

## Influenza Virus-Specific Antibody-Secreting Cells in the Murine Lung during Primary Influenza Virus Infection

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Received 6 January 1986/Accepted 4 August 1986

**An enzyme-linked immunosorbent plaque assay is described which can reliably enumerate influenza virus-specific antibody-secreting cells and exhibits specificity similar to that of the indirect enzyme-linked immunosorbent assay. The assay was used to characterize the development of specific antibody-secreting cells, principally within lung tissue, during primary murine influenza virus infection after intranasal inoculation. Cells secreting influenza virus-specific immunoglobulin M (IgM), IgG, and IgA were detected in greatest numbers in lung tissue, and the data presented indicated that the cells may have originated from specific B-cell precursors in lung tissue which are demonstrable in vitro. At 11 months after infection, cells secreting IgG and IgA were still present in lung tissue. Influenza virus-specific antibody-secreting cells were also detected in spleen tissue and blood. Antibody-secreting cells appeared earlier in spleen than in lung tissue and declined more rapidly in spleen tissue.**

Acute respiratory infection is the major cause of morbidity among and use of health services by children all over the world, and in developed countries viral infections are the leading cause of acute upper and lower respiratory tract diseases (15). Although it is generally accepted that local humoral immunity of the respiratory tract plays an important role in the prevention of viral respiratory tract infections, including influenza, there are important gaps in our understanding of the local B-cell response. The B-cell response to viral infections in the respiratory tract has been largely characterized by measurements of antibody in serum and respiratory secretions. However descriptions of the cellular events underlying the humoral response in the respiratory tract during viral infections are limited. The demonstration of antibody-containing cells by immunofluorescence in lung tissue during influenza virus infection provides indirect evidence that specific antibody in respiratory secretions is locally synthesized (17). More direct evidence for this would be provided by demonstration of antibody secretion at the cellular level. Previous reports of influenza virus-specific antibody-secreting cells (ASCs) detected by the hemolytic plaque assay have provided remarkably little information on events within the lung.

An enzyme-linked immunosorbent (ELISA)-plaque assay recently described for the enumeration of cells secreting antibody specific to soluble antigens (18) is more sensitive than the hemolytic plaque assay and can be adapted to accurately distinguish antibody isotypes. In this study we adapted this technique to enumerate influenza virus-specific ASCs and studied the primary B-cell response, particularly within the lung, to influenza virus infection in mice.

### MATERIALS AND METHODS

**Viruses.** Influenza viruses A/Japan/305/57 (H2N2), A/Port Chalmers/1/73 (H3N2), A/Puerto Rico/8/34 (H1N1), A/Jap-Bel (H2N1) (A/Japan/305/57 [H2N2] × A/Bellamy/42 [H1N1]), and B/Singapore/79 and Sendai virus were grown in embryonated eggs for 40 to 48 h at 35°C. Viruses were

purified by sucrose gradient centrifugation by the method of Laver (9) and disrupted by sodium deoxycholate as previously described (19).

**Mice; immunization and cell preparations.** BALB/c mice, 8 to 12 weeks old, were either immunized intravenously (i.v.) with 1,000 hemagglutination units (HAU) of infectious virus or inoculated intranasally with 50  $\mu$ l containing the required dose of infectious virus under light ether anesthesia.

Spleen cell suspensions were prepared by finely mincing spleens and gently pressing them through a stainless steel sieve. Erythrocytes and dead cells were removed by centrifugation on a Ficoll-Isopaque gradient. Viability counts were performed by the trypan blue exclusion method, and the final concentration of cells was suspended in Eagle minimal essential medium supplemented with 10% fetal calf serum.

Lung cell suspensions were prepared similarly except that finely minced lungs were incubated with collagenase (Boehringer; catalog no. 103586)—4 mg per lung in 2 ml of minimal essential medium—for 30 min at 37°C. This additional step resulted in a 10 to 20-fold increase in the yield of total viable cells from each lung and an approximately 30-fold increase in the number of ASCs detected in the ELISA-plaque assay.

Spleen cell suspensions were depleted of immunoglobulin-bearing cells by the removal of rosettes formed by immunoglobulin-bearing cells and sheep erythrocytes coupled with anti-mouse immunoglobulin (14).

**Spleen and lung cell cultures.** Spleen and lung cell suspensions from normal mice were cultured in minimal essential medium supplemented with 10% fetal calf serum,  $10^{-4}$  M 2-mercaptoethanol, and antibiotics. Flasks were incubated at 37°C in a gas phase of 5% CO<sub>2</sub> in air. Cultures were stimulated by the addition of purified virus.

**ELISA-plaque assay.** Purified whole virus in bicarbonate-carbonate buffer, pH 9.8, was coated onto six-well (3.5 by 1.0 cm; 4 ml per well) plates (Linbro) overnight at 4°C. The wells were washed with phosphate-buffered saline-0.05% Tween 20 three times. Phosphate-buffered saline-0.05% Tween 20-1% bovine serum albumin was added (2 ml per well) and incubated for 1 h at 37°C. Prior to incubation of cell suspensions containing ASCs, Cytodex 1 microcarriers were added to the suspension (1 mg/ml) to improve plaque defini-

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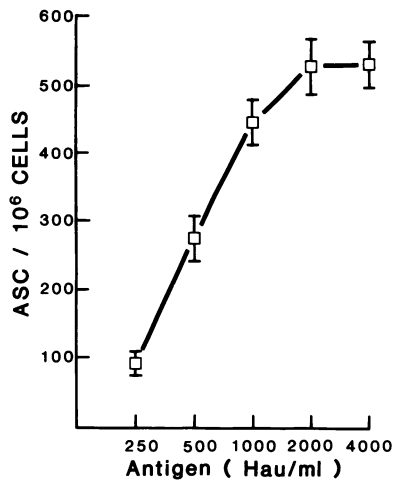


FIG. 1. Antigen titration. IgM ASCs in day 4 spleens after primary i.v. immunization (1,000 HAU of A/Jap). Plates were coated with various concentrations of A/Jap virus.

tion (4). The bovine serum albumin solution was removed, and 1.5 ml of cell suspension was added to the plates, which were placed on a level surface in a gas chamber. The plates were incubated for 3 to 4 h at 37°C in a humid atmosphere containing 10% CO<sub>2</sub>. After three washes with phosphate-buffered saline-Tween 20, 1 ml of goat anti-mouse antiserum of appropriate heavy-chain specificity (Cooper Biomedical, Inc., West Chester, Pa.; diluted 1:2,000 in phosphate-buffered saline-Tween 20-bovine serum albumin) was added, and the plates were incubated for 90 min at 37°C. After washing, 1 ml of rabbit anti-goat immunoglobulin G (IgG) alkaline phosphatase conjugate (Cooper; 1:1,000 dilution) was added and left overnight at 4°C. The substrate solution used was 5-bromo-4-chloro-3-indolyl phosphate dissolved in 2-amino-2-methyl-1-propanol buffer, as described previously (18), in a final concentration of 0.05%. The substrate solution was warmed to 40°C, and 3% agarose solution was added to a final concentration of 0.6% agarose. After the plates were washed, emptied, and placed on a level surface, 1 ml of warmed agarose-substrate mixture was added to each well. After the mixture gelled, the plates were incubated for 2 h at 37°C. Plaques appearing as blue spots were enumerated by using a dissecting microscope ( $\times 12$ ).

## RESULTS

**Standardization of the ELISA-plaque assay.** Preliminary experiments were performed to standardize the ELISA-plaque assay for enumeration of influenza virus-specific ASCs. The titer of purified influenza A/Jap virus was ascertained to determine the optimal amount of viral antigen needed for coating plates (Fig. 1). There was a significant increase in both plaque counts and plaque definition with increasing concentrations of virus up to 1,000 HAU/ml. This concentration was selected for subsequent experiments to achieve efficient use of virus stocks, although the sensitivity of the assay is slightly suboptimal at this concentration. Optimal concentrations of goat anti-mouse antisera, rabbit anti-goat alkaline phosphatase conjugate, and substrate solution were determined by checkerboard titrations.

The precision of the ELISA-plaque assay was estimated by determining intra- and interplate variations in plaque counts by using the same cell suspensions so as to avoid that

source of biological variation. The coefficient of variation for intraplate counts, determined in over 50 experiments, was 9%. Interplate variation, determined in five experiments, was 12%. The total assay variation calculated from these results was 15%.

To demonstrate that plaque formation was dependent on antibody production, various concentrations of cycloheximide were added to primary spleen cell cultures during the final 18 h of incubation and at the time of the plaque assay. A dose-response effect was observed, with plaque formation completely ablated by 1  $\mu$ g cycloheximide per ml. Furthermore, plaque formation was shown to be dependent on the presence of ASCs by depleting immune spleen cell suspensions of immunoglobulin-bearing cells by separating out rosette-forming cells. IgM and IgG plaque counts in the non-rosette-forming fraction were reduced to less than 5% of that of the unseparated cell suspension (Fig. 2). Although plastic-adherent cells are not removed by washing after incubation of cell suspensions, the possibility that plaques were formed by macrophages to which specific cytophilic antibody was bound is unlikely, as plaques do not develop when cell suspensions from immunized mice are incubated on antigen-free plates, despite the presence of adherent cells.

**Specificity of the ELISA-plaque assay.** Several experiments demonstrated the specificity of the ELISA-plaque assay for detecting influenza-specific ASCs (Table 1). Plaques were infrequently detected in spleen and lung cell suspensions from normal unimmunized mice when they were assayed on influenza virus-coated plates. These were mostly IgM plaques ( $<5/10^6$  cells) and only occasionally IgG and IgA plaques ( $<1/10^6$  cells). Plaques were rarely detected when cell suspensions from influenza virus-infected mice were assayed on plates coated with either uninfected allantoic fluid or bicarbonate buffer only. In addition, influenza virus-specific ASCs were not detected in significant numbers in cell suspensions from mice immunized with Sendai virus.

The specificity of the assay was further evaluated by immunizing mice with influenza viruses of different type and subtype specificities. Groups of mice were immunized with A/Jap, A/Jap-Bel, A/Pt Ch, A/PR8, or B/Sing virus. The

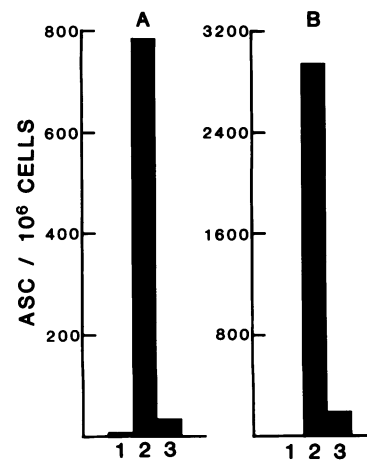


FIG. 2. (A) IgM ASCs in day 4 spleens after primary i.v. immunization (1,000 HAU of A/Jap). (B) IgG ASCs in day 4 spleens after secondary i.v. immunization (two injections of 1,000 HAU of A/Jap each) 1, Normal spleens; 2, suspensions of unseparated cells from immunized mice; 3, cell suspensions depleted of immunoglobulin-bearing cells from immunized mice.

TABLE 1. Detection of influenza virus-specific ASCs by ELISA-plaque assay

Virus used for immunization	Virus on plate	Mean no. ( $\pm$ S.D.) of ASCs per $10^6$ cells <sup>a</sup>		
		IgM <sup>b</sup>	IgG <sup>b</sup>	IgA <sup>c</sup>
None	A/Jap	2 $\pm$ 1	0	0
A/Jap	None (buffer only)	0.5	0	0
A/Jap	None (normal allantoic fluid)	4	0	0
A/Jap	A/Jap	118 $\pm$ 6	98 $\pm$ 3	81 $\pm$ 7
Sendai	A/Jap	4 $\pm$ 2	0	0

<sup>a</sup> Values represent means  $\pm$  standard deviations of triplicate assays.  
<sup>b</sup> Day 6 spleens after primary i.v. immunization (1,000 HAU).  
<sup>c</sup> Day 12 lungs after primary intranasal inoculation.

ELISA-plaque assay was performed on spleen and lung cell suspensions by using plates coated with the above viruses (Table 2). The greatest number of plaques for all isotypes was seen with the homologous combinations. However, significant cross-reaction in each isotype was seen within the type A influenza viruses. A very small number of cross-reactive plaques, of the IgM isotype only, was seen with the type B virus.

The observation that some cross-reaction was seen between viruses with different surface antigens suggested that this was due to the recognition of non-surface antigens. This possibility was supported by the demonstration that plaque formation was completely inhibited by adding virus disrupted by detergent to the cell suspension during the assay but was still observed in the presence of intact virus (Fig. 3).

**ASCs in the primary response to influenza.** Mice were inoculated intranasally with  $10^4$  50% egg infective doses (EID<sub>50</sub>s) of infectious A/Jap virus, causing peak lung consol-

TABLE 2. Effect of influenza virus type and subtype on ELISA-plaque assay specificity

Virus used for immunization	Immunoglobulin isotype	Mean no. of ASCs per $10^6$ cells on plates coated with <sup>a</sup> :				
		A/Jap	A/Jap-Bel	A/PC	A/PR8	B/Sing
A/Jap (H2N2)	IgM <sup>b</sup>	382	171	22	39	12
	IgG <sup>c</sup>	116	91	20	0	0
	IgA <sup>c</sup>	170	14	5	5	0
A/Jap-Bel (H2N1)	IgM	129	243	44	11	6
	IgG	175	184	76	6	0
	IgA	128	170	85	4	0
A/PC (H3N2)	IgM	365	61	600	180	11
	IgG	55	26	172	16	0
	IgA	216	23	299	10	0
A/PR8 (H1N1)	IgM	53	23	25	422	13
	IgG	31	20	12	88	0
	IgA	102	8	0	158	0
B/Sing	IgM	9	4	6	13	110
	IgG	0	0	0	0	40
	IgA	0	0	0	0	70

<sup>a</sup> Values represent means of duplicate assays.  
<sup>b</sup> Day 5 spleens after primary immunization (1,000 HAU).  
<sup>c</sup> Day 14 lungs after primary intranasal inoculation.

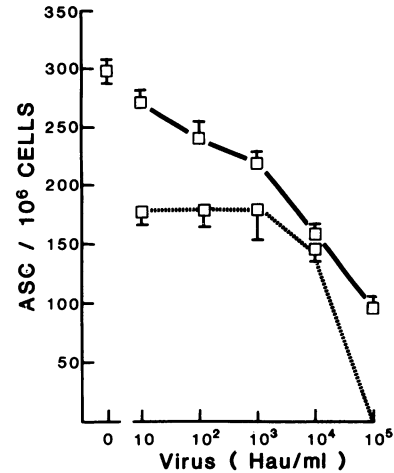


FIG. 3. IgM ASCs in day 4 spleens after primary i.v. immunization (1,000 HAU of A/Jap). Various concentrations of either purified intact A/Jap virus (solid line) or detergent-disrupted virus (broken line) were added to cell suspensions at the time of assay.

idation of  $45 \pm 20\%$  and 5 to 10% mortality. The mean lung virus titer measured at day 3 was  $6.6 \log_{10}$  EID<sub>50</sub>s. Class-specific ASCs to influenza virus were enumerated in lung, spleen, and blood by the ELISA-plaque assay (Fig. 4). ASCs were more numerous in lung than in spleen tissue for each isotype; in each organ, ASCs producing IgM were the predominant cell type found. IgM ASCs were first detected in lung tissue at day 5 and subsequently peaked at day 10. IgG and IgA ASCs were not detected in lung tissue until the week 2, and thence both peaked during week 3 with slightly more IgG than IgA ASCs. At 1 month after infection, numerous ASCs of each isotype were still present in the

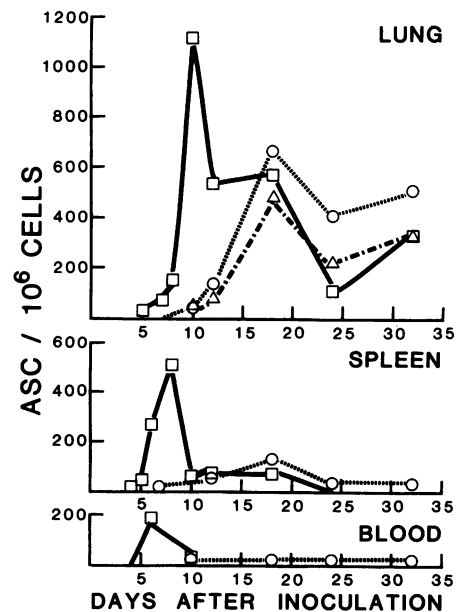


FIG. 4. ASCs in lung, spleen, and blood after primary intranasal inoculation ( $10^4$  EID<sub>50</sub>s of A/Jap). Values represent mean results from triplicate assays performed on suspensions of pooled cells from 3 to 4 mice. Symbols: □, IgM; ○, IgG; △, IgA.

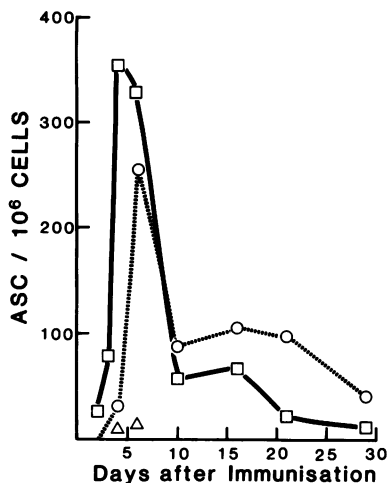


FIG. 5. ASCs in spleen after primary i.v. immunization (1,000 HAU of A/Jap). Values represent mean results from triplicate assays performed on suspensions of pooled cells from 3 to 4 mice. Symbols: □, IgM; ○, IgG; △, IgA.

lung. The onset and peak of the splenic IgM response preceded the pulmonary IgM response by 24 to 48 h. Splenic IgG ASCs were first detected at day 6 in very small numbers and peaked by day 18. A very small number of IgA ASCs ( $7/10^6$  cells) was detected in the spleen during week 3. At 1 month, only IgG ASCs ( $30/10^6$  cells) were detected in the spleen. ASCs producing IgM also constituted the major response in blood; IgM ASCs appeared at day 6 and declined by the end of week 2. IgG and IgA ASCs also circulated during weeks 2 and 3 in small numbers ( $<20/10^6$  cells).

In mice infected with a 10-fold lower dose of A/Jap virus, the onset of the response in both lung and spleen was delayed by 48 h compared with that in mice infected with the higher dose. The pattern of the response was subsequently similar though of lesser magnitude. Peak responses in both lung and spleen for each isotype were 40 to 60% lower than those shown in Fig. 4.

The persistence of cells continuing to secrete antibody in the absence of any further exogenous antigenic challenge was examined 11 months after primary intranasal inoculation with  $10^3$  EID<sub>50</sub>s of infectious A/Jap virus. In the lungs, ASCs producing IgG were still present in considerable numbers ( $157/10^6$  cells); IgA ASCs persisted as well, but to a lesser extent ( $31/10^6$  cells). IgM ASCs were present in only small numbers ( $8/10^6$  cells). In contrast, in the spleen only a small number of ASCs producing IgG ( $9/10^6$  cells) was found.

ASCs were also enumerated after primary i.v. immunization with 1,000 HAU of infectious A/Jap virus. The mean virus titer in lungs 3 days after immunization was  $4.5 \log_{10}$  EID<sub>50</sub>s. The splenic response was characterized by earlier appearance of IgM ASCs at 48 h and relatively greater IgG response compared with that observed after intranasal inoculation (Fig. 5). Although the peak IgM response in the lung after i.v. immunization was only one-third that of the splenic IgM response, IgA ASCs were more numerous in lung than spleen (data not shown).

**ASCs in primary in vitro cultures.** The primary response to influenza virus was further evaluated by stimulation of lung and spleen cell cultures from unprimed mice with influenza virus in vitro.

The titer of purified A/Jap virus was ascertained to determine the optimal concentration for in vitro stimulation. The

maximum number of IgM ASCs detected in both lung and spleen cell cultures followed stimulation with 20 HAU/ml. The number of ASCs decreased with increasing concentrations of virus above 20 HAU/ml. ASCs producing IgM were detected in spleen cell cultures after 48 h of incubation and in lung cell cultures at 72 h. The peak IgM response developed in both cell cultures at 4 days and was slightly greater in the spleen cell culture (Fig. 6). IgG ASCs were not detected in either cell culture, whereas a small number of IgA ASCs ( $5/10^6$  cells) was detected in the lung cell culture. Similar results were obtained with either an H1 (A/PR8) or a B/Sing virus, indicating that the generation of ASCs was not due to any nonspecific mitogenic activity associated with the H2 virus. Furthermore, the specificity of ASCs generated in vitro was similar to that of ASCs obtained in vivo.

ASCs were not detected in cultures of peripheral blood lymphocytes stimulated under the same conditions, indicating that ASCs found in lung cell cultures were not derived to any major extent from precursors in the pulmonary vasculature.

## DISCUSSION

The appearance and development of specific ASCs in lung tissue during infection has now been described for the first time. The only previous detailed descriptions of specific ASCs in lung tissue were after local immunization with foreign erythrocytes (6–8, 12). There was also a single earlier finding of direct plaque-forming cells detected by the hemolytic plaque assay in tracheobronchial cell preparations from influenza virus-infected ferrets (10). We preferred to adapt a recently described technique for detecting ASCs by an ELISA technique, and this has been found to be eminently suitable for the purpose. Just as the ELISA technique for antibody detection can be used to measure antibodies of any specificity, the ELISA plaque technique could most likely be adapted to estimate the numbers of cells secreting antibody to any infectious agent and probably in any host organ.

A variety of experiments attest to the reliability and reproducibility of the ELISA-plaque assay for enumerating cells secreting influenza-specific antibodies of different isotypes. Plaque formation was shown to be dependent on antibody production and on the presence of cells secreting antibody. The assay specifically detected influenza virus-

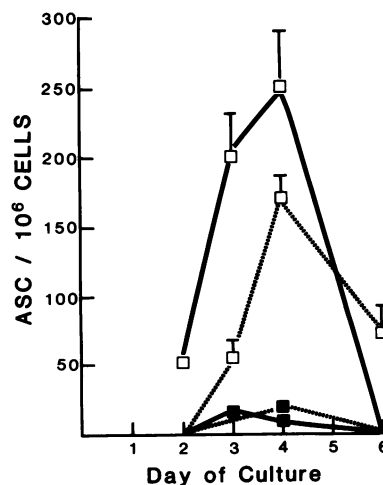


FIG. 6. IgM ASCs in primary spleen (solid line) and lung (broken line) cell cultures either stimulated with 20 HAU of A/Jap virus per ml (□) or unstimulated (■).

specific ASCs essentially only in mice immunized with influenza virus. Cells producing type-specific antibodies, but not cells producing antibodies of different subtype specificities, could be clearly distinguished. This latter result is similar to previous experiences with the indirect ELISA in which whole virus was used as the antigen for measuring anti-influenza virus antibody (1, 5) and is due to the recognition of common antigenic determinants, principally on the internal proteins. These determinants are exposed in the well, probably as a result of physical alteration to the virus structure during adsorption to the solid phase, an interpretation supported by the complete inhibition of plaque formation only by detergent-disrupted virus.

In contrast to previous failures to detect antibody-secreting cells in the lungs of mice after primary local immunization with foreign erythrocytes (6, 12), we readily detected cells secreting IgM, IgG, and IgA in the lungs of mice during primary influenza virus infection. Thus, an earlier report (17) of an increase in immunoglobulin-bearing cells, detected by immunofluorescent labeling, in the lungs of influenza virus-infected mice was not only confirmed but extended to demonstrate their specificity for antigen. Our results provided the most direct evidence yet obtained that influenza virus-specific antibody of each isotype in respiratory secretions is most likely locally produced. Although ASCs were detected earlier in spleen than in lung tissue, the subsequent response in lung tissue was of greater magnitude for each isotype, especially for the IgA component. The time of onset and magnitude of the pulmonary response were also dose dependent. ASCs have been previously detected by the hemolytic plaque assay in the spleens and intrathoracic lymph nodes of influenza virus-infected mice and ferrets, displaying kinetics in the splenic response similar to that described here (10, 11, 16).

The primary response to infection was also characterized by a more rapid decline in the numbers of ASCs in spleen than in lung, possibly relating to the persistence of viral antigen in lung tissue (2). Furthermore, a greater proportion of cells secreting IgG and IgA persisted in lung tissue up to 11 months postinfection. We are currently investigating in greater detail the persistence of cells spontaneously secreting antibody in the absence of any exogenous antigenic stimulation.

In contrast to the considerable difference observed in the sizes of the pulmonary and splenic primary *in vivo* responses, the primary IgM response generated *in vitro* in lung cell cultures was only slightly less in magnitude than that in spleen cell cultures. However, as *in vivo*, ASCs appeared earlier in spleen cell cultures than in lung cell cultures. Previous work comparing the immune response in the spleens and lungs of mice inoculated intranasally with parental and cold-adapted preparations of influenza virus (13) indicated that a splenic response was due to antigen spillover of virus replicating in the lungs; i.e., the antigenic challenge in the spleen is both delayed and of smaller magnitude in the spleen than in the lungs. These findings, taken together, indicate that the antigen-presenting mechanisms in the spleen are qualitatively or quantitatively different from those in the lungs.

What is the origin of ASCs in the lungs during influenza virus infection? The proposition that these cells may arise from influenza virus-specific B-cell precursors in the lungs is directly supported by two findings in this study: (i) the generation of cells secreting specific IgM in stimulated cultures of lung cells from unprimed mice; and (ii) the virtual absence of an IgA response in spleen tissue despite the

prominent response in lung tissue after both intranasal and *i.v.* immunization. Do any circulating B-cell precursors or ASCs derived from extrapulmonary lymphoid tissue contribute to the lung response? The only evidence which might support this is the finding *in vivo* that the IgM response develops earlier in the spleen than in the lungs, thus allowing an opportunity for some migration to the lungs. If this does occur, then further migration is arrested, as indicated by the persistence of ASCs in the lungs. Previous studies on the response to foreign erythrocytes has led to the suggestion, based on indirect evidence, that the major mechanism for the appearance of ASCs in the lungs was through the recruitment of sensitized cells (3, 6), though it has also been shown that, whereas the spleen contributes to the pulmonary response, it is not essential (6). Of course, migration of other cell types such as helper T lymphocytes from the spleen and other extrapulmonary lymphoid tissue could occur.

Whereas these findings have extended our understanding of the B-cell response to a respiratory infection, additional areas which need to be defined and which are presently being studied include the persistence of ASCs and the generation of B memory cells in the lungs after primary infection and the local and systemic cellular responses to reinfection.

#### ACKNOWLEDGMENT

We thank P. G. Holt for his advice and assistance in the use of the ELISA-plaque assay.

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