INFECTION AND IMMUNITY, July 1979, p. 202-207
0019-9567/79/07-0202/06$02.00/0

Vol. 25, No. 1

Antibody Responses and Interferon Titers in the Respiratory Tracts of Mice After Aerosolized Exposure to Influenza Virus

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Received for publication 17 April 1979

We studied the temporal appearance of immunoglobulins (immunoglobulins G1, G2, M, and A) and interferon in lung lavage fluids of mice after aerosol exposure to influenza virus in six animal groups in which mortality rates ranged from 0 to 24%. Immunoglobulin levels in the lung lavage fluids were markedly higher in mouse groups with higher mortality rates (16, 20, and 24%) than in those with low mortality rates (0, 2.5, and 7.5%). Analysis of serum albumin in the respiratory secretions as an index of edema indicated that increased immunoglobulin G levels during the early phase of infection were due to increased vascular permeability. The detection of virus-neutralizing antibodies and antibodies reactive with influenza virus antigens in the lavage fluids at 6 to 8 days postinfection suggested local immunoglobulin synthesis as a result of antigenic stimulation. Both systemic and local antibody productions contributed to immunoglobulin levels in the respiratory secretions after aerosolized influenza virus infection. Peak levels of interferon in the lavage fluids were reached before detection of significant levels of virus-neutralizing antibody in the serum or the lung lavage.

Exposure of the respiratory tract to viruses has been shown to induce increases in immunoglobulin and interferon levels by examination of nasal secretions (3, 9, 10). It has been shown that viral vaccines may be used to stimulate these responses and raise the host resistence to infection (12, 21, 22). The secretory immune response appears to play an important role in respiratory viral infections (14, 18, 23).

There have been relatively few studies on the secretions from the lower respiratory tract due to a limitation of effective bronchial lavage techniques, and the analysis of time-related events during the disease process has necessarily been limited. Reynolds and Newball (15) analyzed the proteins and cells from the bronchial-alveolar lavage fluid of human lungs by transnasal fiber optic bronchoscopy. Using the technique of bronchopulmonary lavage, Waldman et al. (21) demonstrated immunoglobulin G (IgG) and IgA in bronchoalveolar fluids from human volunteers immunized with inactivated influenza virus vaccine by the aerosol route. Recently, Scott and Sydiskis (19) reported immunoglobulin levels in mice immunized with influenza virus. In the present study the severity of influenza infection was related to quantitative changes in the serum albumin and immunoglobulin contents of respiratory secretions. Virus-reactive antibodies and interferon were assayed in lung lavage fluid.

MATERIALS AND METHODS

Virus. Influenza A_0 virus (WSN) was propagated in Madin-Darby bovine kidney (MDBK) cells as described by Choppin (5).

Mice. Male Swiss-Webster mice (10 to 13 weeks old) were used (Horton Lab, Oakland, Calif.), except for the specific pathogen-free mice (Hilltop Lab Animals, Inc., Scottdale, Pa.) which were used in the trial producing 16% mortality. Mice were housed in an isolation room with 15 air exchanges per h at a temperature of approximately 22°C.

Aerosol exposure of mice to influenza virus. Groups of mice (usually 65) were exposed to nebulized influenza virus in a TRI-R airborne infection apparatus (TRI-R Instruments, Inc., New York, N.Y.) for 30 min. The relative humidity in the exposure chamber was approximately 50% at 22°C.

Virus assay. Lungs from six mice for each sampling day were harvested aseptically, and a 10% lung homogenate was prepared in Eagle medium. The tissue suspensions were centrifuged at 400 × *g* for 15 min, and the supernatant fluid was assayed for infectious virus in MDBK cells by the plaque method under a 1.0% agar overlay.

Collection of lung lavage and serum samples. Pulmonary lavage was performed on anesthesized mice (pentabarbital sodium), which were bled out by severing the renal artery. Blood was collected for serum analysis. The lung lavage technique has been described in detail elsewhere (11). The lavage procedure immediately followed blood collection and was completed within 5 to 10 min after death. The lung fluids from groups of 18 mice were pooled and chilled.

The samples were centrifuged to remove cells and then concentrated about 50 times by vacuum dialysis with a collodion bag apparatus (Schleicher & Schuell Co., Keene, N.H.). Concentrated fluids from normal lung lavages contained approximately 0.3 mg of protein per mouse. The pooled lung lavage fluids and serum samples were stored at −20°C.

Immunodiffusion. Quantitative assays for proteins in the lung lavage fluids and sera were performed with the single radial diffusion method. Goat antisera to the heavy chains of mouse IgG1, IgG2, IgA, and IgM (Meloy Labs, Springfield, Va.) or anti-mouse albumin (Cappel Labs, Inc., Cochraneville, Pa.) were incorporated into 1% agarose. Tests of the antisera against mouse serum indicated the purity of the reagents.

Single radial diffusion tests were run in rectangular dishes and analyzed in triplicate to obtain the precipitin ring diameters. Standard curves were prepared for the immunoglobulin assays from an ascites fluid standard and for albumin from a mouse serum albumin preparation.

Interferon assay. Pooled bronchopulmonary lavage fluids were assayed for interferon activity by the method for vesicular stomatitis virus plaque reduction in L cells (6). Interferon units were expressed as the reciprocal of the dilution causing 50% plaque reduction. A standard reference mouse interferon (catalog no. G00-90A-511; National Institutes of Health) was included in each assay. One unit of interferon in our assay was equivalent to two units of the reference interferon.

Identification of the virus-inhibiting substance in the bronchopulmonary lavage fluids as interferon was based on the following criteria: host species specificity, trypsin sensitivity, non-dialyzability, stability at pH 2.0, failure to sediment at 100,000 × *g*, and inhibition by actinomycin D.

Neutralizing antibody assay. Neutralizing antibody titers in pooled lung lavage fluids and pooled sera were determined by the 50% plaque reduction technique (20).

Indirect fluorescent antibody assay. Immunoglobulin class-specific influenza virus antibodies in lung lavages were demonstrated by the indirect fluorescent antibody method (2). Slide cultures of BHK cells were infected with influenza virus and fixed with acetone. Concentrated lung lavage samples were diluted with saline, and each dilution was applied to duplicate slide cultures. The slides were incubated at 35°C for 1 h and washed twice with phosphate-buffered saline (pH 7) for 10 min. The slides were stained with fluorescein-conjugated antisera prepared in goats against the heavy chains of mouse IgG1, IgG2, IgA, and IgM (Meloy). The slides were washed, mounted in 25% glycerin in phosphate-buffered saline (pH 7.6), and examined with a Zeiss microscope using epi-illumination.

RESULTS

Course of influenza virus infection in mice. The study examined influenza infection at a severity causing deaths in no more than 25% of the animals. It was found that a virus concentration of 10^7 plaque-forming units per ml would result in 100% mortality (unpublished data). In experiments reported here a concentration of 5×10^4 plaque-forming units per ml was used on the group where no deaths occurred, and the other five groups were exposed to an aerosolized virus suspension of 5×10^5 plaque-forming units per ml. In each of the six experiments, approximately 200 mice were exposed to aerosolized virus. In all instances, deaths of infected mice occurred between 9 and 12 days after virus exposure. Mice were periodically removed for assays as the experiments progressed so that the exposed population was reduced to approximately 90 mice at the time mortalities began to occur. Mortality rates were then determined on the basis of the number of animals in the experiment when deaths began. Fatal infections were produced at levels of 0, 2.5, 7.5, 16, 20, and 24% of these populations.

The influenza virus titers increased rapidly in lung tissue and appeared to reach a maximum on day 4 after exposure (Fig. 1). Virus replication occurred to a greater extent in those groups with higher mortality rates (7.5, 16, 20, and 24%). Thus, at the end of day 1, there was a difference of nearly 2 logs between the virus titers of the 0 and 24% mortality groups. This general relationship still existed on day 4. Virus titers tended to persist at 10^3 to 10^4 plaque-forming units per g of lung tissue through day 8. The virus was undetectable in mice that survived for 16 days.

Serum albumin in respiratory secretions. The levels of serum albumin in lung lavage fluid were monitored as an index of edema. The albumin content in the respiratory secretions of uninfected mice had been determined to be ap-

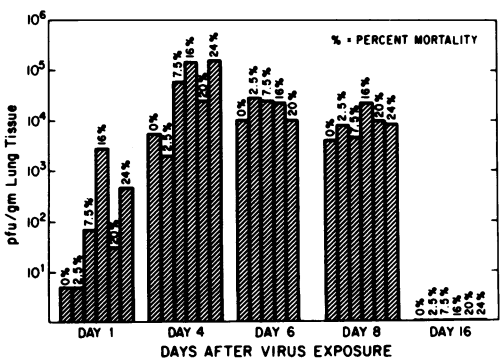

FIG. 1. *Assays for influenza virus in the lungs of mice after aerosolized exposures. The percent mortality for each group is indicated. Each assay was performed on a lung homogenate from six mice. pfu, Plaque-forming units.*

proximately 240 μg of precipitable protein per mouse. This content was presumed to be the transudation level of albumin in respiratory secretions. The edema levels began to rise slowly during the early days of the process, and by day 6 the mean level in six experiments had risen to 760 μg/mouse, an increase of more than threefold (Fig. 2). Edema was pronounced on day 8 when the process was about to result in fatalities. At that time the mean level was 2,370 μg/mouse, and in the group destined to experience 20% mortality the albumin level reached 5,150 μg/mouse. In contrast, the group that experienced no mortality had a serum albumin level of only 1,040 μg/mouse on day 8. Edema levels had declined markedly by day 16, but the level of albumin tended to remain somewhat above normal through 28 days of observation.

Immunoglobulins in lung lavage fluid. Graphs of the IgG1 and IgG2 content in lung lavage fluids resembled the albumin curve and suggested that much of antibody of the IgG classes in respiratory secretions was accumulated as a result of increased vascular permeability (Fig. 3). The amounts of IgG1 and IgG2 present again reflected the severity of the disease process, as shown by the levels for the 0% mortality group versus the 20% mortality group.

The levels of IgM were always low, but it was noted that the plotted curves did not follow those obtained for the IgG classes (Fig. 3). When edema was at its peak on day 8, the IgM levels were only moderately increased and they tended to peak on day 16. The findings indicated that serum IgM was not a common constituent of the edema fluid, and that levels of IgM accumulating later were likely the result of local antigenic stimulation in the lungs.

Similar results were obtained for the analysis of IgA in the respiratory secretions (Fig. 3). The mean value at day 6, when edema fluid was accumulating, was less than 2 μg of IgA per mouse. On day 8 at the height of the exudative phase, the IgA level had risen to little more than 5 μg/mouse. Increases in IgA were detected on day 16 when the 20% mortality group showed more than 15 μg of IgA per mouse. The evidence was compatible with local antigenic stimulation and local IgA synthesis.

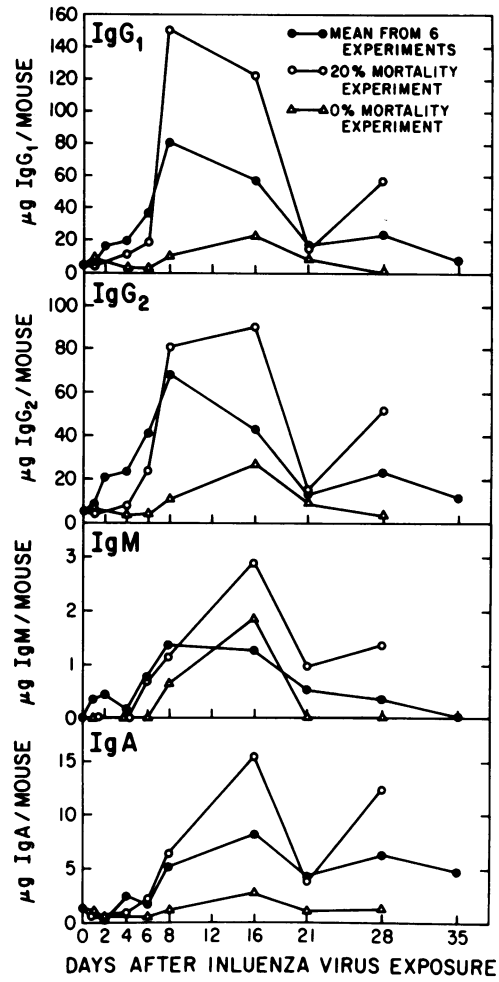

FIG. 3. *Immunoglobulin content in lung lavage fluid from influenza virus-infected mice. Normal mice were used to determine the zero-time value.*

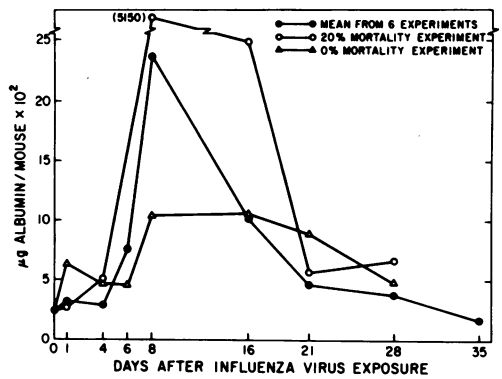

FIG. 2. *Serum albumin content in lung lavage fluid from influenza virus-infected mice. Normal mice were used to determine the zero-time value.*

Virus-neutralizing antibody titers. Antibodies neutralizing influenza virus were present in the sera and respiratory secretions of mice from all of the experimental groups. As Fig. 4 shows, these antibodies were detected on day 8 of the infection but not on day 6. Titers were rising through day 28 and were still present on day 35.

Antibodies to influenza virus among the

immunoglobulin classes. The indirect fluorescent antibody method was used to detect virus-reactive antibodies in respiratory secretions according to each immunoglobulin class. This method was more sensitive than the virus plaque reduction technique for neutralizing antibodies. Thus, antibodies were detected on day 6 in two of the tested groups (Table 1). On day 8 all of the experimental groups had indirect fluorescent antibody titers. At that time one-half of the groups showed IgM responses and one-half showed IgA responses. In five of the six experiments, one or both IgG subclasses were represented on day 8. Titers were rising through day 16, and by day 21 all immunoglobulin classes in all of the experiments contained antibodies directed against influenza virus. By day 35 it was apparent that the titers were falling.

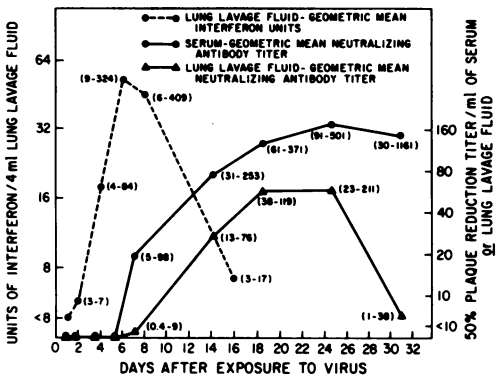

FIG. 4. *Interferon in lung lavage fluid and influenza virus-neutralizing antibodies in serum and lung lavage fluid. Standard deviations are expressed as titers or units in parentheses. Data are from six experiments.*

Interferon in respiratory secretions. Interferon titers were determined in the respiratory secretions. On day 4 after exposure to virus, interferon activity was present in three (2.5, 16, and 24% mortality) of four (2.5, 16, 20, and 24% mortality) tested groups (Fig. 4). All groups became positive, with the highest titers obtained from days 6 to 8. Experimental groups with greater severity of infection tended to have higher interferon levels. By day 16 the mean titer had declined to a low level.

DISCUSSION

Immunoglobulin levels and interferon in external secretions have been shown to be important in several respiratory viral infections. We

TABLE 1. *Anti-influenza virus titers among immunoglobulin classes in lung lavage fluids of mice after infection by the aerosol route*

% Mortality in group	Immunoglobulin class	Titer of antibody to influenza virus at the following no. of days after virus exposure:[a]									
		1	2	4	6	8	12	16	21	28	35
0	IgM	—[b]	—	0[c]	2	8	—	—	32	32	—
	IgG1	—	—	0	8	8	—	—	16	32	—
	IgG2	—	—	0	0	8	—	—	8	8	—
	IgA	—	—	0	0	8	—	—	32	64	—
2.5	IgM	0	—	0	0	0	—	8	8	64	—
	IgG1	0	—	0	0	0	—	0	8	32	—
	IgG2	0	—	0	0	0	—	0	8	64	—
	IgA	0	—	0	8	8	—	8	8	64	—
7.5	IgM	0	0	0	0	0	16	16	32	8	0
	IgG1	0	0	0	0	0	0	64	32	32	16
	IgG2	0	0	0	0	4	16	32	128	64	32
	IgA	0	0	0	0	0	4	16	256	16	4
16	IgM	—	0	0	0	0	—	64	128	64	32
	IgG1	—	0	0	0	0	—	16	16	64	32
	IgG2	—	0	0	0	4	—	256	256	128	64
	IgA	—	0	0	0	0	—	0	16	16	16
20	IgM	—	—	0	—	2	—	—	32	—	—
	IgG1	—	—	0	—	2	—	—	32	—	—
	IgG2	—	—	0	—	0	—	—	32	—	—
	IgA	—	—	0	—	0	—	—	8	—	—
24	IgM	0	—	—	—	8	—	32	—	—	—
	IgG1	0	—	—	—	16	—	32	—	—	—
	IgG2	0	—	—	—	2	—	8	—	—	—
	IgA	0	—	—	—	8	—	8	—	—	—

[a] Titers are expressed as the reciprocal of the highest dilution showing fluorescence by the indirect fluorescent antibody method. Samples were pooled and concentrated 50 times.

[b] —, Not tested.

[c] A value of zero indicates that the titer was less than 2.

undertook this study to analyze the events after aerosol exposure of mice to the human influenza virus in six animal groups in which mortality rates ranged from 0 to 24%. Our previous studies demonstrated that low concentrations of certain immunoglobulins were present in normal mouse lung lavage fluids (13). The mean values for IgG1, IgG2, and IgA in normal mouse lung lavages were 4.09, 5.45, and 2.36 μg/mouse, respectively. IgM and interferon were not detected. These values may be compared with an analysis for protein content in normal mouse serum (milligrams per milliliter): albumin, 35.1; IgG1, 2.53; IgG2, 1.85; IgA, 0.65; and IgM, 0.06. After influenza virus infection, a significant elevation of each immunoglobulin class and interferon was noticed, although the extent of response was substantially influenced by the severity of the infectious process.

In experiments with low mortality rates, immunoglobulin levels in the lung lavage fluids were minimal. The lower virus titers in the lung tissues of these animals may have been responsible for the lack of immunological responses, since there appears to be an association between the amount of antigen present and the level of immunoglobulin response in the respiratory tract (16). Marked increases in IgG1, IgG2, IgA, and IgM levels occurred in experiments with higher mortality rates (16, 20, and 24%). Immunoglobulin concentrations became detectable on day 6 after exposure to virus and reached their peaks on day 8 or 16, depending on the class of immunoglobulins. Maximum viral titers in these experiments were observed on day 4 after exposure. These findings are in close agreement with those of studies of coxsackie virus A-21 respiratory infections in human volunteers carried out by Rossen et al. (17). They demonstrated that the largest amount of virus was shed in nasal secretions 4 days after infection and that 2 days later a sharp elevation in total nasal wash proteins was observed.

There was much IgG in the respiratory secretions during the early phases of the process, but most of this was associated with increased vascular permeability and accumulations of serum proteins as part of the pneumonitis induced by the infection. Acute respiratory inflammation with its concomitant exudation of plasma proteins into the respiratory tract has also been observed with other infectious agents (1, 18). Analysis for serum albumin indicated peak levels on day 8, with high levels persisting through day 16. Fatal influenza infections clustered between days 9 and 12 postinfection. Albumin levels tended to persist somewhat above normal values during the repair processes in surviving mice. These serum protein levels served to indicate that normal pulmonary function had not completely returned during 35 days of observation. During the early days of the process, it was found that the level of viral replication was less in animal groups destined to have low mortality levels, and these same groups also had reduced evidence of edema, as shown by lower serum albumin and IgG levels in respiratory secretions.

Virus-neutralizing antibodies were found on day 8 in both serum and respiratory secretions. The titer was low at the time in the secretions, but much of the antibody available then would be expected to bind to viral antigen and thus become unavailable to assay as free antibody.

Antibodies detected as virus neutralizing and those designated by immunoglobulin class as reactive with influenza virus (by the indirect fluorescent antibody method) were the result of antigenic stimulation from the virus. These antibodies were detected 6 to 8 days into the process and at a time shortly before the deaths, which began on day 9. Titers for these antibodies tended to peak 3 to 4 weeks after the virus exposure, which indicated continued antibody synthesis from the stimulation of viral antigen that had been eliminated from the tissues 2 weeks previously.

Interferon has been presumed to play a major role in recovery from viral respiratory infection, especially in the primary infection with a specific respiratory virus (4, 7, 8). Early events with regard to the availability of interferon and the rapid production of antibodies were probably key determinants in governing the severity of the pneumonitis in different sets of mice. Early antibody to virus was detected on day 6 in the respiratory secretions of the groups experiencing 0 and 2.5% mortality. Both systemic and local antibody production would be expected to contribute to immunoglobulins in the respiratory secretions. The assay curves (Fig. 3) for IgM and IgA suggested that much of these immunoglobulins came from local synthesis. Histological examination of influenza virus-infected lungs has revealed large numbers of IgM-, IgA-, and IgG-containing cells (unpublished data). The present study, as well as the study conducted by Iwasaki and Nozima (8), shows that in the primary infection of mice with influenza aerosols, the peak levels of interferon in lung lavages were reached before detection of significant levels of neutralizing antibody against influenza virus in the serum or in the lung lavage. Interferon began to appear in lung lavages at a time when viral titer was maximal, but it did not reach its peak concentration until the viral titer in the lung tissues began to decline. Higher interferon levels were

found in those experiments with higher mortality rates. It is apparent that the production of interferon is a direct consequence of virus replication in the lung tissues of infected animals. Nevertheless, the precise role of interferon in the recovery of viral respiratory infections still remains to be determined.

ACKNOWLEDGMENTS

This investigation was supported in part by Public Health Service research grant ES 628 from the National Institute of Environmental Health Sciences and by California Air Resources Board grant A7-179-30.

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