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Immunoglobulin Class-Specific Antibody Response in Serum, Spleen, Lungs, and Bronchoalveolar Washings After Primary and Secondary Sendai Virus Infection of Germfree Mice

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Immunoglobulin class-specific antibodies were measured by a solid-phase radioimmunoassay in serum, bronchoalveolar washings (BAW), lung cell lysates, and spleen cell lysates in germfree mice after intranasal (i.n.) and intraperitoneal (i.p.) primary and secondary 10^5, 10^4, and 10^3 mean tissue culture infective doses ($TCID_{50}$) of live parainfluenza 1 (Sendai) virus. The earliest antibody detected in lungs after i.n. virus challenge was immunoglobulin G (IgG), followed by IgM and, lastly, IgA. The local IgA response after both primary and secondary i.n. virus challenge was lowest after the severest infection. It is suggested that the delayed appearance of IgA antibody and the lower response after severe lung damage may be related to a temporary local secretory component-producing cell deficiency. The lungs were a major source of serum IgG antibody after both primary and secondary i.n. virus challenge. Only IgG and IgM antibodies were detectable in lung cell lysates after the i.n. 10^3 $TCID_{50}$ secondary response. A secondary response was detected in IgG, IgA, and IgM after secondary i.n. challenge with the other two doses. The lung response to all of primary and secondary i.p. doses of virus was exclusively IgG and IgM. Calculation of radioimmunoassay antibody per microgram of IgG, IgA, and IgM in serum and BAW after both i.n. and i.p. virus challenges showed that, when BAW antibody was present, the ratio in BAW was always higher than that in serum. This finding in the i.n. mice, together with the presence of IgA antibody-containing cells in the lungs, strongly indicates local manufacture and secretion of IgA antibodies in these animals and suggests that the same conclusion could apply to local IgG and IgM antibodies after both i.n. and i.p. challenges.

Virus inoculation of the respiratory tract results in production of local secretory immunoglobulin A (IgA), IgG (2, 21), and, sometimes, trace amounts of IgM (7, 18) antibody in bronchoalveolar washings (BAW). Studies of the immunoglobulin class of lung cells after respiratory virus infection in rodents have shown an initial IgG accumulation with few IgM-containing cells and a somewhat later IgA accumulation (6). Local cellular accumulation has been documented as early as day 2 postinfection (6), and local specific antibody has been detected by day 3 (5). The immunoglobulin class of the early antibody response is not known. It has been shown that free antibody in BAW after day 8 was both IgG and IgA; however, IgA antibodies predominated later (2, 21). The appearance of antibody in local immune complexes at day 3 postinfection has been shown to be associated with the disruption and desquamation of infected cells, accumulation of polymorphs, and severe bronchial basement membrane damage (3, 4). In addition, lungs have been shown to be anticomplementary at this time (9). There is no convincing data to show that secretory IgA can evoke such tissue-damaging responses or activate the complement bypass mechanism in vivo. It thus seemed likely that the early local antibody would be predominantly IgG, which could mediate what appears to be a local type III immunological reaction.

The studies reported in this paper set out to determine the immunoglobulin class of the earliest detectable specific antibody after respiratory virus infection and to document the subsequent class distribution and the effects of a secondary challenge. A parallel study of systemic virus administration and studies of the effect of viral dose were also undertaken.

MATERIALS AND METHODS

Experimental design. Six hundred female germfree random-bred Swiss albino mice, weighing 20 to 24 g, were purchased from Charles Rivers Ltd., Mon-

treal, Canada. Three hundred mice were infected with live Sendai virus intranasally (i.n.) (5), and 300 were infected intraperitoneally (i.p.). The mice in each of the two challenge groups were divided into three subgroups. The i.n. group received 0.04 ml and the i.p. group received 0.08 ml of 10^5, 10^4, and 10^3 mean tissue culture infective doses ($TCID_{50}$) of virus. Experiments were performed in duplicate, each subgroup consisting of 100 mice. Five mice from each subgroup were killed on each experimental day by ether anesthesia and exsanguination. On day 35 the remaining mice were rechallenged with the same dose by the same route as in the initial challenge, and animals were killed as for the primary challenge studies.

Virus. Parainfluenza 1 (Sendai) virus was raised in 10-day-old chicken embryos. The allantoic fluid was harvested after 72 h and clarified by centrifugation at 12,000 × *g* in a Sorvall RC-2B refrigerated centrifuge. The clarified fluid was stored at −70°C and used for infection of mice and as the source of viral antigen for the solid-phase micro-radioimmunoassay (RIA). Virus for RIA was titrated by the micro-hemagglutination technique. $TCID_{50}$ was measured by the hemadsorption technique in primary African green monkey kidney cells. End points were calculated according to the method of Reed and Muench (15).

Antisera. Rabbit anti-Sendai serum was prepared as previously described (5). Rabbit antisera specific for mouse IgG, IgM, and IgA were prepared by injecting purified mouse myeloma proteins (Litton Bionetics Inc., Kensington, Md.) in Freund complete adjuvant into 3-month-old female rabbits at 4-week intervals. Sheep anti-rabbit IgG serum was purchased from Joseph De Rose Associates, Downsview, Ontario, Canada. All antisera were made monospecific by adsorption and elution from immunoadsorbent columns (cyanogen bromide-activated Sepharose 4B; Pharmacia Fine Chemicals, AB, Uppsala, Sweden) prepared from appropriate purified mouse proteins or from rabbit IgG fraction II (Pentex Biochemical, Kankakee, Ill.). The antisera, assessed by immunoprecipitation techniques, were specific. The sheep anti-rabbit IgG serum was iodinated with ^{125}I (12) and conjugated with fluorescein-isothiocyanate (5) as described elsewhere.

Histopathology. Cold alcohol fixation of tissues was used for both conventional and immunofluorescent staining (17), and parallel sections of the lungs of two mice from each subgroup were stained by immunofluorescence for Sendai virus as previously described (5).

Preparation of cell lysates, BAW, and serum. Sera from five mice in each group on each experimental day were pooled and stored at −20°C and used for determination of specific antibody titers. BAW were obtained from the lungs of three exsanguinated mice in each group by lavage with 0.5 ml of phosphate-buffered saline containing 0.1% sodium azide (PBS). The lavages on each day were pooled and clarified by low-speed centrifugation, and the supernatants were stored at −20°C for further study.

Cell lysates were prepared from the spleen and lungs removed from each of the three mice used for BAW. These organs were stripped of any attached tissue and placed in 2 ml of PBS at 4°C. Cell suspensions were prepared by finely mincing and aspirating the tissues into 3-ml disposable syringes. The cell suspensions were washed three times in 10 ml of cold PBS with intermittent centrifugation at 4°C. After the final rinse the pelleted cells were suspended in PBS to give a weight/volume ratio of 1:4 for spleen and 1:2 for lungs. The suspensions were frozen and thawed once and then homogenized in a Dounce homogenizer at 4°C. The lysates were stored at −20°C until used. When required for testing they were thawed and clarified by centrifugation at 12,000 × *g* for 10 min.

RIA procedure. The RIA procedure was as reported in detail elsewhere (8). Microtiter plates were coated by air-drying 8 hemagglutinating units of virus in each well and subsequent alcohol fixation. Control plates were coated with uninfected allantoic fluid. Twenty-five-microliter test samples were serially diluted, applied to duplicate wells, and incubated for 1 h at 37°C. After careful saline washing, 25 μl of rabbit antiserum specific for mouse IgG, IgA, or IgM, appropriately diluted, was added. The plates were incubated for 1 h at 37°C and were then washed three times in saline. Finally, 25 μl of ^{125}I-labeled sheep anti-rabbit IgG (10,000 cpm) was added, and, after 1 h at 37°C, the plates were washed 10 times with cold tap water. The wells were cut out and counted in an automatic gamma counter (Searle automatic gamma counter, model 1285). The technique was able to detect 10^{-10} g of IgG and 10^{-11} g of IgA and IgM.

Calculation of RIA results. The reference standard for mouse IgG and IgM anti-Sendai antibodies consisted of whole pooled serum of mice hyperimmunized with killed Sendai virus. The reference standard for IgA anti-Sendai antibodies was pooled BAW of mice after recovery from i.n. Sendai virus infection. These standards were chosen for their high concentrations of class-specific antibody titers established by RIA testing. RIA standard curves were plotted from 12 doubling dilutions of each standard preparation, tested in duplicate. A 1:50 dilution of the IgG and IgM standard and a 1:2 dilution of the IgA standard were arbitrarily assigned 1,000 U of antibody activity. The units of antibody for each dilution of the standard was calculated from the initial dilution. Standard curves were prepared by plotting counts per minute against the units of antibody at each dilution. Six dilutions of each test sample were made and examined by RIA, and the counts per minute were obtained. The counts per minute at each dilution were applied to the standard curves, and the units of antibody obtained were corrected for sample dilution and averaged to give the units of class-specific anti-Sendai antibody for each sample tested.

The RIA technique permitted measurement of immunoglobulin class-specific antibody in cell lysates from lungs and spleens, and the results reflect the numbers of cells containing antibody in these organs. The serum and BAW concentrations of antibody were measurements of free antibody.

Protein determination. The total protein concentrations in BAW were determined by the method of Lowry et al. (13).

Quantitation of serum and BAW total IgG, IgM, and IgA. The total IgG, IgM, and IgA in serum

and BAW were determined by the Mancini technique (14).

RESULTS

Lung histology. Primary i.n. challenge with the three doses of live Sendai virus resulted in respiratory infection in the germfree mice. The lung histology of 10^5 and 10^3 $TCID_{50}$ of virus was mainly limited to the ciliated epithelial cells, with little alveolar involvement, as previously reported (16). The 10^4 $TCID_{50}$ resulted in a 10% mortality rate, with extensive alveolar infection with pneumonia. Fluorescent antibody staining of the lungs showed that viral antigens were present in all groups at days 3 and 6 but not at day 10 or thereafter.

Secondary i.n. challenge with only 10^5 and 10^4 $TCID_{50}$ of virus resulted in minimal infection of bronchial epithelial cells on days 3 and 6.

Lung histology after i.p. challenge showed normal bronchi and a slow progressive increase in peribronchial mononuclear cells. The increase was not dose dependent, but there appeared to be more mononuclear cells in challenged mice than in controls. A search for viral antigens in the lungs by immunofluorescence was uniformly negative.

Antibody levels after i.n. 10^5 and 10^3 $TCID_{50}$ (Fig. 1). (i) Primary response. The primary antibody responses in all locations were similar after 10^5 and 10^3 $TCID_{50}$ of virus. The immunoglobulin classes of antibody detected in the lung lysates were IgG at day 6 followed by IgG and IgM at day 10 and IgG, IgM, and IgA at day 24. These results indicate that the cellular sequence of the different antibody classes in the lungs was IgG followed by IgM followed by IgA.

A spleen response was not detected until day 24, when all three antibody classes were present. The late spleen cell response suggests that it could not have contributed directly to the earlier antibody responses in serum, BAW, or the lungs.

Antibody was not detected before day 10 in the BAW, when all three antibody classes were found. At days 24 and 35 10^3 $TCID_{50}$ resulted in twice the concentration of BAW IgA antibody compared with that after 10^5 $TCID_{50}$ infection.

(ii) Secondary response. A secondary response followed the 10^5 $TCID_{50}$ secondary challenge in all locations. The response involved all three antibody classes in lung lysates. The secondary IgG and IgM responses peaked earlier than IgA. Secondary IgG and IgA antibody responses were detected in the spleen cell lysates. The IgA response occurred earlier and at higher levels than the IgG response. Secondary responses in all three antibody classes were observed in BAW. The peak response was at day 45. By day 59 the antibody levels had dropped approximately sixfold. Secondary rises in serum IgG and IgM antibodies were observed, but serum IgA antibody was never detected.

After the 10^3 $TCID_{50}$ rechallenge a detectable rise in IgG and IgM antibody production was observed in the lung. A secondary response was not evident in any other immunoglobulin class or location.

Antibody levels after i.n. 10^4 $TCID_{50}$ (Fig. 1). (i) Primary response. The severe lung involvement in mice infected with 10^4 $TCID_{50}$ of virus was associated with a different pattern of antibody response compared with the milder infections. The lung and spleen lysates at 10^4 $TCID_{50}$ were quantitatively identical to each other. IgG and IgM antibodies were first detected on day 10. IgA antibody was not detected until day 24.

The BAW contained IgG antibody at day 6. From day 10 through day 35 all three antibody

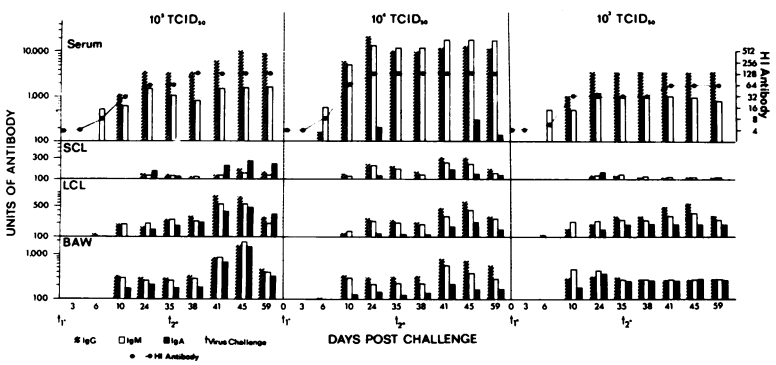

FIG. 1. *Immunoglobulin class-specific antibody levels in serum, spleen cell lysates (SCL), lung cell lysates (LCL), and BAW, as measured by the RIA technique, after primary (1°) and secondary (2°) i.n. live Sendai virus challenge in germfree mice. Hemagglutination inhibition (HI) antibody levels in serum are also depicted.*

classes were present. The levels of IgA antibody in the lung lysates and BAW were lower than the levels observed in the 10^5 and 10^3 $TCID_{50}$ groups.

The serum antibody response was characterized by the presence of IgG and IgM antibodies at day 6. The concentrations of serum IgG and IgM antibodies by day 24 were approximately five times greater than those observed after 10^5 and 10^3 $TCID_{50}$. Serum IgA antibody was detected on day 24, an observation not made after the other two doses.

(ii) Secondary response. A secondary antibody response was observed by day 41 in all locations and in all antibody classes. The secondary IgA antibody response did not display as great a rise as that observed in the 10^5 $TCID_{50}$ group. A very poor secondary IgA response was also evident in BAW. The levels of serum antibody remained high. The IgM levels were higher than after the primary challenge, whereas the IgG levels were not. Serum IgA antibody was detected again at day 45 and at a higher concentration that that observed after the primary infection.

Antibody levels after i.p. 10^5 and 10^3 $TCID_{50}$ (Fig. 2). (i) Primary response. The antibody responses in all locations were higher after 10^5 $TCID_{50}$. The earliest antibody response was in the spleen, where antibody was detected at day 3. IgG and IgM antibodies were detected in the 10^5 $TCID_{50}$ group, whereas only IgG was found in the 10^3 $TCID_{50}$ group. In the lungs IgM antibody peaked earlier than IgG. IgA antibody was not detected in any location before secondary challenge.

(ii) Secondary response. After the 10^5 $TCID_{50}$ rechallenge a secondary antibody response was observed in all locations. It was detected on day 38 in the spleen and serum but not until 3 days later in the lung. The challenge with 10^3 $TCID_{50}$ resulted in a minimal secondary response, most evident in the lung lysates. IgA antibody was detected in the serum of the 10^5 $TCID_{50}$ group.

Antibody levels after i.p. 10^4 $TCID_{50}$ (Fig. 2). (i) Primary response. IgG and IgM antibodies were first detected simultaneously in the lung, spleen, and serum on day 6. The serum levels of antibody were several times higher than those after 10^5 and 10^3 $TCID_{50}$ of virus. The 10^4 $TCID_{50}$ was the only dose in which the spleen cell response was clearly greater than the lung cell response when the different dilution factors were taken into account.

(ii) Secondary response. Secondary antibody responses were detected in all locations at day 41 and were most marked in lungs, spleen, and serum. The secondary response peaked earlier in the spleen than in the lungs or serum. IgA antibody was detected in the serum and spleen lysates. The level of IgA antibody in the serum was five times higher than that observed after 10^5 $TCID_{50}$.

Hemagglutination inhibition antibody response. Hemagglutination inhibition antibody titers in serum after i.n. and i.p. challenges are depicted in the graphs superimposed on the serum histograms in Fig. 1 and 2, respectively. They show that after i.n. infection the highest antibody response followed 10^4 $TCID_{50}$ and that the 10^5 and 10^3 $TCID_{50}$ responses followed in descending order. Significant secondary responses were not detected. After i.p. challenge the hemagglutination inhibition titer was highest after the highest dose and lowest after the lowest dose. The only significant secondary rise was noted after 10^5 $TCID_{50}$.

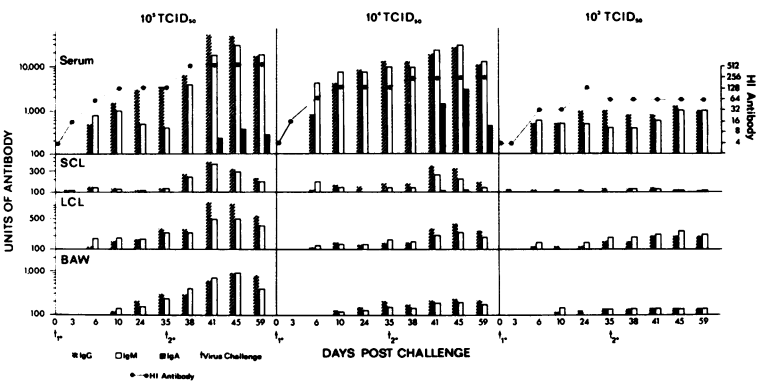

FIG. 2. *Immunoglobulin class-specific antibody levels in serum, spleen cell lysates (SCL), lung cell lysates (LCL), and BAW, as measured by the RIA technique, after primary (1°) and secondary (2°) i.p. live Sendai virus challenge in germfree mice. Hemagglutination inhibition (HI) antibody levels in serum are also depicted.*

Comparison of antibody units per microgram of immunoglobulin between serum and BAW. The serum and BAW concentrations of IgG, IgA, and IgM were obtained. The ratios of antibody units to micrograms of immunoglobulin were calculated for both serum and BAW (Fig. 3 and 4). Much higher ratios were observed in BAW than in serum after both i.n. and i.p. challenge. These observations suggest that local manufacture, concentration, or selective secretion was occurring.

DISCUSSION

Germfree mice were used in these experiments, as a stock of normal Sendai-free mice was unavailable. Intraperitoneal challenge did not result in any apparent lung infection, whereas i.n.-challenged animals had pathological and serum hemagglutination inhibition antibody responses essentially similar to those reported in normal Sendai-free mice (5, 16). A few fatalities occurred in the 10^5 and 10^4 $TCID_{50}$ ranges, which did not occur in normal animals. The 10^4 $TCID_{50}$ primary infection was characterized by the most severe and extensive lung damage, with alveolar infection. This may be attributed to efficient and maximal infectious virus production, whereas the production of incomplete, noninfectious virus probably followed 10^5 $TCID_{50}$.

Previous studies (5) have shown that antigen-antibody complexes were present in infected mouse lungs as early as 3 days post-Sendai infection, associated with submucosal immunoglobulin-containing cells. The failure to detect specific antibody in lung cell lysates before day 6 may be due to excess viral antigen from lysed infected mucosal cells combining with antibody released from lysed plasma cells.

The earliest local antibody detected after infection was IgG, followed by IgM and, lastly, IgA. The appearance of IgG before IgM has also been demonstrated in normal mice following Sendai virus infection (unpublished data). A similarly delayed appearance of IgA antibody followed nonlethal influenza A infection in normal mice (18). In contrast, some reports on infections in humans indicate that local IgA antibodies may appear exclusively (21). The discrepancy may be related to the sensitivity of the methodology we have used to detect immunoglobulin class-specific antibodies.

The late appearance of IgA antibody may be due to loss of mucous membrane cells and, hence, a local secretory component deficiency. Support for this concept can be assembled from various observations. Certainly infected bronchial mucosal cells become necrotic and desquamate by day 3 postinfection. The inverse relationship between BAW IgA antibody and the severity of lung damage ($10^4 > 10^5 > 10^3$ $TCID_{50}$) in the experiments reported here could be related to excessive loss of secretory component-producing cells. The bronchi of the 10^4

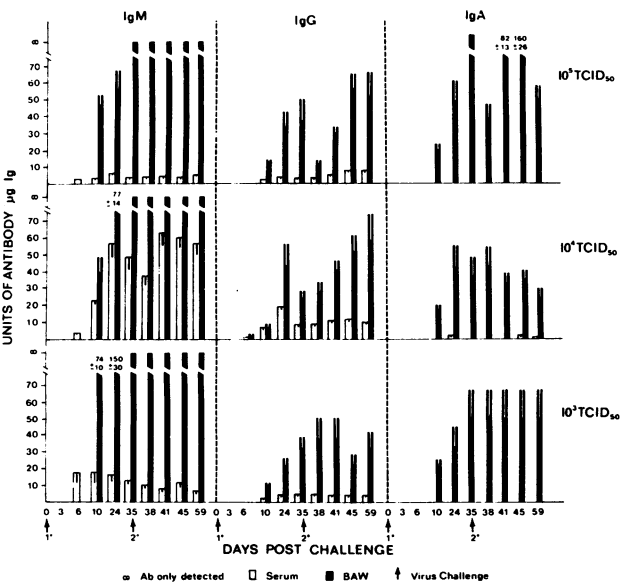

FIG. 3. *Serum and BAW units of antibody per microgram of immunoglobulin after primary (1°) and secondary (2°) i.n. live Sendai virus challenge in germfree mice. Where the ratio is infinity (∞), antibody (Ab) was detected but immunoglobulin was below the level of quantitation.*

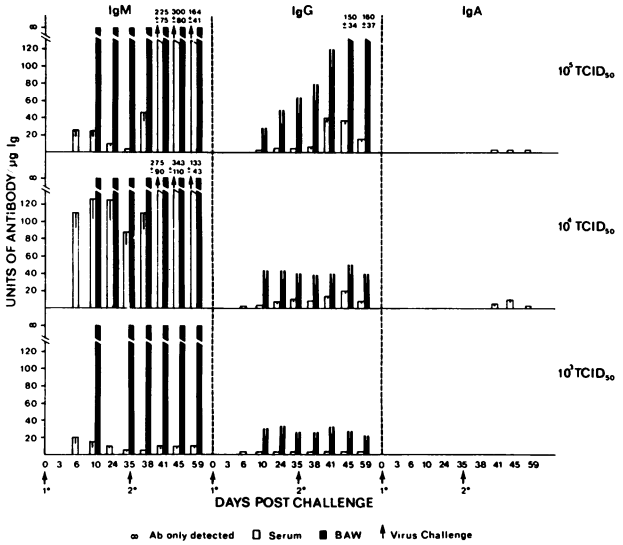

FIG. 4. *Serum and BAW units of antibody per microgram of immunoglobulin after primary (1°) and secondary (2°) i.p. live Sendai virus challenge in germfree mice. Where the ratio is infinity (∞), antibody (Ab) was detected but immunoglobulin was below the level of quantitation.*

$TCID_{50}$ animals at the time of secondary challenge were affected by considerable squamous metaplasia. It is not known whether such cells are deficient in secretory component; however, the BAW secondary IgA response in this group was less than the persisting levels in the 10^3 $TCID_{50}$ group in spite of a substantial serum IgA response. Several studies (10–12) have indicated that the lower respiratory tract, which lacks a secretory epithelium, is deficient in IgA response compared with the upper respiratory tract. Finally, an individual with secretory component absent (19) was found to have very small amounts of IgA in external secretions and was unable to synthesize local IgA in the intestinal mucosa.

It seems likely that we observed a true local secondary IgA response. After secondary 10^5 and 10^4 $TCID_{50}$ i.n. infections the IgA antibody titers doubled in 6 days in both BAW and lung cell lysates, whereas the initial responses took 10 and 24 days, respectively. Since the secondary infections resulted in minimal viral replication, the rapid increase in antibody titer must certainly have followed a much smaller total antigenic challenge. The prompt decay of the local IgA secondary response was different from the sustained levels seen in secondary serum antibody responses. The difference may be attributed to at least two peculiarities of the local environment. Firstly, viral antigens do not persist locally, whereas antigens have been detected in the spleen up to 49 days after infection (5). Thus, there is no continuing local antigenic drive. Secondly, antibodies produced into external secretions do not recirculate, but drain away with the local secretions.

The results for both primary and secondary infections indicate that the lungs were a major, and probably the first, site for production of IgG antibodies and that local secondary IgG and IgM responses may occur without other evidence of reinfection.

Live virus inoculated by the parenteral route resulted in a spleen response before the serum and lung responses. The lung response was exclusively IgG and IgM and was dose dependant. A dose-related lung IgG and IgM secondary response was detected, also without IgA. This exclusive appearance of lung IgG and IgM antibodies is in contrast to other studies where BAW IgA antibody has followed systemic challenge (1, 20). In these studies the subjects were not germfree, and it is therefore possible that the local IgA response after systemic challenge may have been the result of stimulating an immunologically experienced secretory surface.

The most impressive difference between i.n. and i.p. infection clearly involved the lack of appearance of IgA antibodies in lung cell lysates and BAW following i.p. virus challenge. It should be emphasized that these findings are in germfree mice and cannot readily be extrapolated to normal mice or humans.

The calculations of RIA antibody-immunoglobulin ratios in serum and BAW showed that the latter were higher than the former at all times, regardless of the route of challenge. This

would indicate that, although serum exudation certainly occurred with acute inflammation, as indicated by the BAW immunoglobulin concentrations, either a local antibody-concentrating mechanism, selective secretion of antibody into the respiratory fluid, or local manufacture must also have occurred. The presence of IgA antibody in secretions and its total absence from serum after i.n. challenge clearly support local manufacture and secretion, a conclusion further supported by the presence of IgA antibody-producing cells in the lungs.

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