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Dynamics of B-Lymphocytes in the Lungs of Mice Exposed to Aerosolized Influenza Virus

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Immunoglobulin-containing cells were revealed by immunofluorescence in lung sections from mice infected with influenza virus by the aerosol route. The numbers of immunoglobulin A (IgA)- and IgM-containing cells were increasing by day 3 of the infection, whereas IgG-containing cells appeared a few days later. The responding B-cell populations appeared in two principal locations: along major airways and in consolidated lesions within lung parenchyma. IgA-containing cells were the most numerous isotype, occurring predominantly in the lamina propria of the airways. IgG-containing cells were the least frequently encountered class along airways and appeared most often within consolidated lung lesions in clustered groupings. Cells staining for mu chain appeared along the airways and in lung lesions. The population of IgM-containing cells declined approximately 30 days after infection. Cells producing alpha and gamma chains were still numerous on day 46. Assays for virus-reactive antibodies in lung secretions were positive on day 8 of the infection. The IgM titers were the first to decline, but virus-binding antibodies for all classes were still present on day 33. The implications of immune responses in viral pneumonitis were considered.

Lungs in the normal or unstimulated state contain a sparse amount of lymphoid tissue (2), but immunocytes of the respiratory tract can be activated by a variety of antigenic stimuli, including inhaled infectious disease agents and inert sensitizing antigens (3, 14). Immunological events in the respiratory tissues are of interest in understanding lung diseases and in the elaboration of immunoprophylactic procedures.

Pertinent immunological studies on the respiratory tissues of mice include an analysis of the serum-related proteins in respiratory secretions of normal mice (17) and the detection of specific antibody in secretions 2 to 3 weeks after exposure to aerosolized influenza virus (20). Antibody responses and interferon titers in mice infected with influenza virus were reported by Zee et al. (28). Lymphocytes freed from the lungs of influenza virus-infected mice included an increased number of cells bearing surface immunoglobulins (21). Rises in the numbers of immunoglobulin A (IgA)-containing cells in lung tissue were encountered when mice were exposed to ozone as a mild irritant to the respiratory membranes (18).

This study presents information on lymphocyte biology by examining the pneumonitis arising from influenza virus infection in mice. The timing of responses by immunocytes synthesizing mu, gamma, and alpha heavy chains was investigated. Rising and declining cell populations were correlated with the antibody content of lung secretions.

MATERIALS AND METHODS

Animals. Specific-pathogen-free Swiss-Webster mice at 10 weeks of age were used (Hilltop Lab Animals, Inc., Scottdale, Pa). The mice were housed in an isolation room with 15 air exchanges per h at a temperature of ≈22°C.

Aerosol exposure of mice to influenza virus. Influenza A_0 virus (WSN) was propagated in Madin-Darby bovine kidney cells as described by Choppin (7). Groups of 50 to 60 mice were exposed for 30 min to nebulized influenza virus in a TRI-R airborne infection apparatus (TRI-R Instruments, Inc., New York, N.Y.). The relative humidity in the exposure chamber was approximately 50% at 22°C. Virus-exposed mice were maintained in cages under microbiological filters and held in an isolation room. Normal control mice were similarly maintained in a separate isolation room.

Sectioning and fixing lung tissues. Mice were anesthetized with pentabarbital sodium and exsanguinated. Lungs were infused in situ with an inert, water-soluble supporting medium for frozen tissues (Tissue-Tek, Labtek Products, Miles Laboratories, Naperville, Ill.). Approximately 0.7 ml of Tissue-Tek was infused through the trachea, which was then tied off to prevent escape of the viscous fluid. The left lung was cut longitudinally down the main bronchus, frozen in Tissue-Tek, and stored in liquid nitrogen (18).

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Lung sections of 6-μm thickness were cut on a cryostat. Serial sections of the left lung, oriented longitudinally through the lobar bronchus, were placed on clean slides. The slides were dried, acetone fixed for 30 min, and stored at −20°C until they were stained and examined.

Enumeration of antibody-containing cells. Fluorescein-conjugated antisera to the heavy chains of mouse immunoglobulins were used to detect antibody-forming cells in lung tissue by the direct staining technique (8). Antisera were produced in goats from mouse myeloma proteins (Meloy Laboratories, Springfield, Va.).

The conjugated antisera were diluted in phosphate-buffered saline (pH 7.2) and tested against mouse spleen cells as positive controls. Negative controls were obtained from normal lung tissue and preparations of alveolar macrophages. When we assayed for antibody-forming cells in lung tissue, the conjugated anti-immunoglobulins, including anti-IgA, anti-IgM, and anti-IgG, were applied, respectively, to the first, second, and third serial sections on a slide. Slides were incubated for 1 h at 37°C with the conjugates. Sections were washed and then covered with mounting medium (1:1 glycerol–phosphate-buffered saline, pH 8.3) and a cover slip.

Examinations were made on a Zeiss microscope, using epi-illumination. A 16× objective and a 10× eyepiece were used to photograph regions of the lung used for cell counting. Counts were made on 18 photographed fields per section, comprising an area of 2.09 mm^2. The 18 microscopic fields were designated as a lung unit (LU), which was located along a 4.0-mm section of the lobar bronchus, and the adjacent tissue, out to the pleural surface. Counts were made from three tissue locations. (i) The first location included six fields along the bronchus, with "membrane-related cells" counted as those located in the lamina propria. By definition, membrane-related cells were within photographed fields containing the major airway. Antibody-containing cells that were clearly located in adjacent alveolar tissue were scored as lung parenchyma-related cells. (ii) The second location was composed of six fields in areas of cellular hyperplasia or infiltration. These regions included consolidated lung lesions and areas of lymphoid cell expansion around blood vessels and at branch points of the airways. (iii) The third location encompassed six fields of alveolar tissue that had normal, or near normal, cellularity. The rationale for this procedure is presented below. Cells counted showed cytoplasmic staining.

Microscopic fields were photographed on Tri-X panchromatic film (Eastman Kodak Co., Rochester, N. Y.). To facilitate counting, the 35-mm negatives were projected on a screen containing grid lines.

Collection of lung lavages. The lung lavage technique has been described in detail elsewhere (15, 17). Lung lavage fluids were collected from groups of 18 mice. The lavage fluids were pooled, chilled, freed of cells, and concentrated by vacuum dialysis. The pooled lung lavage fluids were stored at −20°C.

Immunodiffusion. Quantitative assays for proteins in the lung lavage fluids were made by using the single radial diffusion method. Goat antisera to the heavy chains of mouse IgG1, IgG2, IgA, and IgM (Meloy Laboratories) or to antimouse albumin (Cappel Laboratories, Cochraneville, PA.) were incorporated into 1% agarose. Purity of the reagents was determined by tests of the antisera against mouse serum (17).

Indirect fluorescent-antibody assay. Immunoglobulin class-specific influenza virus antibodies in lung lavages were demonstrated by the indirect fluorescent-antibody method (28).

RESULTS

Antibody-containing cells in the lungs of uninfected mice. Counts for antibody-containing cells were made on the lungs of 20 normal mice. Very few cells containing IgM or IgG were present (mean values, 9 and 4 cells per LU, respectively), and in the majority of instances no cells containing these classes were observed. Cells containing IgA were more numerous, however, with a mean count of 40 cells per LU (17). The IgA-containing cells were located predominantly in the lamina propria of the bronchus (58%).

Experiment A. (i) Early phase of the infection. The viral pneumonitis in this experiment led to 43% mortality in the mice after exposure for 30 min to an aerosol generated by 8 ml of virus suspension containing 3.8×10^5 plaque-forming units/ml. As the infection progressed, observations were made on the B-cell populations of surviving animals. Samples were examined beyond the period of fatalities to trace the pattern of events in mice that had mounted a successful immunological response.

On day 1 after virus exposure, gross lesions were not apparent, and antibody-containing cell numbers were comparable to those of normal animals. By day 3 there were evidences of tissue destruction such as necrosis of respiratory epithelium (27). The number of cells containing IgA was starting to increase along the airways on day 3 (Fig. 1) Increasing numbers of IgM-containing cells were also detected (Fig. 2). By day 5 IgG-containing cells were present in small clusters among the accumulating mononuclear cells found in parenchymal lesions.

(ii) Phase of maximum disease. Deaths occurred during days 6 to 10. The titer of influenza virus exceeded 10^5 plaque-forming units/ml of 10% lung homogenate on day 7, but no virus was detected on day 13 or later. Extensive lung consolidation developed, with lesions sometimes involving 50% of the lung section (Fig. 3). A large increase of IgG-containing cells was seen on day 9 as these cells formed packed clusters within consolidated lesions (Fig. 4). The number of IgA-containing cells increased along the airways and in consolidated lesions in lung parenchyma. Cells containing IgM also increased dramatically in

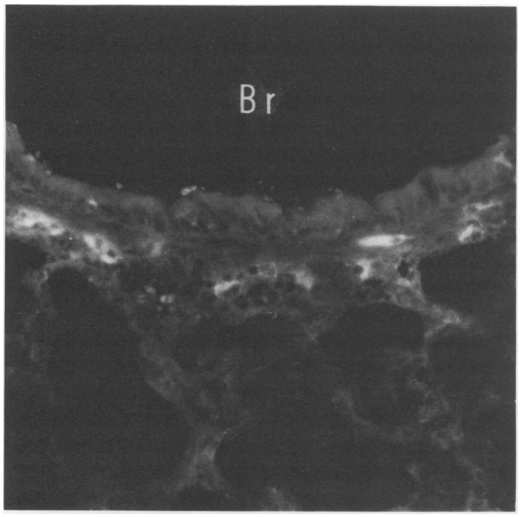

FIG. 1. *Increased numbers of IgA-containing cells along the main bronchus (Br) on day 3 of influenza infection (experiment A). The cells were revealed by fluorescein-labeled antibodies to the alpha heavy chain. ×240.*

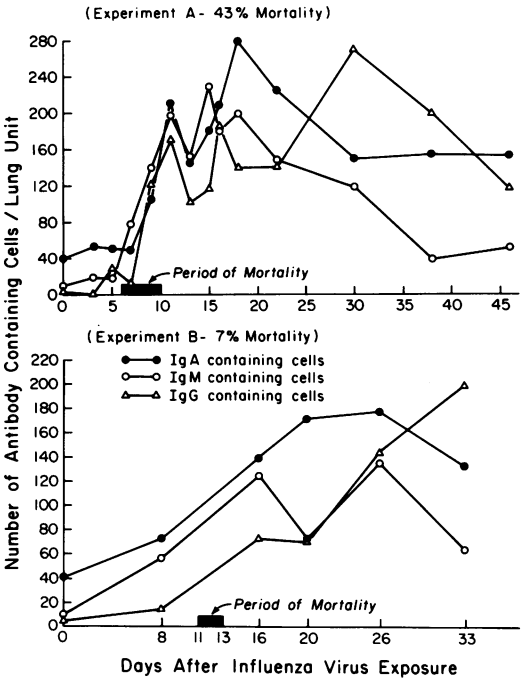

FIG. 2. *Response patterns of immunoglobulin-containing cells per LU in mice exposed to aerosolized influenza virus. Plotted values for experiment A (43% mortality) are averaged counts from two mice. Plotted values for experiment B (7% mortality) are averaged counts from four mice. Day 0 values are mean counts from 20 normal mice.*

samples taken from days 9 to 15, appearing as single cells or small clusters throughout consolidated areas and along the airway membranes. The IgM production reached a peak on day 15 (Fig. 5).

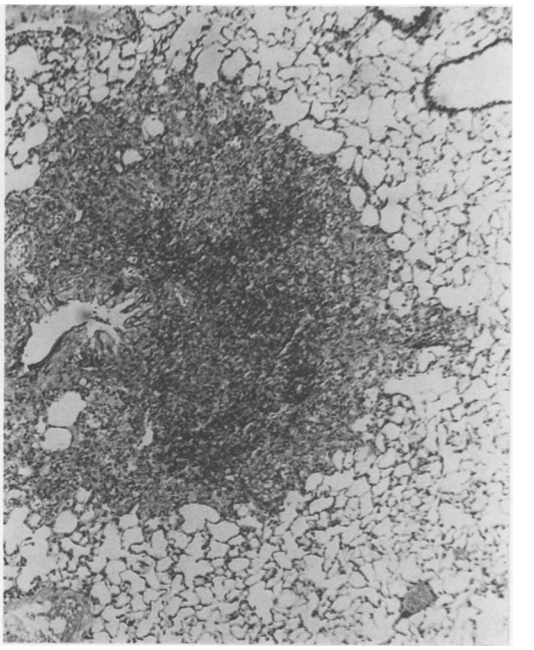

FIG. 3. *Extensive consolidated lesion around a small airway on day 13 of influenza infection (experiment A, 43% mortality). ×60.*

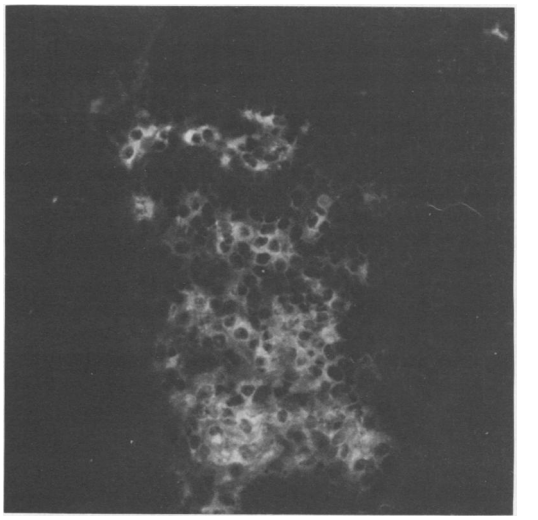

FIG. 4. *Cluster of IgG-containing cells (day 9 of infection). These colony-like cell groups occurred in consolidated lesions. The cells were revealed by fluorescein-labeled antibodies to the gamma heavy chain. ×240.*

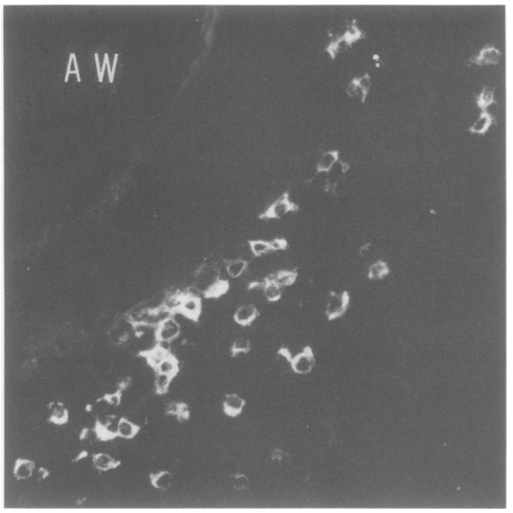

FIG. 5. *Airway (AW) on day 15 of influenza pneumonitis showing dispersed IgM-containing cells. Fluorescein-labeled antibodies to the mu heavy chain were used. ×240.*

(iii) Phase of recovery. The maximum number of IgA-containing cells was seen on day 18 postinfection, 1 week after the cessation of mortalities. Forty-five percent of the cells counted at that time contained IgA, and an elevated number of IgA-containing cells was still apparent 46 days after infection (Fig. 2). The numbers of IgM-producing cells had greatly diminished by day 33 postinfection, but IgG-containing cells did not reach peak levels until day 30 (Fig. 2). Synthesis of IgG remained at a high level, and lung tissue still contained regions of cellular infiltration where antibody-forming cells could be found after 46 days (Fig. 6).

Experiment B. (i) Phases of maximum disease and recovery. By using a smaller infecting dose of virus, a milder influenza process was induced in experiment B (7% mortality). Mice were exposed for 30 min to an aerosol containing 8 ml of virus suspension with a titer of 3.0×10^5 plaque-forming units/ml. The extent of lung damage was much less than had been encountered in experiment A, with mortalities occurring on days 11 to 13. Cellular responses were somewhat slower in developing, and the number of plasmacytes in the tissue was fewer than had been encountered in the more severe infection of experiment A (Fig. 2). The average number of antibody-containing cells in an LU was 453 in experiment A (mean of counts from days 7 through 46), compared with 327 in experiment B.

Consistent cellular response patterns in both experiments were early increases in IgA- and IgM-producing cells and the delayed rise in IgG-containing cell responses. IgM-containing cells were the first to decline, whereas many IgA- and IgG-producing cells persisted well beyond the time when virus could be isolated from lung tissue.

The importance of IgA synthesis was shown by the dominance of that set of B-cells in the process of viral pneumonitis (12). Table 1 shows that 38.3 and 42.2% of the cells counted in experiments A and B, respectively, were of the IgA class. Throughout the infection cells containing IgA were closely related to the lamina propria

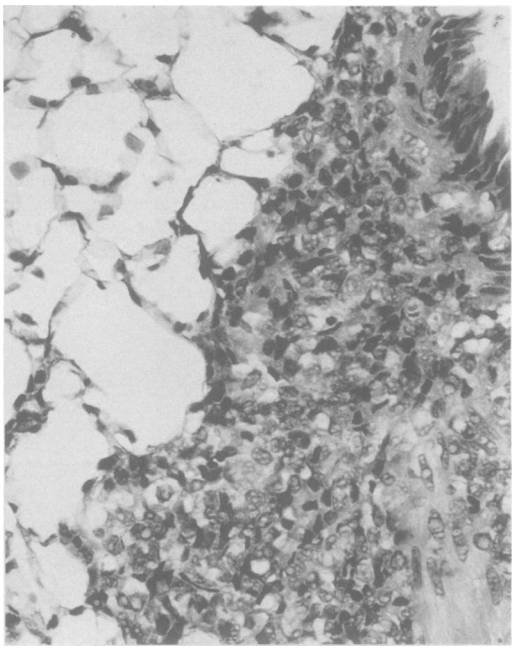

FIG. 6. *Persisting lesion in the lung of a mouse 46 days after infection with influenza virus. Antibody-containing cells were still present in such lesions. ×240*

TABLE 1. *Proportions of antibody-containing cells in the lungs of mice infected with influenza virus*

Immunoglobulin class	Antibody-containing cells			
	Expt A (43% mortality)[a]		Expt B (7% mortality)[b]	
	Total no.	%	Total no.	%
IgM	3,150	30.5	1,792	27.4
IgG	3,226	31.2	1,992	30.4
IgA	3,954	38.3	2,760	42.2

[a] Cells counted in 26 LU collected at 13 times during the infection (two mice per sampling time).

[b] Cells counted in 20 LU collected at five times during the infection (four mice per sampling time).

of the major airways and tended to be dispersed as individual mononuclear cells in connective tissue (Table 2). The clustering IgG cells were seen mainly in consolidated lesion sites or in nearby alveolar tissue. These cells were the least frequently encountered class in sites associated with the airways (Table 2).

(ii) Immunoglobulins in respiratory secretions. Changes in the immunoglobulin content of respiratory secretions were studied in a search for further evidence of local antibody synthesis. Pulmonary edema was to be anticipated as a consequence of inflammatory changes resulting from the infection. Since serum albumin in respiratory secretions arises from vascular sources, the amounts of albumin above normal transudation levels may be used as an index of edema. Increased vascular permeability associated with edema naturally leads to increasing levels of antibodies arising from the circulation. An impression of the extent of local antibody production can be gained from the relative change in immunoglobulin content of respiratory secretions, independent of exudated immunoglobulin levels, by comparing the ratio of immunoglobulin/albumin in the stressed state (viral pneumonitis) to the ratio found in the resting state (normal lung).

The high level of edema encountered on day 8 of the infection (Fig. 7) had also been observed in our previous studies on the influenza process (28). As expected, the immunoglobulin levels were greatly elevated at the period of maximum tissue damage and exudation. Much of the antibody in the respiratory secretions at that stage of the process resulted from increased vascular permeability. However, the ratio changes shown in Fig. 8 indicated a "real" increase of immunoglobulins in the respiratory secretions. The increase was greater on day 16 than it had been on day 8, even though infective virus had been eliminated from the tissues by day 16. The continued higher levels of IgG1, IgG2, and IgA classes suggested continued local synthesis. IgM was present in the secretions on days 8 and 16, but it had declined below the level of detection with the single radial diffusion method by day 20.

On day 8 of the infection, all immunoglobulin classes tested (IgG1, IgG2, IgA, and IgM) showed antibodies to influenza virus (Table 3). The titer for the IgM class continued to rise through day 16 and then declined. Peak titers for the IgG and IgA classes were reached a few days later (day 20). Thirty-three days after infection, antibodies to influenza virus of the various immunoglobulin classes could still be found in respiratory secretions.

DISCUSSION

Pneumonitis, induced by influenza virus, was studied here to observe B-cell responses. Influenza in humans is most often limited to virus attack on epithelial cells, leading to desquamation of epithelium in the nasal passages and throughout the tracheobronchial tree (6). How-

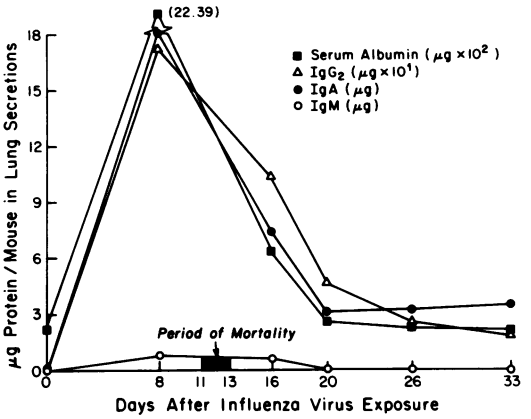

FIG. 7. *Immunoglobulin and serum albumin content in lung lavage fluid from influenza virus-infected mice (experiment B, 7% mortality). Graph points represent pooled samples from approximately 18 mice. Normal mice were used to determine the zero-time value.*

TABLE 2. *Location of antibody-containing cells in the lungs of mice at times of maximum increase during influenza virus pneumonitis*

Immunoglobulin class	Expt A (43% mortality)			Expt B (7% mortality)		
	Day of peak cell no.[a]	% Membrane related	% Parenchymal lesion related	Day of peak cell no.	% Membrane related	% Parenchymal lesion related
IgM	15	16	84	26	28	72
IgG	30	14	86	33	15	85
IgA	18	36	64	26	43	57

[a] Days after exposure to aerosolized influenza virus.

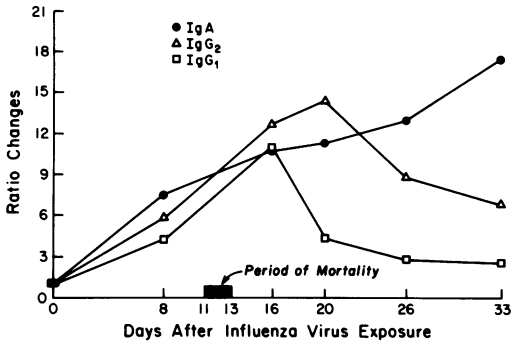

FIG. 8. *Relative changes in immunoglobulin content, independent of exudated serum immunoglobulin, in respiratory secretions of influenza virus-infected mice (experiment B, 7% mortality). The ratio of immunoglobulin/albumin at zero time was set at "1" for each immunoglobulin class. The value is based on the mean of 11 analyses of normal lung lavage fluids. Changes in ratios were calculated as test ratio/normal ratio.*

TABLE 3. *Anti-influenza virus titers in lung lavage fluids of mice*

Days after infection[a]	Titer[b]			
	IgM	IgG1	IgG2	IgA
8	16	2	2	4
16	32	64	64	16
20	16	32	64	32
33	4	8	4	4

[a] Days after exposure to influenza virus aerosol (experiment B, 7% mortality).

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

ever, pneumonitis may also develop, and the disease as it occurs in the human subject is considered to be essentially identical to that occurring in mice (16, 24).

Aerosolized influenza virus is widely distributed in the parenchyma of the lung and along the airways. As the virus enters susceptible cells and proliferates, there is an expansion in the amount of antigen that is locally available. Furthermore, systemic antigenic stimulation follows as virus spills out of the lungs to produce viremia (10). Lymphocytes located in the lung are, no doubt, the first to respond when the viral antigen accumulates along the airways. As virus replication progresses, general recruitment of lymphocytes into the lung would follow as cellular traffic expands into inflammatory sites.

It was beyond the scope of this investigation to enumerate all antibody-containing cells in the infected lungs. However, it was intended to obtain a time-lapse view of events occurring in different regions of the lung. To meet this objective, the LU was devised. Data from cell counts in LUs indicated times when cellular responses were increasing or decreasing. Although the procedure only gave an approximation of quantitative events, it did show the pattern of immunoglobulin class responses, as the proportions of immunoglobulin-containing cells changed during the infection. The regions of the lung in which B-cells producing a given immunoglobulin class were most likely to proliferate were also apparent.

The response patterns of IgM- and IgG-forming cells were reminiscent of the general pattern in lymphatic tissue wherein initial IgM production is followed by a switch to a more sustained synthesis of IgG (9). The observations with respect to IgA synthesis were in accord with the model of Abney et al. (1) for generation of immunoglobulin isotype diversity among developing lymphocytes. Their study implied that expression of IgG or IgA occurs developmentally on separate sublines of precursor surface IgM^+ cells. The specific-pathogen-free mice used in our study encountered the antigens of influenza virus for the first time, and the primary immune response promptly gave rise to IgA-containing cells. Whereas some evidence has been found for an IgG-to-IgA switch (13), the circumstances of our study were compatible with a model in which many of the cells express surface IgM and surface IgA, or simply surface IgA, and commense alpha-chain synthesis soon after antigenic stimulation. The extensive membranes in lung tissue would be expected to offer a favorable environment for this subset of B-cells (5, 11).

Although not studied here, cytotoxic T-cells have been found to be important in the elimination of virus from tissues (4, 22, 23, 25, 26). The extensive parenchymal lesions would be important sites for cytotoxic cell activity, in addition to serving as a major new region for antibody synthesis. Infective virus had been eliminated from the tissues in surviving animals a few days after the last deaths. Whereas all factors contributing to death in influenza pneumonitis are not known, the loss of respiratory exchange tissue from edema and consolidated lesion formation is of major importance. Delay in mounting an immune response could lead to overwhelming of the animal by massive lesion production and the associated inflammatory changes.

The study by Ramphal et al. (19) showed that the availability of injected serum antibodies protected the lungs of mice from severe pneumonitis after intranasal inoculation of influenza virus.

Antibodies neutralized virus and thus prevented further invasion of host cells. However, antibodies did not prevent the destruction of ciliated epithelium in the upper airways. Our studies have shown the presence of virus-specific antibodies in the respiratory secretions 6 to 8 days after infection (28). No doubt much of the antibodies formed early in the process reacted with the abundantly available viral antigen. The amounts of free antibody appearing in secretions during pneumonitis varied with the permeability of the respiratory membranes. In the exudative phase of the infection, the loss of membrane integrity permitted freer passage of antibodies, both these locally formed and those arising from the circulation, into the secretions. During the stages of lung healing and membrane repair, more of the IgG synthesized in the lung would be expected to move from the tissue spaces into lymphatic vessels, and then to the blood, whereas the levels in respiratory secretions would diminish. Such an effect was seen (Fig. 8, Table 3) as the ratio changes declined after 16 to 20 days in the respiratory secretions, and the content of virus-specific antibody was reduced, even though the number of IgG-containing cells in the lungs was elevated.

This study presents evidence that the limited number of immunologically competent cells in normal lungs is subject to great expansion in response to diffuse invasion by an infectious agent. The B-cell events were diverse with respect to class and timing. Antibody was formed in an apparent response by resident immunocytes, as well as by cells recruited from the general lymphocyte traffic into sites of tissue damage and concentration of viral antigen.

Antibody synthesis of the IgG and IgA classes was seen to continue for an extended period beyond the time of pneumonic crisis and after infective virus had disappeared. The special transport mechanism for IgA would maintain virus-specific antibodies in the secretions during convalescence, whereas the locally formed IgG could contribute in a major way to the serum titers, in addition to those of the respiratory secretions.

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