Vaccination with Inactivated Influenza A Virus during Pregnancy Protects Neonatal Mice against Lethal Challenge by Influenza A Viruses Representing Three Subtypes

INNOCENT N. MBAWUIKE,* HOWARD R. SIX,† THOMAS R. CATE, AND ROBERT B. COUCH

Influenza Research Center, Baylor College of Medicine, Houston, Texas 77030

Received 21 August 1989/Accepted 6 November 1989

A single intraperitoneal injection of pregnant mice with a monovalent Formalin-inactivated influenza A virus vaccine protected their offspring against a lethal challenge dose of the same influenza A virus H3N2, H2N2, and H1N1 subtypes, as well as against challenge with the other two subtypes. Degree of protection was vaccine dose related. Cross-fostering of neonates indicated that protection was conferred by breast milk antibodies. Serum virus-specific neutralizing antibodies in the mothers and neonates correlated with resistance to vaccine virus, but were detected against other subtypes only in a complement enhancement test or when high doses of vaccine were given.

Influenza viruses are significant causes of disease in human infants, having been implicated in bronchiolitis, croup, pneumonia, febrile convulsions, and death (8, 19, 32). Formalin-inactivated influenza virus vaccines reportedly provide subtype-specific (i.e., type A H1N1, H2N2, or H3N2) protection in mice (2, 3) primarily, whereas in unprimed mice, they preferentially stimulate cells mediating delayed-type hypersensitivity reactions (1, 13, 15) that can increase the severity of influenza virus pneumonia in the absence of circulating antibody (1, 5, 13). Thus, one should proceed with caution in considering the use of killed virus vaccines in human infants. An alternative to prevention of influenza during the first few months of life could be vaccination of pregnant women with inactivated influenza virus vaccines. A rationale for this approach comes from demonstration that antibody to influenza virus is transferred transplacentally to human infants (17, 20, 28) and that this passively acquired antibody can protect them against serious influenza (22).

Nonlethal influenza A virus infection of pregnant mice (24, 25) and ferrets (10) was shown to confer protection against the same subtype to the offspring. Vaccination of pregnant ferrets with killed virus plus adjuvant, killed virus after infection with a different influenza A subtype, or infection with vaccinia-influenza virus hemagglutinin recombinant also provided subtype-specific protection to offspring (11, 30). Protection in these instances was presumably mediated through antibody in breast milk (10, 11, 24, 25, 30). In the present study, pregnant mice were immunized with various doses of Formalin-inactivated influenza A virus vaccines and resistance or altered responsiveness of the offspring to challenge with three influenza A virus subtypes was assessed.

Vaccine-induced protection against challenge with influenza A viruses. NIH Swiss mice (8 to 12 weeks old) were bred by placing one or two females and one male in a cage for 7 days. Gestating females were then injected once intraperitoneally with 0.2 ml of vaccine or phosphate-buffered saline (PBS). Three doses of vaccine were tested (low, intermediate, and high; HA titer, 1:256, 1:2,560, and 1:25,600, respectively).

Pregnant mice delivered 21 ± 2 days after initiation of mating. Two-week-old neonates were challenged with influenza virus by small-particle aerosol (33) and observed for 14 days for mortality. Protection was expressed as the percent reduction in mortality rates for the experimental group from that of the control group.

Neonates born to mothers vaccinated with an intermediate dose of A/Aichi/68 (H3N2) (an A/HK/68-like virus) vaccine and challenged with 3 50% lethal doses (LD_{50} s) of either A/HK/68 (H3N2), A/Jap/305/57 (H2N2), or A/PR/8/34 (H1N1) exhibited a reduction in the mortality rate of 99, 54, and 56%, respectively, compared with those born to mothers vaccinated with PBS ($P < 0.001$ for each vaccine). Neonates receiving the low-dose vaccine exhibited diminished but statistically significant protection against A/HK/68 (43%; $P < 0.001$) and A/Jap/305/57 (15%; $P < 0.02$) virus challenge (Fig. 1). Infants born to mothers receiving the high-dose vaccine were almost completely resistant (74 to 100% protection) to all three influenza A virus subtypes ($P < 0.001$ for each). Vaccine did not protect neonates against B/Lee/40 virus.

Mothers given an intermediate dose of Formalin-inactivated A/Aichi/68, A/Jap/305/57, or A/PR/8/34 vaccine conveyed the highest protection (96 to 100%; $P < 0.001$ for each) to their offspring against homologous virus challenge (Fig. 2). Protection was also accorded against challenge with other subtypes (32 to 85% protection; $P < 0.080$ to $P < 0.001$), but not B/Lee/40 virus. These unexpected results suggest that when given at an adequate dose, an inactivated influenza virus vaccine consisting of a single subtype can provide immunity to all three influenza A virus subtypes.

Role of breast milk. To assess the mode of maternal-infant transfer of immunity, offspring of immune mothers were transferred to and nursed by nonimmune mothers and vice versa (25, 30). Offspring of mothers given PBS but cross-fostered by mothers immunized with A/Aichi/68 vaccine were almost completely protected (82 to 100%; $P < 0.001$) when challenged with A/HK/68 virus at 2 and 4 weeks of age. Neonates born to immune mothers but nursed by nonimmune mothers were less well protected (51 to 54%; $P < 0.001$) at 2 weeks of age and were not protected at all at 4 weeks of age. Protection of the latter group at 2 weeks could be due to antibodies in colostrum obtained during the first

* Corresponding author.

† Present address: Connaught Laboratories, Swiftwater, PA 18370-0187.

FIG. 1. Effect of vaccine dose on resistance to influenza virus challenge. Seven days after mating, gestating female mice were vaccinated with PBS (■) or a low (▨), intermediate (▩), or high (▬) dose of A/Aichi/68 Formalin-inactivated vaccine. Two-week-old siblings were challenged by small-particle aerosol with 3 LD₅₀s of the indicated virus. The figure shows cumulative mortality rates (23 to 80 mice per group) observed in two to four replicate experiments. A/PR/8/34 and B/Lee/40 virus challenges were not performed for the low-dose vaccine.

few hours of life (2 to 12 h) before separation from their natural mothers. These results suggest that protection is mediated primarily, if not entirely, by breast milk, as previously shown by others (25, 30, 31).

Lung virus. Influenza virus titers in the lungs were determined 3 to 4 days following virus challenge (33, 34). Intermediate-dose A/Aichi/68, A/Jap/305/57, or A/PR/8/34 vaccine induced a significant reduction in the mean lung virus titer in 2-week-old offspring after challenge with homologous virus (Table 1). Smaller but still significant reductions were seen after A/HK/68 challenges following A/Jap/305/57 or A/PR/8/34 vaccines and after A/Jap/305/57 challenge following A/Aichi/68 vaccine, but not after either A/Jap/305/57 challenge following A/PR/8/34 vaccine or A/PR/8/34 challenge following A/Aichi/68 or A/Jap/305/57 vaccine, even though significant protection against death was observed in each case in other mice (Fig. 2). The mean lung virus titer of neonates challenged with A/PR/8/34 after their mothers had been vaccinated with A/Aichi/68 vaccine was slightly but significantly higher than that of neonates whose mothers received PBS. High-dose A/Aichi/68 vaccination, however, resulted in virus being undetectable in lungs of offspring challenged with A/HK/68 or A/Jap/305/57 and reduced in those given A/PR/8/34 virus ($P = 0.06$).

Serum-neutralizing antibody. Sera obtained from mothers and their offspring 2 weeks after delivery (before virus challenge) were heat inactivated and tested for neutralizing antibody (9). Pooled hamster serum (Flow Laboratories, Inc., McLean, Va.) as a complement source was previously shown to enhance neutralizing antibody titers against influenza viruses and was therefore added in some tests (7). Serum virus-specific neutralizing antibody in neonates correlated with resistance to homologous virus ($r = 0.89$; $P < 0.001$). At 2 weeks of age, neonates had neutralizing anti-

body titers very similar to those of the mother that had been nurturing them (data from cross-fostering experiments [not shown]). Mothers immunized with intermediate-dose A/Aichi/68, A/Jap/305/57, or A/PR/8/34 virus vaccine had neutralizing antibody responses to the homologous virus only; the geometric mean titers ($\log_2$) for the homologous viruses were 4.5, 9.8, and 9.5 for A/Aichi/68, A/Jap/305/57, and A/PR/8/34, respectively. In the offspring from mothers given A/Aichi/68 vaccine, low- and intermediate-dose vaccine induced neutralizing antibody to A/HK/68 virus only (Fig. 3). A high-dose vaccine, however, induced significant neutralizing antibody to both A/HK/68 and A/Jap/305/57 virus ($P < 0.05$); titers to A/PR/8/34 virus were low but detectable. Addition of complement to the assay resulted in the enhancement of neutralizing antibody titers to A/HK/68 virus and in the detection of significant titers to A/PR/8/34 in the intermediate- and high-dose vaccine groups, but there was only a slight increase in neutralizing antibody titer to A/Jap/305/57 virus. Neutralizing antibody to B/Lee/40 influenza virus was not detected under any assay conditions.

The present study has demonstrated that immunization of pregnant mice with monovalent inactivated influenza A virus vaccine provides resistance to lethal challenge of their offspring with each of the three influenza A virus subtypes. Serum-neutralizing antibody titers in the offspring correlated with resistance to challenge with homologous virus. Although neutralizing antibody to other influenza A virus subtypes was not initially evident at lower vaccine doses, cross-reactive neutralizing antibody against them was demonstrated by the addition of complement in a neutralizing-antibody assay and when mice were given higher vaccine doses. Our study showed a vaccine dose- and antibody titer-related protection against lung virus titers. It is not known why intermediate-dose vaccine did not produce a

FIG. 2. Specificity of vaccine-induced protection. Gestating female mice were given a single intraperitoneal dose of PBS (■) or Formalin-inactivated zonal purified A/Aichi/68 (▨), A/Jap/305/57 (▩), or A/PR/8/34 (▧) influenza virus vaccine (intermediate dose) 7 days after breeding. Two-week-old neonates born to these mothers were challenged by small-particle aerosol with 10 LD₅₀s of A/HK/68 or 3 LD₅₀s of Jap/305/57, A/PR/8/34, or B/Lee/40 influenza virus. The percent cumulative mortality rates of mice (14 to 38 per group) in two combined experiments are presented. Mice vaccinated with A/Jap/305/57 vaccine were not challenged with B/Lee/40 virus.

greater reduction than that observed in A/HK/68 and A/Jap/305/57 and appeared to enhance A/PR/8/34 virus replication in the lungs on days 3 and 4 after challenge, since significant protection against death was apparent at this dose. However, a similar reduction in mortality rate in the high-dose A/Aichi/68 vaccine groups was associated with undetectable A/Jap/305/57 and a reduction in A/PR/8/34 virus.

The maternal-fetal-neonatal transfer of influenza virus immunity in this study is most probably mediated by breast milk antibodies (10, 25, 30, 31). Since immunoglobulin A and M antibodies do not effectively cross rodent neonatal gut epithelium (16, 31), milk-derived anti-influenza virus immunoglobulin G antibodies most probably mediated the protection of infant mice and ferrets (11, 24, 31). Other possible mechanisms include anti-idiotypic antibodies to maternal anti-influenza antibodies (18, 27) and the presence of T cells in colostrum and milk (26); both are unlikely, however, because the short duration of protection (8 weeks) precludes an active immunity.

Nonlethal influenza virus infection in the mouse induces subtype cross-reactive protection. Since subtype cross-reactive antibodies have generally not been found, this protection has been attributed to T cells (2, 5, 14). In the present study, neutralizing-antibody titer to other subtypes was detected when high-dose vaccine was given or when serum complement was added to increase the sensitivity of the assay; in preliminary studies, we have also detected immunoglobulin G antibodies reactive against all three influenza virus subtypes in a sensitive radioimmunoassay. In most studies comparing live virus infection and inactivated virus,

TABLE 1. Effect of intermediate- and high-dose vaccines on lung virus titers of offspring following challenge with influenza viruses

Vaccine group	Challenge group	Lung virus titer (log ₁₀ GMT + SD) ^a at:	
		Intermediate dose	High dose ^b
PBS	A/HK/68	>6.4 ± 0.1	>6.7 ± 0.0
A/Aichi/68	A/HK/68	5.3 ± 0.2 ^c	≤1.0 ± 0.0 ^d
A/Jap/305/57	A/HK/68	5.9 ± 0.4 ^c	NT ^e
A/PR/8/34	A/HK/68	5.6 ± 0.5 ^c	NT
PBS	A/Jap/305/57	2.8 ± 0.1	2.9 ± 0.3
A/Aichi/68	A/Jap/305/57	2.4 ± 0.1 ^c	≤1.0 ± 0.0 ^d
A/Jap/305/57	A/Jap/305/57	≤1.0 ± 0.0 ^c	NT
A/PR/8/34	A/Jap/305/57	2.7 ± 0.1	NT
PBS	A/PR/8/34	4.3 ± 0.4	3.5 ± 0.5
A/Aichi/68	A/PR/8/34	4.7 ± 0.2 ^c	2.9 ± 0.7 ^f
A/Jap/305/57	A/PR/8/34	3.9 ± 0.3	NT
A/PR/8/34	A/PR/8/34	≤1.0 ± 0.0 ^c	NT
PBS	B/Lee/40	4.2 ± 0.4	NT
A/Aichi/68	B/Lee/40	4.1 ± 0.3	NT
A/Jap/305/57	B/Lee/40	NT	NT
A/PR/8/34	B/Lee/40	4.0 ± 0.2	NT

^a Lungs from 2-week-old neonates challenged with 3 LD₅₀s of virus by small-particle aerosol were collected after 3 to 4 days, homogenized, and tested for virus on MDCK cells (6, 29); results for three separate experiments are presented. The starting dilution was 1:30; a value of 1:10 was assigned to samples with no detectable virus. GMT, Geometric mean titer.

^b High-dose A/Aichi/68 vaccine only was used.

^c Significantly different from PBS control ($P < 0.05$).

^d Significantly different from PBS control ($P < 0.001$).

^e NT, Not tested.

^f $P = 0.06$, not significantly different from PBS control.

FIG. 3. Effect of vaccine dose and complement on the neutralizing-antibody titer of neonates. Sera were obtained from 2-week-old offspring whose mothers had received PBS (■) or a low (▨), intermediate (⊗), or high (▧) dose of A/Aichi/68 vaccine during pregnancy. Hamster serum (complement, C') was added at the 1/200 dilution. The starting dilution of sera was 1:16, a value of 1:8 was assigned to samples with no detectable antibody. GMT, Geometric mean titer; Nt Ab, neutralizing antibody; C', complement.

it was not possible to compare antigen doses (1–3, 23, 36). Thus, the failure to find antibodies reactive against other subtypes may have been related to the immunizing dose and the assay method. In support of our results, inactivated influenza A virus vaccine induced hemagglutinin-mediated antibody responses cross-reactive between H1 and H2 subtypes (21), and children with primary infection developed enzyme-linked immunosorbent assay, but not hemagglutination inhibition, antibodies cross-reactive with noninfecting H1 or H3 and H8 hemagglutinin (4). Thus, our high-dose vaccine could induce cross-reactive antibody directed to a common determinant on the hemagglutinin or neuraminidase (12, 35), and this could be the basis for the cross-protection.

In summary, our results demonstrate that vaccination of pregnant mice with monovalent Formalin-inactivated influenza A virus vaccine can result in transfer through breast milk of influenza A subtype-specific and cross-reactive immunity to the offspring. These results, along with other studies with animals (10, 24, 25, 30, 31), support the suggestion that vaccination of pregnant women against influenza, followed by breast feeding, may be beneficial to the infant in the first few months of life. Definition of the antigens in influenza A virus that induce cross-protection should facilitate the development of a more effective vaccine against influenza A viruses.

We thank Karen Kincade for preparing the manuscript.

This work was supported by Public Health Service contract N01-A1-62517 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. Ada, G. L., K.-N. Leung, and H. Ertl. 1981. An analysis of the effector T-cell generation and function in mice exposed to influenza A or Sendai viruses. *Immunol. Rev.* **58**:5–24.
2. Amerding, D., and E. Liehl. 1981. Induction of heterotypic T- and B-cell immunity with influenza A virus in mice. *Cell. Immunol.* **60**:119–135.
3. Braciale, T. J., and K. L. Yap. 1978. Role of viral infectivity in the induction of influenza virus-specific cytotoxic T-cells. *J. Exp. Med.* **147**:1236–1252.
4. Burlington, D. B., P. F. Wright, K. L. Van Wyke, M. A. Phelan, R. E. Mayner, and B. R. Murphy. 1985. Development of subtype-specific and heterosubtypic antibodies to the influenza A virus hemagglutinin after primary infection in children. *J. Clin. Microbiol.* **21**:847–849.
5. Cate, T. R., and N. G. Mold. 1975. Increased influenza pneumonia mortality of mice adaptively immunized with node and spleen cells sensitized by inactivated but not live virus. *Infect. Immun.* **11**:908–914.
6. Frank, A. L., R. B. Couch, C. A. Griffis, and B. D. Baxter. 1979. Comparison of different tissue cultures for isolation and quantitation of influenza and parainfluenza viruses. *J. Clin. Microbiol.* **10**:23–36.
7. Frank, A. L., J. Puck, B. J. Hughes, and T. R. Cate. 1980. Microneutralization test for influenza A and B and parainfluenza 1 and 2 viruses that uses continuous cell lines and fresh serum enhancement. *J. Clin. Microbiol.* **12**:426–432.
8. Glezen, W. P. 1980. Consideration of the risk of influenza in children and indications for prophylaxis. *Rev. Infect. Dis.* **2**:408–420.
9. Gruber, W. C., S. Z. Wilson, B. J. Throop, and P. R. Wyde. 1987. Immunoglobulin administration and ribavirin therapy:

- efficacy in respiratory syncytial virus infection of the cotton rat. *Pediatr. Res.* **21**:270–274.
10. **Husseini, R. H., C. Sweet, H. Overton, and H. Smith.** 1984. Role of maternal immunity in the protection of newborn ferrets against infection with a virulent influenza virus. *Immunology* **52**:389–394.
 11. **Jakeman, K. J., H. Sweet, and C. Sweet.** 1989. Mechanisms of immunity to influenza: maternal and passive neonatal protection following immunization of adult ferrets with a live vaccinia-influenza virus hemagglutinin recombinant, but not with recombinants containing other influenza virus proteins. *J. Gen. Virol.* **70**:1523–1531.
 12. **Jones, P. D., and G. L. Ada.** 1986. Influenza virus-specific antibody-secreting cells in the murine lung during primary influenza virus infection. *J. Virol.* **60**:614–619.
 13. **Leung, K.-N., and G. L. Ada.** 1980. Cells mediating delayed-type hypersensitivity in the lungs of mice infected with influenza A virus. *Scand. J. Immunol.* **12**:393–400.
 14. **Leung, K.-N., R. B. Ashman, H. C. J. Ertl, and G. L. Ada.** 1980. Selective suppression of cytotoxic T-cell response to influenza virus in mice. *Eur. J. Immunol.* **10**:803–810.
 15. **Liew, F. Y., and S. M. Russell.** 1983. Inhibition by pathogenic effect of effector T-cells by specific suppressor T-cells during influenza virus infection in mice. *Nature (London)* **304**:541–543.
 16. **Mackenzie, N.** 1984. Fc receptor-mediated transport of immunoglobulin across the intestinal epithelium of the neonatal rodent. *Immunol. Today* **5**:364–366.
 17. **Masurel, N., J. I. DeBruijne, H. A. Beuningh, and H. J. A. Schouten.** 1978. Haemagglutination-inhibition antibodies against influenza A and influenza B in maternal and neonatal sera. *J. Hyg.* **80**:13–19.
 18. **Moran, T., Y.-N. C. Liu, J. L. Shulman, and C. A. Bona.** 1984. Shared idiotypes among monoclonal antibodies specific for A/PR/8/34 (H1N1) and X-31 (H3N2) influenza viruses. *Proc. Natl. Acad. Sci. USA* **81**:1809–1812.
 19. **Murphy, T. P., F. W. Henderson, W. A. Clyde, A. M. Collier, and F. W. Denny.** 1981. Pneumonia: an eleven-year study in pediatric practice. *Am. J. Epidemiol.* **113**:12–21.
 20. **Murray, D. L., D. T. Imagawa, D. M. Okada, and J. W. St. Geme.** 1979. Antibody response to monovalent A/New Jersey/8/76 influenza vaccine in pregnant women. *J. Clin. Microbiol.* **10**:184–187.
 21. **Noble, G. R., H. S. Kaye, A. P. Kendal, and W. R. Dowdle.** 1977. Age-related heterologous antibody responses to influenza virus vaccination. *J. Infect. Dis.* **136**:S686–S692.
 22. **Puck, J. M., W. P. Glezen, A. L. Frank, and H. R. Six.** 1980. Protection of infants from infection with influenza A virus by transplacentally-acquired antibody. *J. Infect. Dis.* **142**:844–849.
 23. **Reiss, C. S., and J. L. Shulman.** 1980. Cellular immune responses of mice to influenza virus vaccines. *J. Immunol.* **125**:2182–2188.
 24. **Reuman, P. D., R. M. Kris, E. M. Ayoub, and P. A. Small, Jr.** 1988. Maternal-infant transfer of influenza-specific immunity not detectable by hemagglutination-inhibition. *Microbios* **54**:135–147.
 25. **Reuman, P. D., C. M. A. Paganini, E. M. Ayoub, and P. A. Small, Jr.** 1983. Maternal-infant transfer of influenza-specific immunity in the mouse. *J. Immunol.* **130**:932–937.
 26. **Ruben, F. L., I. R. Holzman, and P. Fireman.** 1982. Responses of lymphocytes from human colostrum or milk to influenza antigens. *Am. J. Obstet. Gynecol.* **143**:518–522.
 27. **Sigal, N. H., M. Chan, M. A. Reale, T. Moran, Y. Beilin, J. L. Shulman, and C. A. Bona.** 1987. The human and murine influenza-specific B cell repertoires share a common idotype. *J. Immunol.* **139**:1985–1990.
 28. **Sumaya, C. V., and R. S. Gibbs.** 1979. Immunization of pregnant women with influenza A/New Jersey/76 virus vaccine: reactogenicity and immunogenicity in mother and infant. *J. Infect. Dis.* **140**:141–146.
 29. **Sun, C. S., S. Z. Wilson, and P. R. Wyde.** 1986. Limited efficacy of aerosolized recombinant interferon against virulent influenza A/HK infection in mice. *Proc. Soc. Exp. Biol. Med.* **181**:298–304.
 30. **Sweet, C., R. A. Bird, K. Jakeman, D. M. Coates, and H. Smith.** 1987. Production of passive immunity in neonatal ferrets following maternal vaccination with killed influenza A virus vaccines. *Immunology* **60**:83–89.
 31. **Sweet, C., K. J. Jakeman, and H. Smith.** 1987. Role of milk-derived IgG in passive maternal protection of neonatal ferrets against influenza. *J. Gen. Virol.* **68**:2681–2686.
 32. **van Voris, L. P., J. F. Young, J. M. Bernstein, W. C. Graham, E. L. Anderson, G. J. Gorse, and R. B. Belshe.** 1984. Influenza viruses, p. 267–297. *In* R. B. Belshe (ed.), *Textbook of human virology*. PSG Publishing Co., Inc., Littleton, Mass.
 33. **Wilson, S. Z., V. Knight, P. R. Wyde, S. Drake, and R. B. Couch.** 1980. Amantadine and ribavirin aerosol treatment of influenza A and B infection in mice. *Antimicrob. Agents Chemother.* **17**:642–648.
 34. **Wyde, P. R., M. R. Wilson, and T. R. Cate.** 1982. Interferon production by leukocytes infiltrating the lungs of mice during primary influenza virus infection. *Infect. Immun.* **38**:1249–1255.
 35. **Yarchoan, R., and D. L. Nelson.** 1984. Specificity of *in vitro* anti-influenza virus antibody production by human lymphocytes: analysis of original antigenic sin by limiting dilution cultures. *J. Immunol.* **132**:928–935.
 36. **Zweerink, H. J., B. A. Askonas, D. Millican, S. A. Countneidge, and J. J. Skehel.** 1977. Cytotoxic T-cells to type A influenza virus: viral hemagglutinin induces A-strain specificity while infected cells confer cross-reactive cytotoxicity. *Eur. J. Immunol.* **7**:630–635.