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Induction of Partial Specific Heterotypic Immunity in Mice by a Single Infection with Influenza A Virus

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ABSTRACT

SCHULMAN, JEROME L. (Cornell University Medical College, New York, N.Y.), AND EDWIN D. KILBOURNE. Induction of partial specific heterotypic immunity in mice by a single infection with influenza A virus. J. Bacteriol. **89**:170–174. 1965.—Mice infected 4 weeks previously with influenza A virus were found to be partially immune when challenged with influenza A2 virus. This partial immunity was demonstrated by reduced titers of pulmonary virus, decreased mortality, and less extensive lung lesions. A specific immunological basis for this protection was suggested by the absence of any protection in animals previously infected with influenza B virus when challenged with A2 virus, or in animals previously infected with influenza A virus when challenged with influenza B virus. Parenteral inoculation with inactivated influenza A virus did not induce partial immunity to A2 virus challenge. An accelerated rise of hemagglutinating-inhibiting antibody after A2 virus challenge was demonstrated in animals previously infected with influenza A virus.

Immunity to influenza is generally considered specific for the viral subtype that induces the infection. In natural human infection, this view is most dramatically illustrated by the vaccine failures of 1947 (Francis, Salk, and Quilligan, 1947) and the pandemic of 1957—both sequels of antigenic mutation of influenza A viruses (A1 and A2) so extreme as to circumvent immunity engendered by the earlier A variants.

In the past decade, studies based on seroepidemiological data (Davenport, Hennessy, and Francis, 1953; Lief and Henle, 1960), as well as serum-absorption studies of experimentally immunized animals (Jensen et al., 1956) have suggested strongly that minor antigens are shared by viral strains of all four subtypes. Thus, the influenza A subtypes differ in the major capsid antigens responsible for specific immunity and immunization but also share minor capsid and nucleoprotein antigens. There is no evidence that the nucleoprotein antigen induces immunity to influenza virus infection (Lennette and Horsfall, 1941), and the concept of significant heterotypic immunity engendered by the minor antigens is at present an attractive hypothesis to explain the apparent increase in immunity with increasing age.

In earlier studies of the viruses of human and swine influenza, it was demonstrated that ferrets, mice, and swine convalescent from infection of human or swine influenza viruses had less extensive pulmonary lesions and decreased mortality following challenge with the heterotypic virus. However, neutralizing antibody to the heterotypic virus was regularly demonstrable in the sera of such animals only after repeated infection or immunization with the homotypic virus (Smith, Andrewes, and Laidlaw, 1933, 1935; Shope, 1935, 1937; Francis and Shope, 1936; Shope and Francis, 1936). In recent investigations by Henle and Lief (1963), it was shown that repetitive homotypic infection may elicit formation of heterotypic viral-specific complement fixing and neutralizing antibodies in guinea pigs. More important, it was also demonstrated that quadruple (but not single) infections of mice with an A1 virus induced heterotypic immunity to all other A subtypes as determined by LD_{50}.

The present studies were designed to investigate further the problem of specific immunity (rather then antibody specificity) in influenza, by use of a murine experimental model in which pulmonary virus titers may be measured with a high level of reproducibility and confidence (Schulman and Kilbourne, 1963*a*), with the expectation that the criterion of lessened viral multiplication after challenge would constitute a

sensitive and definitive indicator of specific heterotypic immunity.

Materials and Methods

Mice. Manor Farm (MF-1) specific pathogen-free male mice, 10 to 16 weeks of age, were employed in all experiments. Mice were housed in stainless-steel boxes in groups of 10, or in pairs in smaller stainless-steel containers with wire-mesh floors.

Lungs were removed aseptically at designated intervals and ground in glass tubes in accordance with techniques previously described (Schulman and Kilbourne, 1963*a*).

Viruses. The Stuart-Harris neurovirulent variant of WS virus (NWS) and PR8 virus were employed as strains of influenza A virus, and an unadapted, inhibitor-sensitive strain of virus, isolated at The Rockefeller Institute ($RI/5^+$; Choppin and Tamm, 1959), and mouse-adapted Jap. 305 virus were used as influenza A2 viruses. In some experiments, the Lee strain of influenza B virus was also used.

Viral titrations. Virus content of ground suspensions of individual mouse lungs was measured by techniques previously described (Schulman and Kilbourne, 1963*a*).

Hemagglutinating-inhibiting (HI) antibody titrations. HI antibody was titrated in individual mouse sera at appropriate intervals, by use of the mouse-adapted A2 (Jap. 305) virus as the antigen. Control sera from uninfected mice were collected at each time interval, and used to exclude the possibility of serum inhibitor affecting the results. It was found in preliminary tests that, with this antigen, trypsin or periodate treatment of serum was not necessary. Sera were heated at 56 C for 30 min, and then serial twofold dilutions of 0.2 ml of heated serum were made in phosphate-buffered saline (PBS). A 0.2-ml amount of the mouse-adapted Jap. 305 virus containing 16 to 32 hemagglutinating units was added to each tube. Then 0.4 ml of human "O" red cells was added, and after 50 min at room temperature the tubes were observed for the absence or presence of agglutination.

Scoring of pulmonary lesions. A modification of the maximal score method (Horsfall, 1939) was used in which the extent of pulmonary lesions was expressed as a percentage of total lung surface.

Aerosol procedure. The apparatus and the technique used to generate an aerosol mist of infective virus has been described elsewhere (Schulman and Kilbourne, 1963*b*).

Mice were exposed during a 30-min period to an estimated 100 MID_{50} of each of the viruses employed.

Results

Induction of heterotypic immunity by exposure of mice to aerosols of live virus. Table 1 summarizes the results of one experiment in which mice were infected by aerosol with 100 MID_{50} of the viruses indicated, and challenged 4 weeks later with aerosol mists of Jap. 305 (A2) or influenza B (Lee) viruses. Control animals were exposed to an aerosol of saline 4 weeks prior to challenge. Five mice in each group were autopsied 72 hr after challenge, and pulmonary titers of infective virus were determined by separate titration of each lung in eggs. Observations for mortality were conducted on 15 animals initially infected with NWS virus and then challenged with Jap. 305 virus, and 15 control animals challenged with Jap. 305 virus. The results indicated that prior infection of mice with two strains of influenza A virus (NWS and PR8) induced some cross-protection to challenge with A2 (Jap. 305) virus. This was demonstrated by reduced pulmonary virus titers compared to control animals 3 days after challenge, and, in the case of animals previously infected with NWS virus, reduced mortality 7 days after challenge. The effect appears to be immunologically specific, because prior infection with influenza A (PR8 or NWS) virus did not lead to reduced pulmonary virus titers upon challenge with influenza B (Lee) virus. Conversely, prior infection with influenza B (Lee) virus had no effect upon challenge with A2 (Jap. 305) virus. Immunity resulting from prior infection with homologous virus was absolute after a 4-week interval, in that no virus was demonstrable in lungs of mice infected with one A2 strain ($RI/5^+$), and challenged with another (Jap. 305).

TABLE 1. *Effect of previous infection of mice with heterotypic or heterologous virus on subsequent challenge with influenza A2 virus*

Initial infection	Challenge infection*	Pulmonary virus titers (72 hr)†	Mortality
None	A2 (Jap. 305)	7.9 ± .49	15/15 (100%)
B (Lee)	A2 (Jap. 305)	7.4 ± .43	ND‡
A (PR8)	A2 (Jap. 305)	6.5 ± .38	ND
A (NWS)	A2 (Jap. 305)	5.5 ± .54	2/15 (13.3%)
A2 ($RI/5^+$)	A2 (Jap. 305)	<1.0	
None	B (Lee)	6.0	ND
A (PR8)	B (Lee)	6.1	ND
A (NWS)	B (Lee)	6.0	ND

* At 4 weeks after initial infection.

† Log_{10}, EID_{50}, mean of individual titers (five animals in each group).

‡ Not determined.

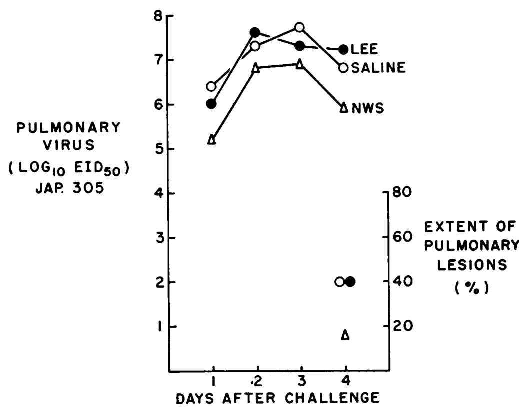

FIG. 1. *Pulmonary virus titers* (EID_{50}) *and pulmonary lesions in animals previously infected with heterotypic or heterologous viruses and challenged with Jap. 305 virus.*

TABLE 2. *Effect of parenteral immunization with inactivated influenza A virus upon subsequent challenge with A2 virus*

Injection[a]	Challenge[b]	Pulmonary virus titers[c] (72 hr after challenge)
Saline	A2 (Jap. 305)	7.5
A (PR8)[d]	A2 (Jap. 305)	7.7
Saline	A (NWS)	7.2
A (PR8)[d]	A (NWS)	5.6
Saline	A (PR8)	7.0
A (PR8)[d]	A (PR8)	<4.7[e]

[a] Three intraperitoneal injections of 0.2 ml at weekly intervals.
[b] At 1 week after final injection.
[c] EID_{50}, $\log_{10}$, five animals in each group.
[d] Injection of 200 CCA units/ml; HI antibody titer to PR8 virus = 1:128.
[e] No virus at 10^{-5} dilution in any of five lungs.

In another similar experiment, pulmonary virus titers were measured at daily intervals over a 4-day period to determine whether cross-protection was demonstrable at more than one time period after challenge with A2 virus. The results (Fig. 1) indicated that at every interval, pulmonary titers of A2 virus were lower in animals previously infected with influenza A (NWS) virus than in mice previously infected with influenza B (Lee) virus or previously exposed to a saline aerosol. (The *P* value of the differences is less than 0.05 in six of the eight possible comparisons.) An additional group of mice was infected with PR8 virus and challenged 4 weeks later with the Jap. 305 virus. After 72 hr of infection, these mice had a mean pulmonary virus titer of $10^{6.8}$ (not shown in Fig. 1). This single observation was in accord with the decreased viral titers induced by prior infection with NWS—also of the influenza A subtype. The reduced pulmonary virus titers in mice previously infected with NWS virus were associated with less extensive pulmonary lesions. Of eight animals in each group observed for 7 days, there were eight deaths in the Lee and saline groups and no deaths in the NWS group. In the same experiment, similar groups of mice were challenged with aerosols of NWS or Lee virus. Prior infection with NWS or PR8 virus had no effect upon subsequent challenge with Lee virus, as contrasted to the effect on Jap. 305 challenge (Table 2). In this experiment, prior infection with A2 (RI/5$^+$) virus did not modify pulmonary virus titers 72 hr following NWS challenge.

Attempted induction of heterotypic immunity by parenteral inoculation of inactivated virus. It was of interest to determine whether a partial, heterotypic immunity could be induced by parenteral immunization with inactivated virus, as well as by exposure to live virus infection. Mice were inoculated intraperitoneally at weekly intervals with 0.2 ml of formalin-inactivated PR8 virus (200 chick cell agglutinating units per ml) for a total of three injections. One week after the last injection, they were challenged with Jap. 305, NWS, or PR8 virus at a time when mean homotypic HI antibody titer was 1:128 (Table 2). No protection (as evidenced by reduction in pulmonary virus titers) was afforded to mice immunized with inactivated PR8 virus and then challenged with A2 (Jap. 305) virus infection. In contrast, PR8-immunized animals were at least partially protected upon challenge with either the PR8 or NWS strains of influenza A virus. Thus, homotypic but not heterotypic immunity was elicited by parenteral immunization in this experiment.

Antibody response to challenge infection as an indication of heterotypic immunity. Attempts were also made to determine whether the partial immunity to A2 virus challenge afforded by prior NWS virus infection was associated with a more rapid appearance or greater titer of HI antibody to A2 virus after challenge. Mice were infected with NWS or Lee virus by aerosol, or were exposed to an aerosol of saline. They were challenged with Jap. 305 virus by aerosol 4 weeks later. HI antibodies to Jap. 305 virus were measured in individual sera of five animals from each group (Fig. 2). HI antibodies to this virus were not demonstrable before day 7 in any of the three groups. On day 7, however, all of the mice previously infected with NWS virus had demon-

strable titers of antibody, whereas only one out of five animals in each of the other two groups had detectable antibody. Despite this accelerated immune response in animals previously infected with NWS virus, reduced titers of virus were demonstrable in the lungs of these animals as early as 24 to 28 hr after challenge with Jap. 305 virus, long before circulating antibody was detectable.

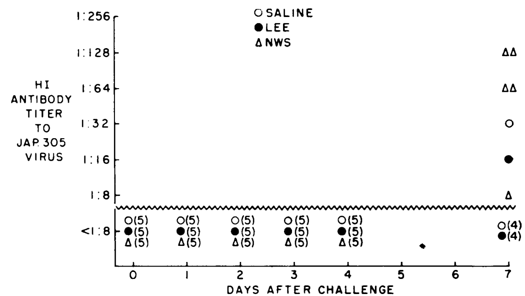

FIG. 2. *HI antibody response after challenge with Jap. 305 virus in animals previously infected with heterotypic or heterologous viruses.*

DISCUSSION

The present studies demonstrate that significant immunity may be induced in mice by a single aerosol-mediated infection with an influenza A virus, heterotypic, but not heterologous, to the virus subsequently used for challenge. This partial immunity is reflected by 10- to 100-fold reduction in the titers of infective challenge virus in the lungs, by diminution of macroscopically discernible pulmonary lesions, and by reduction in mortality. That this partial immunity is specific is strongly indicated by the failure of antigenically heterologous (influenza B) virus either to induce partial immunity, or to indicate immunity when used in the challenge infection. Further, heterotypic (influenza A), but not heterologous (influenza B), virus infection led to an accelerated homotypic antibody response to the challenge virus—suggesting specific antigenic recall, and hence, the sharing of antigens operative in immunity.

It is unlikely that the partial immunity induced by heterotypic infection is a nonspecific effect. The course of the initial immunizing infections with the several viruses employed was quite comparable, because all but the RI/5^{+} strain of influenza A2 virus were mouse-adapted and multiplied to comparable levels in the lung, and induced gross lesions of comparable degree. Viral interference may be excluded as a likely mechanism of immunity in the present studies, because earlier studies (Schulman and Kilbourne, 1963*a*) of influenza virus infection in the mouse lung have shown this to be a phenomenon of relatively brief duration. Interference is inducible also with heterologous virus, and no effect was observed with heterologous (Lee) virus in the present investigation.

The present studies have been concerned with the ultimate expression of immunity as evidenced by reduction in viral replication and disease, and not with the quality of the antibody response. Nevertheless, they offer confirmation of the work of Henle and Lief (1963), and extend their observations on heterotypic protection after repeated monotypic infection by demonstrating that substantial immunity may be engendered by a single heterotypic infection. In accord also with the earlier studies of cross-immunity with human and swine influenza viruses, protection in the absence of detectable heterotypic antibody was observed in our experiments.

The failure of repeated parenteral injections of noninfective virus to provide heterotypic immunity is of interest, particularly because high levels of circulating homotypic antibody were induced. This observation is consistent with earlier evidence that the antibody formed in response to parenteral injection of nonreplicating virus is more strain-specific than that produced after infection (Francis, 1959). However, it may simply reflect a lesser antigenic mass provided by parenteral injection compared to that elaborated during infection. Alternatively, the greater efficacy of immunization with live virus may be related to the priming of antibody-forming cells in proximity to the respiratory tract. This latter hypothesis is consistent with our finding that mice previously infected with heterotypic virus have accelerated antibody response to the challenge virus despite reduced concentrations of virus in the lung after challenge. Thus, reduced viral replication may be the sequel of prompt local antibody response, primed by earlier heterotypic infection.

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LITERATURE CITED

CHOPPIN, P. W., AND I. TAMM. 1959. Two kinds of particles with contrasting properties in influenza A virus strains from the 1957 pandemic. Virology **8**:539–542.

DAVENPORT, F. M., A. V. HENNESSY, AND T. R. FRANCIS, JR. 1953. Epidemiologic and immunologic significance of age distribution of antibody

to antigenic variants of influenza virus. J. Exp. Med. **98**:641–656.

FRANCIS, T., JR. 1959. Influenza, p. 633. *In* Thomas M. Rivers and Frank L. Horsfall, Jr. [ed.], Viral and rickettsial infections of man, 3rd ed. J. B. Lippincott Co., Philadelphia.

FRANCIS, T., JR., J. E. SALK, AND J. J. QUILLIGAN, JR. 1947. Experience with vaccination against influenza in the spring of 1947. Amer. J. Public Health **37**:1013–1016.

FRANCIS, T., JR., AND R. E. SHOPE. 1936. Neutralization tests with sera of convalescent or immunized animals and the virus of swine and human influenza. J. Exp. Med. **63**:645–653.

HENLE, W., AND F. S. LIEF. 1963. The broadening antibody spectra following multiple exposures to influenza viruses. Amer. Rev. Respirat. Dis. **88**:379–386.

HORSFALL, F. L., JR. 1939. Neutralization of epidemic influenza virus. J. Exp. Med. **70**:209–222.

JENSEN, K. E., F. M. DAVENPORT, A. V. HENNESSY, AND T. FRANCIS, JR. 1956. Characterization of influenza antibodies by serum absorption. J. Exp. Med. **104**:199–209.

LENNETTE, E., AND F. L. HORSFALL. 1941. Studies on influenza virus: CF antigen of influenza A and swine influenza viruses. J. Exp. Med. **73**:581–599.

LIEF, F. S., AND W. HENLE. 1960. Antigenic analysis of influenza viruses by complement fixation. VI. Implication of age distribution of antibodies to heterologous strains following infection and vaccination. J. Immunol. **85**:494–510.

SCHULMAN, J. L., AND E. D. KILBOURNE. 1963*a*. Induction of viral interference in mice by aerosols of inactivated influenza virus. Proc. Soc. Exp. Biol. Med. **113**:431–435.

SCHULMAN, J. L., AND E. D. KILBOURNE. 1963*b*. Experimental transmission of influenza virus infection in mice. I. The period of transmissibility. J. Exp. Med. **118**:257–266.

SHOPE, R. E. 1935. The infection of mice with swine influenza virus. J. Exp. Med. **62**:561–572.

SHOPE, R. E. 1937. Immunological relationship between the swine and human influenza viruses in swine. J. Exp. Med. **66**:151–168.

SHOPE, R. E., AND T. FRANCIS, JR. 1936. The susceptibility of swine to the virus of human influenza. J. Exp. Med. **64**:791–799.

SMITH, W., C. H. ANDREWES, AND P. P. LAIDLAW. 1933. A virus obtained from influenza patients. Lancet **2**:66–68.

SMITH, W., C. H. ANDREWES, AND P. P. LAIDLAW. 1935. Influenza: experiments on the immunization of ferrets and mice. Brit. J. Exp. Pathol. **16**:291–302.