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Persistence of Influenza as an Immunogen in Pulmonary Antigen-Presenting Cells

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Influenza antigens inoculated into the lung induce local immune responses. It has been proposed that this induction might be partly regulated by local antigen-presenting cells. The purpose of the current study was to inoculate heat-inactivated influenza virus into the tracheae of guinea pigs and determine the quantity of antigens that became cell-associated. Second, we determined how long antigen-presenting bronchoalveolar cells that had taken up virus in vivo retained their ability to specifically stimulate virus-immune T lymphocytes. Radioiodinated heat-inactivated influenza virus was inoculated into the tracheae of guinea pigs. The animals were killed from 30 min to 14 days after intratracheal inoculation, and radioactivity was determined in cells isolated from lung tissue. At least one-third of the radioactivity in the lungs was cell-associated from 1 to 14 days post-inoculation. In separate studies, heat-inactivated virus was inoculated into the airways of guinea pigs, and animals were killed at various times thereafter. Bronchoalveolar cells from these animals were compared with those from uninoculated controls in their ability to specifically stimulate virus-immune T cells to proliferate in vitro. Bronchoalveolar cells from virus-inoculated animals specifically stimulated T lymphocytes for up to 7 days after virus inoculation. These studies suggest that immunogenic virus persists in the lung within antigen-presenting cells for at least 1 week and possibly for up to 2 weeks. The persisting immunogenic stimulus after the termination of viral infections might be critical in ensuring the development of a local protective immune response.

Immunization via the respiratory route results in the accumulation of both specific T lymphocytes and immunoglobulin-secreting B cells in bronchoalveolar spaces and pulmonary parenchyma (4, 10, 17, 29, 32, 39). It is not known whether the generation of specific cells can occur locally or whether it occurs exclusively in draining hilar lymph nodes (HLN) and other extrapulmonary lymphoid tissue. If generated solely in extrapulmonary sites, the specific T and B lymphocytes would require a mechanism to return to the lung. Residual antigen after the initial intratracheal inoculation could provide this mechanism, since antigen has been shown to influence cell traffic into the lung by both specific and nonspecific mechanisms (3, 9, 18, 22). Whether local or extrapulmonary induction occurs, the persistence of antigen in the lung should facilitate the local accumulation of immune cells. Thus, it was important to determine the time course of antigen retention in the lung after local deposition of an immunogenic dose.

We have shown that guinea pig alveolar macrophages (AM) are antigen-presenting cells in vitro (23). In addition, antigen-containing bronchoalveolar cells (BAC; consisting of 72% macrophages) inoculated into the trachea can induce an immune response characterized by (i) the development of positive skin tests and (ii) the induction of T lymphocytes in HLN and peritoneal exudates that demonstrate specific proliferative responses to antigen in culture (22). Lastly, when antigen-containing BAC (as well as antigen-containing, adherence-purified AM) are inoculated into the trachea, they induce the selective accumulation of specific T lymphocytes in the lung (25). Thus, antigen-containing AM can regulate pulmonary immunity by both inducing primary immune responses and selectively recruiting circulating specific T cells into the lung. It is likely that the long-term retention of antigen in the lung in association with antigen-presenting cells is important in the development of local immunity after intratracheal inoculation.

The purpose of the current studies was to determine the time course for retention of influenza antigens in the lung and to assess the

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quantity that was cell associated. Furthermore, we determined whether cell-associated antigen was able to stimulate pulmonary immune responses. First, radioactive heat-inactivated (HI) influenza virus was inoculated into the tracheae of guinea pigs, and the rate of loss from the lung of cell-associated and trichloroacetic acid (TCA)-precipitable counts was measured. These measurements were compared with those in which the antigen was inoculated in the footpad, a route that fails to produce pulmonary accumulation of immune cells. In addition, HI virus was inoculated into the lung, and at various times thereafter the ability of BAC to restimulate immune T lymphocytes in vitro was determined.

MATERIALS AND METHODS

Animals. Strain 2 and 13 guinea pigs bred from animals originally provided by Stewart Schlossman (Harvard Medical School, Boston, Mass.) were used in most of the studies. It has been previously shown that both strain 2 and strain 13 guinea pigs respond immunologically to influenza virus inoculated into the lungs (25). Some of the radioactive antigen clearance studies were performed with outbred Hartley female guinea pigs obtained from Simonsen Laboratories (Gilroy, Calif.). There were no detectable differences in clearance among inbred and outbred animals. Therefore, data were pooled for the indicated time points.

Viral antigen. Sucrose gradient-purified PR/8/34 strain influenza virus was a generous gift of M. Phelan of the Bureau of Biologics, Bethesda, Md. Virus was inactivated by heating for 1 h at 56°C. Hemagglutinating units (HAU) were determined by a standard technique (8).

Radioiodination of virus. HI virus was radioiodinated by the chloramine T method to a specific activity of 1 μCi per 256 HAU (33). It has been shown that this procedure radioiodinates the hemagglutinin as well as some internal proteins of the influenza virus (33). The concentration of reagents was selected to assure that hemagglutinating activity was not diminished by the procedure. The radioiodinated virus was separated from the unbound iodine by Sephadex G-25 chromatography. The percent radioactivity precipitated with TCA was 59 ± 3%. It is probable that the 40% nonprecipitable radioactivity reflected iodination of viral membrane lipid, labeling of small peptides, or trapping of free radioactive iodine within the viral envelope. Centrifugation of selected samples at 186,000 × *g* precipitated all of the radioactivity. Each virus sample was tested for hemagglutinating activity after the radioiodination procedure. A sample was not used if the hemagglutinating activity was decreased by the radioiodination procedure. Negative stains of several of the preparations revealed intact virions by transmission electron microscopy.

Radioactive virus clearance studies. Animals were anesthetized, and the tracheae were exposed via surgical incision. Influenza virus (256 HAU in 0.1 to 0.2 ml of phosphate-buffered saline [PBS]) was injected directly into the trachea by using a syringe and a 25-gauge needle. Before inoculation, air was drawn into the syringe to assure proper placement of the needle. In some animals, the same quantity of virus was injected directly into the left footpad. A 256-HAU dose of influenza antigen has been shown previously to be the minimal dose to consistently induce an immune response in guinea pigs when inoculated intratracheally (IT) (22). At various times after inoculation, the animals were killed, tissues were collected, and radioactivity was determined in various organs. Before removal of the lung, blood in the pulmonary tree was removed by perfusing saline through the pulmonary arteries from the right ventricle. Lung tissue was finely minced, pressed through a wire mesh, strained through cotton gauze to remove visible particles, and washed twice. This technique yielded 50 to 70% macrophages, 30 to 50% lymphocytes, and less than 2% neutrophils and alveolar epithelial or interstitial cells (15). Lymph nodes were minced on a wire mesh screen, and the cells were collected and washed twice. All of the wash fluid from the mincing and washing steps was saved and designated the supernate fraction. The radioactivities in both the cell and supernate fractions were determined in a gamma counter. Lung or lymph node was not minced if the radioactivity in the organ was less than 250 or 100 cpm, respectively. In experiments where radioactive virus was inoculated IT, only 44 ± 3% of the lung and 53 ± 5% of the HLN radioactivity was recovered in the cell-plus-supernatant fractions. Therefore, the percent cell-associated radioactivity was determined as the counts per minute in the cell pellet divided by the counts per minute in the whole organ and represented the minimal amount of radioactive virus in the cells. Protein precipitation with 10% TCA was performed on both the cell and supernate fractions to determine the percentage of radioactivity in peptides of >3,000 molecular weight and, therefore, of sufficient size to be potentially immunogenic in guinea pigs (40).

Pulsing BAC with antigen in vitro and in vivo. For in vitro antigen-pulsing experiments, BAC were recovered from nonimmune guinea pigs that had been anesthetized and exsanguinated by cardiac puncture. A catheter was placed in the trachea, and cells were recovered by lavaging with room temperature PBS. For antigen pulsing, BAC (10^7 per ml) were incubated in complete medium (RPMI 1640 with 25 mM HEPES [*N*-hydroxyethylpiperazine-*N'*-Z-ethanesulfonic acid]; 300 μg of glutamine, 200 μg of penicillin, 100 μg of streptomycin, and 25 μg of gentamicin sulfate per ml; and 10% fetal calf serum) with HI virus (128 HAU/ml) and 40 μg of mitomycin C per ml for 1 h at 37°C in a CO_2 incubator. Cells were washed five times with Hanks balanced salt solution and resuspended in complete medium for use in culture. Control or "mock-pulsed" BAC were treated similarly except that they were not exposed to virus.

In a second set of experiments, BAC were antigen pulsed in vivo by the inoculation of HI virus (256 HAU/0.1 ml) directly into the trachea as described above for the inoculation of radioactive virus. This inoculation was performed at several time points before testing the ability of BAC from the animals to stimulate immune T lymphocytes. On the day of testing, in vivo-pulsed BAC were obtained by lavage, utilizing a catheter secured into the trachea and repetitive 5- to 10-ml washes of warm PBS for a total lavage of 50 ml.

In a third set of experiments, BAC were pulsed in vivo by the inoculation of HI virus (256 HAU per 0.1

ml) into a single lung lobe. This was accomplished by puncturing the trachea with an 18-gauge needle and inserting a catheter through the needle to about 4 mm past the tracheal bifurcation. Radioiodinated tobacco mosaic virus (TMV; ≤0.02 μCi and <0.1 μg) was inoculated with the virus as a marker of the inoculated lobe. On day 0, animals were sacrificed, and all lung lobes were counted in a gamma counter. Intrabronchial lavage of the inoculated lobes yielded 10×10^6 to 25×10^6 BAC per lobe, which was used to stimulate a proliferative response in syngeneic immune T cells. The radioiodinated TMV did not interfere with the proliferation assay, i.e., BAC (10^5 per well) without lymphocytes gave a response of <200 cpm. Furthermore, TMV does not cross-react with influenza nor was it used in a quantity sufficient to be immunogenic.

BAC from a nonimmune animal were harvested on day 0 as controls for the BAC that had been pulsed in vivo. Control BAC were split into two pools; one pool was pulsed in vitro as described above and the second pool was mock pulsed. All BAC, including those pulsed in vivo, were treated with mitomycin C (40 μg/ml) for 1 h followed by washing to prevent any contribution from immunized T cells in the BAC in the subsequent proliferation assay. The percentage of AM, identified in a hemacytometer as large cells with cytoplasmic granules, was always >60%. It was previously shown that BAC contain on the average 72% AM, 14% eosinophils, and 14% lymphocytes (23).

T cell responses to pulsed BAC. Immune T cells were obtained from syngeneic animals immunized 3 weeks previously in the footpads with HI influenza (256 HAU) emulsified in complete Freund adjuvant. T cells were isolated from mineral oil-induced peritoneal exudates by purification on a nylon wool column as previously described (16).

Immune T cells (10^5 per well) were cultured with BAC (10^5 per well) for 3 days, pulsed for 18 h before harvest with 0.5 μCi of [*methyl*-^{3}H]thymidine (5 Ci/mmol; Amersham Radiochemical Centre, Amersham, England), harvested on an automated microharvester, and counted in a scintillation counter. Results were expressed as raw counts per minute and as a stimulation index (SI = counts per minute in cultures with pulsed BAC/counts per minute in cultures with control BAC). A stimulation index of ≥2.0 has been shown previously to indicate a specific immune response (22).

RESULTS

Retention of radioiodinated influenza within the lung. It has been shown that HI influenza virus (256 HAU) inoculated into the trachea of guinea pigs resulted in the development of specific T cells in lung and HLN by 5 days, with a peak response at 14 days. Although identical quantities of HI virus inoculated into the footpads induce immune responses in draining popliteal lymph nodes (PLN), footpad inoculation fails to induce specific T cells in the lung at any time for 4 weeks after inoculation (22). These observations could be accounted for by the difference in antigen concentration in the lung after the two methods of immunization. However, in these previous studies, a quantitative comparison of antigen deposited in the lung from the two routes of inoculation was not done. Therefore, we performed radioactive viral clearance studies utilizing both routes of inoculation to help elucidate the role of pulmonary antigen in the accumulation of specific immune cells in the lung.

HI influenza virus was radioiodinated and inoculated into the tracheae of guinea pigs (256 HAU per animal) in five separate experiments involving three to seven animals per experiment. At various times after inoculation, animals were killed, and organs were removed to determine radioactivity. The organs studied included lung, trachea, HLN, axillary lymph nodes, inguinal lymph nodes, PLN, spleen, stomach, gallbladder, liver, kidneys, and urinary bladder. Samples of blood were also examined. Table 1 shows the data on all animals from the five experiments. At 30 min, 45% of the inoculated counts was accounted for in the tissues examined. However, by day 1 only 20% of the counts was recovered. By day 14, only 0.9% of the inoculated radioactivity was recovered. By 24 h, one-third of the radioactivity recovered from the lungs was in cells (Table 1). Furthermore, the majority of the radioactive virus within cells was TCA precipitable (74% at 30 min and 95 to 97% at 1 to 14 days). Thus, some of the inoculated virus persisted in the lung within cells, and the viral protein was of sufficient size to be immunogenic. TCA precipitability suggests the presence of a peptide greater than 3,000 molecular weight, and heptapeptides have been shown to be immunogenic in guinea pigs (40). A measureable amount of potentially immunogenic virus was also present in HLN (Table 1).

Inoculation into the left rear footpad resulted in the accumulation of radioactivity in the left footpad and draining PLN comparable to the accumulation of radioactivity in lung and HLN after IT inoculation, e.g., 3.6% in the footpad and 0.05% in the PLN after footpad inoculation versus 4.7% in lung and 0.03% in HLN after IT inoculation at day 1 (Tables 1 and 2). However, the percentage of antigen inoculated into the footpad that reached the lung was very small, e.g., 0.06% at 30 min and 0.01% at day 1 (Table 2). Furthermore, very little of the antigen that reached the lung from the footpad was retained in cells, i.e., 4% in the one animal in which there was enough radioactivity at 24 h to allow this analysis (Table 2).

The amount of antigen present in HLN after IT inoculation was slightly less than that present in PLN after footpad inoculation for all time points except 30 min. However, both routes of immunization lead to the development of immune T cells in their respective draining lymph node (22).

In summary, as would be suspected, inocula-

TABLE 1. Persistence of radiolabeled influenza in lung and HLN after IT inoculation[a]

Time after inoculation	No. of animals	^{125}I-influenza in lung[b]		^{125}I-influenza in HLN[c]	
		% Of inoculum ± SEM[d]	% Of lung cpm in cells ± SEM[e]	% Of inoculum ± SEM[d]	% of HLN cpm in cells ± SEM[e]
30 min	4	18.0 ± 2.0	8 ± 1	0.15 ± 0.05	4 ± 1
1 day	5	4.7 ± 2.0	34 ± 4	0.03 ± 0.02	30 ± 6
2 days	4	6.3 ± 0.9	33 ± 2	0.01 ± <0.01	28 ± 3
4 days	4	5.7 ± 0.6	35 ± 3	0.01 ± <0.01	25 ± 2
5 days	2	2.5 ± 0.9	27 ± 10	<0.01	ND[f]
7 days	3	1.4 ± 0.1	24 ± 4	<0.01	ND
14 days	3	0.7 ± 0.1	35 ± 3	<0.01	ND

[a] The data shown represent five separate experiments involving three to seven animals each.
[b] The TCA-precipitable radioactivity in cells in the lung was 78% at 30 min but 95 to 97% at 1 to 14 days. In the supernatant fraction of the lung mince the TCA-precipitable radioactivity was 36% at 30 min but 84 to 94% thereafter.
[c] The TCA-precipitable radioactivity in cells from the HLN varied from 54 to 86% and in the noncellular fraction of HLN was 22% at 30 min but 50 to 85% at 1 to 4 days.
[d] Percentage of inoculum = counts per minute in tissue/total counts per minute inoculated.
[e] Percentage of lung or HLN counts per minute in cells = counts per minute in cells harvested from the lung or HLN/counts per minute in the intact organ.
[f] ND, Not done. The counts in HLN were too low, i.e., ≤100 cpm, to make this assessment reliable.

tion of antigen via the trachea resulted in a marked difference in pulmonary antigen concentration as compared with the concentration in the lung after footpad inoculation. Furthermore, after the IT inoculation of radioactive virus, TCA-precipitable radioactivity persisted in the lung within cells for at least 2 weeks in contrast to the absence of any detectable cell-associated radioactivity in the lung 24 h after footpad inoculation.

Persistence of immunogen in BAC. It was important to determine whether the persisting cell-associated antigen in the lung could stimulate an immune response. We first determined whether BAC exposed to virus in vitro and subsequently cultured for several days would

TABLE 2. Persistence of radiolabeled influenza in footpad, PLN, and lungs after footpad inoculation[a]

Time after inoculation	No. of animals	^{125}I-influenza in:				
		Footpad	PLN		Lung	
		% Of inoculum ± SEM[b]	% Of inoculum ± SEM[b]	% Of PLN cpm in cells ± SEM[c]	% Of inoculum ± SEM[b]	% Of lung cpm in cells ± SEM[c]
30 min	3	16.8 ± 3.7	0.05 ± 0.01	2 ± 1	0.06 ± <0.01	3 ± 1
1 day	3	3.6 ± 0.4	0.05 ± 0.02	16 ± 6	0.01 ± <0.01	4[d]
2 days	2	1.5 ± 0.6	0.08 ± 0.02	15 ± 6	0.01 ± <0.01	ND[e]
5 days	2	1.1 ± 0.1	0.04 ± 0.01	19 ± 6	0.01[f]	ND
7 days	2	1.0 ± 0.1	0.03 ± 0.01	18[d]	<0.01	ND
14 days	1	0.3	0.01	15	<0.01	ND

[a] Radioiodinated HI influenza virus (256 HAU) was inoculated into the left rear footpad. Radioactivity in the footpad was determined by using the footpad severed at the ankle. The draining PLN was also collected. The data represent four separate experiments on three or four animals each.
[b] Percentage of inoculum = counts per minute in tissue/total counts per minute inoculated.
[c] Percentage of PLN or lung counts per minute in cells = counts per minute in cells harvested from the PLN or lung/counts per minute in the intact organ. Counts that were TCA precipitable varied from 73 to 95% in PLN cells and from 52 to 95% in the PLN supernatant fraction. TCA precipitable counts were 70 to 75% from 30 min to 2 days in lung cells and 35 to 49% in the supernatant fraction of the lung mince. Counts in lung were negligible thereafter.
[d] Only one of the animals in the group had enough radioactivity in its lungs, i.e., ≥250 cpm, to determine cell-associated counts.
[e] ND, Not done. The counts in HLN were too low, i.e., ≤100 cpm, to make this assessment reliable.
[f] Only one of the animals had any detectable antigen in its lungs at 5 days.

retain their ability to specifically stimulate T lymphocytes. BAC were recovered from nonimmune inbred guinea pigs and cultured in multiple portions for 4 successive days, i.e., days 4 to 1. Each day, one portion was incubated in vitro with HI influenza (128 HAU per ml) for 1 h, washed, reincubated overnight, washed, and reincubated. On the last day, day 0, one portion was pulsed with virus and one was mock pulsed with medium. All portions were treated for 1 h with mitomycin C and cultured with T lymphocytes from virus-immune syngeneic guinea pigs. In vitro-pulsed BAC retained the capacity to stimulate immune T lymphocytes for at least 4 days (Table 3).

We then determined whether BAC exposed to an IT inoculation of an immunogenic dose of HI influenza would become pulsed in vivo, i.e., develop the capacity to specifically induce immune T cells to proliferate in culture. For up to 4 days after an IT inoculation of virus, BAC were able to stimulate a specific proliferative response in immune T lymphocytes (Table 4). In two other experiments (not shown), BAC were able to stimulate specific responses for 2 and 3 days.

In the radioactive viral clearance studies, it was determined that antigen inoculated IT was usually deposited in the two lower and right accessory lobes. Since lavage removes BAC from the entire lung, some of the BAC in the lung lavages were probably not exposed to antigen in vivo. Thus, the in vivo-pulsed BAC would be diluted by unpulsed BAC, reducing the sensitivity of the proliferation assay to demonstrate the retention of immunogen in BAC.

To address this problem, animals were inoculated with HI virus by passing a catheter into a single lobe. BAC were harvested from the inoculated lobe at the times indicated in Table 5 by lavage through an intrabronchial catheter. As described above, the inoculated lobe could be readily identified by the inoculation of a small quantity of radiolabeled TMV together with the influenza virus. The rationale of this approach was that this inoculation and harvest technique would provide a greater percentage of BAC that would have been exposed to influenza in vivo for the T cell proliferation assay. Thus, the concentration of immunogen per BAC would be higher, and it might be possible to demonstrate the retention of immunogen in lung cells for a longer period of time after deposition via the airways. With this technique, BAC harvested 5 to 7 days after an intralobar inoculation were able to specifically stimulate T lymphocyte proliferation (Table 5).

TABLE 3. Retention of immunogenic antigen in BAC after in vitro pulsing

Time (days) before test of in vitro pulse of BAC[a]	Proliferative response of immune T cells[b]	
	cpm ± SEM	SI[c]
4	8,012 ± 541	6.1
3	3,838 ± 333	2.9
2	9,396 ± 180	7.2
1	10,465 ± 568	8.0
0 (pulsed)	12,531 ± 430	9.6
0 (mock pulsed)	1,310 ± 279	

[a] BAC were harvested from an inbred animal 4 days before testing with immune peritoneal exudate lymphocytes. At the times indicated, portions were incubated with 128 HAU of HI influenza virus per ml, washed, incubated overnight, washed, and reincubated. On day 0, all portions were treated with mitomycin C and cultured with syngeneic immune peritoneal exudate lymphocytes.

[b] Immune T cells were obtained by filtering peritoneal exudate cells from influenza-immune animals through a nylon wool column. In vitro culture of T cells with soluble influenza virus (128 HAU/ml) and no BAC resulted in 318 ± 95 cpm (SI = 2.0).

[c] SI = counts per minute in cultures with in vitro-pulsed BAC/counts per minute in cultures with mock-pulsed BAC. It has been previously shown that an SI ≥ 2.0 is significant.

TABLE 4. Retention of immunogenic antigen in BAC after IT inoculation of virus

Time (days) before testing of in vivo pulse of BAC[a]	Proliferation responses of immune T cells[b]	
	cpm ± SEM	SI[c]
5	3,733 ± 134	<1.0
4	9,269 ± 434	2.6
3	6,348 ± 386	1.8
2	10,058 ± 1,388	2.8
1	17,147 ± 1,489	4.7
0 (in vitro pulsed)	32,832 ± 1,215	9.1
0 (mock pulsed)	3,622 ± 876	

[a] BAC were obtained by using an intratracheal catheter to lavage the whole lung.

[b] Immune T cells cultured with HI influenza virus (128 HAU/ml) and no BAC resulted in 7,951 ± 935 cpm (SI = 25.0).

[c] SI = counts per minute in cultures with either in vivo- or in vitro-pulsed BAC/counts per minute in cultures with mock-pulsed BAC.

DISCUSSION

Two major findings emerged from these studies. First, animals inoculated IT with radiolabeled inactivated virus retained TCA-precipitable radioactivity in the lung for at least 2 weeks. At least one-third of the retained radioactivity in the lung was in cells. In contrast, after footpad inoculation of radiolabeled virus, minimal radioactivity was found in the lung. The difference in pulmonary concentration of radioactivity after

TABLE 5. Retention of immunogenic antigen in BAC after intralobar inoculation

Time before testing of in vivo pulse of BAC[a]	Proliferative responses of immune T cells[b]	
	cpm ± SEM	SI[c]
Expt 1		
5 days	22,103 ± 2,302	2.4
4 days	14,279 ± 1,128	1.6
3 days	21,712 ± 1,390	2.4
0 day (in vitro pulsed)	49,480 ± 5,018	5.4
0 day (mock pulsed)	9,124 ± 730	
Expt 2		
10 days		
No. 1	866 ± 343	0.3
No. 2	1,078 ± 103	0.4
7 days		
No. 1	6,144 ± 921	2.3
No. 2	3,998 ± 627	1.5
5 days		
No. 1	10,034 ± 1,727	3.8
No. 2	6,819 ± 609	2.6
0 day (in vitro pulsed)	25,591 ± 3,569	9.7
0 day (mock pulsed)	2,625 ± 167	

[a] In vivo-pulsed BAC used to stimulate responses were harvested by an intrabronchial catheter and lavage of the single inoculated lobe.

[b] In experiment 1, immune T cells with soluble virus but no BAC incorporated 21,784 ± 2,472 cpm (SI = 9.6). In experiment 2, T cells incorporated 3,458 ± 759 cpm (SI = 8.2).

[c] See footnote *c* in Table 4.

the two routes of inoculation is consistent with the observations that IT immunization with influenza virus leads to the development of immune T cells in bronchoalveolar washes and in cells from lung homogenates, whereas footpad immunization fails to produce immune cells in the lung (22).

Second, BAC from animals that were inoculated with virus via the lung were able to stimulate specific T cells in vitro for up to 7 days postinoculation. We propose that these in vivo-pulsed BAC might help explain the presence of immune T cells in the lung after IT inoculation (22, 29, 32). These studies demonstrated that antigen inoculated IT reaches the HLN in quantities comparable to those that reach the PLN after footpad inoculation. It was previously shown that immune T cells are detected in HLN by 5 days (22). Furthermore, IT immunization also resulted in the initial appearance of immune T cells in the lung at 5 days (22). Since lymphoblasts enter the circulation from stimulated lymph nodes 3 to 5 days after initial antigen inoculation (11) and BAC pulsed with antigen can selectively recruit specific T cells into the lung (25), we suggest the following: IT inoculation leads to generation of immune T cells in HLN, and for 5 to 7 days local antigen-presenting cells specifically recruit the circulating immune cells into the lung and stimulate their further proliferation.

Many studies have looked at the clearance of antigens from the lung; only a few have attempted to correlate clearance with the development of an immune response in lungs and HLN or to determine whether an immunogenic form was retained in lung cells (19). Soluble protein antigens are rapidly removed from the lung after deposition in the airways. The major route is across the alveolar-blood capillary membrane (2, 5, 38). Inflammation may increase the uptake of soluble molecules via lymphatics (34). Particulates are retained for long periods in the lung, and one route of removal is via the lymphatics with a resultant accumulation in HLN (28). Lauweryns and Baert studied the clearance of carbon and ferritin from the lung by electron microscopy and found that the three major mechanisms for removal of these substances were (i) direct mucociliary clearance, (ii) macrophage uptake and destruction, with some cellular movement up the airways, and, although quantitatively less important, (iii) lymphatic clearance (21). Thus, a number of previous studies show that both soluble and particulate antigens reach HLN after IT inoculation. The current studies confirm these observations. However, the clearance of microorganisms from the lung depends predominantly on the uptake and killing by alveolar macrophages or recruited neutrophils or both (12). No studies have directly addressed the question of whether antigen uptake by mononuclear phagocytes might result in pulsing the antigen-presenting cell, enabling it to stimulate local immune responses. Our radioactive viral clearance studies indicate that uptake by cells in the lung is an important mechanism for clearing inactivated viral particles. Furthermore, we determined that microbial antigens could be retained in an immunogenic form within antigen-presenting cells in the lung.

Whether there are antigen-presenting cells in the lungs of all species and, if so, whether AM fulfill this function is a controversial question. Antigen-presenting cells are required for the specific stimulation of the proliferative and effector functions of T lymphocytes (36). It has been reported that there are antigen-presenting cells in bronchoalveolar washes from humans (20), although there is a more recent report that conflicts with the earlier observation (26). In mice, AM present antigen, but less effectively than other murine antigen-presenting cells (31, 35, 37). In several species, suppressor activity by AM interferes with the expression of antigen presentation, at least in vitro (1, 13, 30, 41). In the guinea pig, AM do not suppress in vitro immune responses and are as effective as perito-

neal exudate macrophages in presenting antigen (14, 22). Furthermore, we have shown that AM are important regulators in the development of local immune responses in vivo (25). The present studies suggest that after the uptake and killing of virus, pulmonary antigen-presenting cells could continue to stimulate a local immune response.

A second controversy, which also may reflect species differences, is whether antigen specifically recruits immune lymphocytes into the lung or whether the recruitment phenomenon is simply a result of nonspecific inflammatory influences. Several authors have shown that inflammation or preprogrammed homing tendencies to mucosal surfaces or both play important roles in the accumulation of circulating lymphocytes in the lung (3, 6, 7, 9, 11, 24, 27). However, several studies indicate that local antigen is also specifically involved over and above its inflammation-provoking capability (9, 18, 22). These studies suggest that specific recruitment by antigen is a possibility in an immune animal for up to a week after a pulmonary reexposure to an antigen. Such specific recruitment would not necessarily rule out a concomitant accumulation of lymphocytes by nonspecific inflammatory mechanisms.

In summary, after the inoculation of influenza virus, cells in the lung demonstrate the ability to specifically stimulate T cells in vitro for up to 1 week and retain TCA-precipitable viral peptides for at least 2 weeks. Therefore, antigen might continue to exert an immunogenic stimulus to prime T cells locally or to recruit circulating T cells into the lung or both for an extended time after the resolution of an infection or other antigenic exposure. Furthermore, the potential exists for in vivo-pulsed antigen-presenting cells to continually trigger lymphoproliferation and lymphokine release from local specific T cells. These events might result not only in enhanced protective responses against a large number of intracellular organisms but also might lead to damaging hypersensitivity reactions.

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