80 (4/5)	90 (18/20)	83 (10/12)	100 (8/8)

[a] Mock infected.
[b] ND, Not done.

in buffered glycerol (PBS-glycerol, 1:9). Observations for antigens were made with a Zeiss microscope equipped with an epifluorescence condenser.

RESULTS

Influenza A alone at doses of 10^2 or 10^3 EID_{50} did not induce lethal infection, whereas a dose of 10^4 EID_{50} killed 21% of the CD-1 mice (Table 1). Antecedent infection with 10^3 EID_{50} of influenza A increased mortality in mice secondarily inoculated with 10^1, 10^2, or 10^4 CFU of GBS from 0 to 60%, 15 to 69%, and 21 to 94% respectively. At an input dose of 10^2 CFU of GBS, an apparent dose-response relationship between the input dose of influenza A and percent mortality was observed. Also, in both single and combined infections, a direct relationship between input dose of GBS and mortality was observed.

Using the same experimental protocol, we did experiments to ascertain whether influenza A and type Ia GBS would induce a lethal synergistic phenomenon in BALB/c mice (Table 2). Concurrent influenza A and type Ia GBS infection effected a lethal synergism. This was most dramatically evident in mice inoculated with 10^2 EID_{50} of influenza A and 10^2 CFU of type Ia GBS; 76% of these mice died, whereas death occured in only 2 and 16% of the mice singly infected with type Ia GBS or influenza A, respectively. If the input doses of influenza A and type Ia GBS are expressed as 50% lethal dose per mouse, the synergism effected by concurrent infection with these agents becomes more evident; 10^2 CFU of type Ia GBS and 10^2 EID_{50} of influenza A correspond to a 50% lethal dose of 0.01 when assayed in BALB/c mice. As such, either agent alone should theoretically kill 0.5% of the mice inoculated.

An inverse relationship was observed between input dose of GBS and mean time to death in CD-1 mice with either a single or combined infection. Although influenza A infection potentiated the lethality of GBS infection, no significant differences were noted in the mean time to death between CD-1 mice singly infected with GBS and those dually infected with the same input dose of GBS. Also, no significant differences were noted in the mean time to death for GBS-inoculated BALB/c mice relative to the input dose of influenza A.

Experiments were then carried out to assess the kinetics of infection in mice undergoing single and combined infections with influenza A and type Ia GBS. Using the protocol described above, we inoculated male BALB/c mice i.n. with 10^2 EID_{50} of influenza A. At the same time, other mice were mock infected with PBS. After 48 h, one-half of the influenza A-infected mice and all of the mock-infected mice were inoculated i.n. with 10^2 CFU of type Ia GBS. The remaining influenza A-infected mice were mock infected with an equal volume of PBS. On days 1, 3, 5, and 7 after GBS infection, the mice were sacrificed, and their spleens, livers, and lungs were harvested and assayed for viable GBS and infectious influenza A. The kinetics of GBS replication in the lungs of the mice are shown in Fig. 1. Replication of GBS was significantly potentiated in the influenza A-infected mice on all days ($P < 0.01$, Student's t test), whereas in mice infected with GBS only the CFU of GBS decreased to less than 10% of the input dose.

TABLE 2. Mortality of BALB/c mice

Virus dose (EID_{50})	% Mortality (no. dead/no. inoculated) at GBS dose (CFU):			
	PBS[a]	10^1	10^2	10^3
PBS[a]	0 (0/16)	0 (0/16)	2 (1/50)	0 (0/16)
10^1	13 (2/16)	19 (3/16)	44 (7/16)	31 (5/16)
10^2	16 (8/50)	19 (3/16)	76 (38/50)	63 (10/16)
10^3	38 (6/16)	50 (8/16)	63 (10/16)	100 (16/16)

[a] Mock infected.

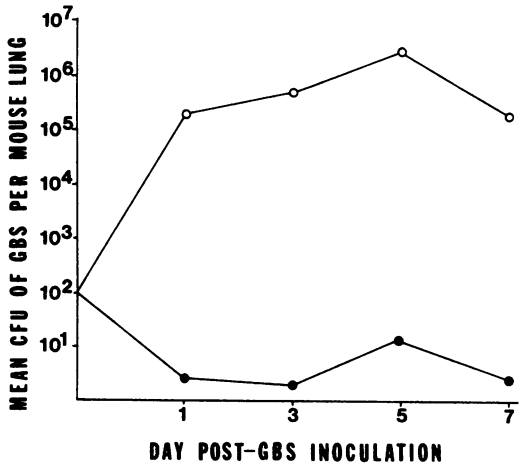

FIG. 1. Kinetics of type Ia GBS growth in the lungs of singly (●) and dually (○) infected mice. On days 1 (day 3 after influenza A infection) 3, 5, and 7 after GBS inoculation, the lungs from five or six mice per group were harvested, homogenized, and individually assayed for GBS by conventional plating on blood agar medium.

The presence of high levels of GBS in the lungs of dually infected mice on day 5 after GBS inoculation was confirmed serologically by an indirect immunofluorescence assay procedure and frozen lung sections. Supporting evidence for enhanced GBS growth in the lungs of dually infected mice also came from the observation of a 10-fold increase on day 5 in the mean total number of polymorphonuclear neutrophils in the lungs of dually infected mice relative to the number in mice singly infected with GBS (W. T. Jones and J. H. Menna, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, E91, p. 75). Extrapulmonary dissemination of GBS was not apparent. No GBS were detected in the spleen or liver in 24 dually infected mice or in the same organs in 24 mice singly infected with GBS.

To confirm the finding that extrapulmonary infection with GBS did not occur in dually or singly infected mice, animals inoculated as described above were serially bled at the same times after GBS inoculation via the retroorbital plexus, and the blood samples were assayed for viable GBS. No GBS were detected in the blood of 10 dually infected or 5 singly infected BALB/c mice.

The kinetics of influenza A replication in the lungs of singly and dually infected mice is shown in Fig. 2. No significant differences were noted in the kinetics of influenza A replication between these groups. Extrapulmonary dissemination of virus was not observed.

Additional evidence for the lack of viremia and bacteremia in dually infected mice was the absence of influenza A or GBS antigen in frozen sections of spleen and liver tissues prepared on day 5 after GBS inoculation, when these tissues were assayed by the indirect immunofluorescence procedure.

DISCUSSION

The lethal synergism effected by concurrent influenza type A virus and type Ia GBS infection in mice is most likely a complex phenomenon involving the interplay of many factors, including microbial virulence factors and the innate and immunologically mediated resistance factors of the host.

Perhaps central to the capacity of influenza A to enhance the lethality of secondary GBS infection is an influenza virus-induced defect in the lung phagocytic system. The three major functions of polymorphonuclear leukocytes and macrophages, i.e., chemotactic response, phagocytosis, and intracellular killing, have been shown by various investigators (1, 2, 9, 13) to be significantly impaired by influenza virus infection. It is apparent, therefore, that a cause and effect relationship may exist between influenza A-induced depression of alveolar macro-

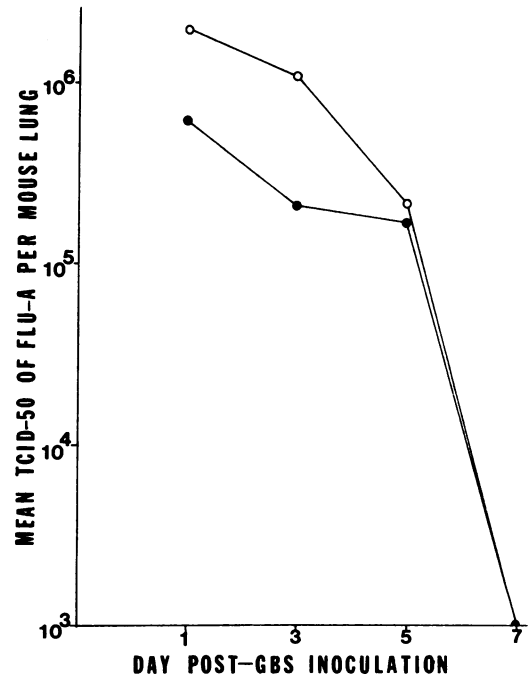

FIG. 2. Kinetics of influenza A replication in the lungs of singly (○) and dually (●) infected mice. On days 1 (day 3 after influenza A infection), 3, 5, and 7 after GBS inoculation, the lungs from five or six mice per group were harvested, homogenized, and individually assayed for infectious influenza A (FLU-A). TCID-50, 50% tissue culture, infective dose.

phage and polymorphonuclear leukocyte phagocytic or microbiocidal activity and our observations of enhanced growth of GBS in the lungs of influenza A-infected mice and enhanced lethality.

Bacterial adherence to the surface of cells as a function of influenza A infection may be a contributing factor by which influenza predisposes the host to secondary bacterial infection of the respiratory tract. Sanford and co-workers (14, 15) documented enhanced adherence of type Ia GBS to cells in vitro as a function of influenza A infection, the adherence presumably mediated by a virus gene-coded cell surface receptor(s). Jones and Menna (6) extended the findings of Sanford et al. (14, 15) by documenting a 120-fold increase in the adherence of type 1a GBS to mouse tracheal tissue in vivo as a function of influenza A infection. The specificity of this adherence was shown by the finding that GBS adherence was reduced 94% by homologous antiviral antiserum and 0% by heterologous antiserum.

Based on the finding of Wennerstrom (17) that mortality in mice inoculated i.n. with type Ia GBS is associated with fulminant septicemia not preceded by detectable multiplication of GBS in the lung, it appears that bacterial dissemination is negated in concurrent influenza A and GBS infection as a result of the adherence of GBS to influenza A-infected lung tissue. This hypothesis is based on the present finding of greatly increased numbers of GBS in the lungs of dually infected mice in the absence of bacteremia and on the finding of Jones and Menna (6) of influenza A-enhanced GBS adherence to mouse tracheal tissue in vivo. The numbers of GBS in the lungs of mice during combined infection when the agents are inoculated separately i.n. would certainly lead to septicemia, because Wennerstrom (17) reported that lower input doses of GBS resulted uniformly in fulminant septicemia. The findings of Wennerstrom (17) were confirmed in this study. When male BALB/c mice were given an input dose of type Ia GBS i.n. equal to 10% of the maximal pulmonary levels observed in dually infected mice, they developed fulminant lethal septicemia.

Antecedent influenza A infection has been shown to potentiate the growth of *Haemophilus influenzae* (10, 11), *Streptococcus pneumoniae* (5), and *Staphylococcus aureus* (4) in the lungs of rodents secondarily inoculated i.n. with these agents. The present finding of enhanced GBS growth in the lung after influenza A infection is consistent with the aforementioned observations (4, 5, 10, 11). Of interest, however, was the lack of bacteremia in influenza A-infected, GBS-inoculated mice, a finding inconsistent with the observation of bacteremia in mice secondarily inoculated with *Streptococcus pneumoniae* (5) and rats secondarily inoculated with *Haemophilus influenzae* (10, 11).

The present finding of enhanced GBS growth in the lungs of influenza A-infected mice, together with our previous finding (6) of influenza A-potentiated adherence of type Ia GBS to mouse tracheal tissue in vivo, suggests that the lethal synergism may be due in part to enhanced colonization with GBS after their adherence to influenza A-infected respiratory tract epithelium.

Although the lethal synergism effected in mice by influenza A and GBS may lack direct clinical relevancy because GBS is not commonly associated with infections secondary to influenza in humans, this animal model may be applicable to the study of other more common causes of post-influenza infections, e.g., *Staphylococcus aureus*, as Davison and Sanford (3) have shown that *Staphylococcus aureus* adheres to influenza A-infected cell cultures in vitro.

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