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Virus-Specific Antigen Presentation by Different Subsets of Cells from Lung and Mediastinal Lymph Node Tissues of Influenza Virus-Infected Mice

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Immune responses at mucosal sites are thought to be initiated in the draining lymph nodes, where dendritic cells present viral antigens and induce naive T cells to proliferate and to become effectors. Formal proof that antigen-presenting cells (APC) do indeed localize to the regional lymph nodes has been lacking for viral infections of the respiratory tract. Influenza virus was detected in the draining mediastinal lymph nodes (MLN) early after intranasal inoculation, with peak virus titers in this tissue measured at 2 days postinfection. Virus-specific cytotoxic T-lymphocyte responses were first detected in the MLN 1 day later. Macrophages, dendritic cells, and B lymphocytes were isolated from influenza virus-infected mice and assayed for the capacity to stimulate a major histocompatibility complex class I-restricted virus-specific T-cell hybridoma. All APC populations from lungs and MLN contained virus and thus had the potential to present antigen to $CD8^+$ T cells. The APC recovered from the lungs of influenza virus-infected mice and dendritic cells from the MLN were able to stimulate virus-specific responses. The lack of a virus-specific T-cell response to B cells corresponds to the small number of virus-positive B lymphocytes in the MLN. These results indicate that dendritic cells and macrophages are antigen positive in mice acutely infected with an influenza A virus and that dendritic cells are probably responsible for initiating the cytotoxic T-lymphocyte response to influenza virus in the draining lymph nodes.

Influenza virus-specific $CD8^+$ cytotoxic T lymphocytes (CTL) are responsible for the clearance of infected cells from pneumonic lungs (6, 21) and for the termination of infection in the absence of the $CD4^+$ T-cell subset (1). Compensatory mechanisms are able to effect clearance, although somewhat slower, in the absence of $CD8^+$ T cells (7). Primed $CD8^+$ CTL also provide some protection against heterologous influenza A viruses since the response is often to a conserved viral antigen that is synthesized intracellularly and presented as a peptide in association with major histocompatibility complex (MHC) class I molecules. For example, the $CD8^+$ T-cell response to influenza A virus infection in C57BL/6 (B6) mice is predominantly specific for a peptide of the conserved nucleoprotein (NP) molecule presented by H-$2D^b$ (35).

Hence, the events required to initiate a virus-specific CTL response are crucial to the establishment of protective immunity. Foreign intestinal antigens are first presented to T cells in draining Peyer's patches, with activated T cells then circulating to the effector site (19). The immune response in the lungs is thought to be initiated in a similar fashion (1). The premise is that $CD8^+$ T cells are primed in the regional mediastinal lymph nodes (MLN) that drain the lungs and then migrate back to the site of predominant virus growth in the respiratory epithelium. Although intranasal (i.n.) infection with influenza virus X-31 does not result in systemic infection, a small amount of replicating virus has been detected by PCR analysis of mRNA 2 days after infection in the MLN (8). Within 2 to 3 days, the MLN enlarge considerably and virus-specific $CD8^+$ T cells can be recovered by culture (1).

Professional antigen-presenting cells (APC) include dendritic cells, macrophages, and B lymphocytes. The superior quality of dendritic cells, compared with macrophages and B cells, has been clearly shown in other experimental systems. In particular, the ability of dendritic cells to stimulate antigen-specific T cells from a pool of naive lymphocytes seems to be unique (20, 33). Hence, it is believed that these are the APC responsible for initiation of the immune response in vivo. We have examined this by identifying the APC populations that harbor virus after i.n. infection of B6 mice with the X-31 influenza A virus and by determining their capacities to stimulate T-cell hybridomas specific for the D^b-restricted NP epitope.

MATERIALS AND METHODS

Mice. Female B6 (*H-2*^{b}) and BALB/c (B/c; *H-2*^{d}) mice were purchased from The Jackson Laboratory (Bar Harbor, Maine) and housed under specific-pathogen-free conditions at St. Jude Children's Research Hospital (SJCRH).

Antibodies and flow cytometry. Single-cell suspensions were counted and stained as follows: 2×10^5 cells were incubated with an isotype-matched control antibody or the corresponding cell surface antigen-specific monoclonal Ab (MAb) for 20 min at 4°C. Fluorescein isothiocyanate-conjugated RA3-6B2 (3), a rat MAb specific for B220; biotinylated M1/70.15 (31), a rat MAb specific for Mac-1 (CD11b); phycoerythrin-conjugated 30-H12 (17), a rat MAb specific for Thy1.2; and phycoerythrin-conjugated H57-597 (15), a hamster MAb specific for αβ T-cell receptor, were purchased from Pharmingen, San Diego, Calif. Fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G was purchased from Southern Biotechnology Associates (Birmingham, Ala.). The hamster MAb, N418 (24), specific for CD11c, a dendritic cell marker, was used as culture supernatant from hybridoma cells (American Type Culture Collection, Rockville, Md.). Cells were washed and (in the case of M1/70.15 and N418) subsequently stained with streptavidin-phycoerythrin (Southern Biotechnology Associates) or fluorescein isothiocyanate-conjugated goat anti-hamster immunoglobulin G (Southern Biotechnology Associates), respectively. Following a second wash step, cells were examined by flow cytometry; 10,000 events were analyzed by using a FACScan and the Lysis II program (Becton Dickinson and Co., Mountain View, Calif.).

Rat antibodies 53-6.72 (17; specific for mouse CD8a), H129.19 (26; specific for mouse CD4) and RA3-6B2 were used as culture supernatant from hybridoma

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cells (American Type Culture Collection). These three MAbs were used in the depletion steps of the mixed lymphocyte reaction described below.

Viral peptides. The influenza A virus and Sendai virus NP peptides, residues 365 to 380 (IASNENMETMESSTLE) and 324 to 332 (FAPGNYPAL), respectively, were synthesized at the Center for BioTechnology at SJCRH. G. Cole (SJCRH) kindly provided the latter peptide.

Cell lines and T-cell hybridomas. The LIE 13.14 (*H-2*^{b}) macrophage cell line was established by culturing cells lavaged from the lungs of B6 mice in the presence of colony-stimulating factor 1 with subsequent cloning by limiting dilution (37). This cell line was maintained on polymethylpentene petri dishes (Nalgene, Rochester, N.Y.) in Dulbecco's modified Eagle medium (Bio Whittaker, Walkersville, Md.) supplemented with 10% fetal calf serum (FCS; Atlanta Biologicals, Norcross, Ga.) and 20% supernatant from cultures of Ladmac cells (30). Both cell lines were obtained from W. Walker (SJCRH).

T-cell hybridomas 11.2.2, 12.64, 12.91, and E19.3 recognize NP peptide residues 365 to 380 in association with H-2D^b (5). M3.4H3, a T-cell hybridoma specific for Sendai virus NP peptide residues 324 to 332 restricted by H-2K^b (4), was obtained from G. Cole. These hybridomas were maintained in S minimal essential medium (Life Technologies, Grand Island, N.Y.) containing 10% FCS and supplements (13).

Viral infection and titration. The X-31 (H3N2) influenza A virus (14) was grown in the allantoic cavities of embryonated chicken eggs. Virus stocks were shown to be free of bacteria and endotoxin. Eight- to twelve-week-old B6 mice were anesthetized with avertin (2,2,2-tribromoethanol) and inoculated intranasally with 240 hemagglutinating units (HAU) of virus (30 μl of phosphate-buffered saline [PBS] containing a 1/5 dilution of X-31 stock). For virus titration, mice were killed and both MLN and lungs removed. Tissues were then homogenized in 1 ml of PBS, and 100-μl aliquots of serial 10-fold dilutions were inoculated into the allantoic cavities of 10-day-old embryonated chicken eggs. Alternatively, isolated APC suspensions were counted, and 10-fold dilutions containing known numbers of cells were used to inoculate eggs. After 48-h of incubation at 35°C, the allantoic fluid from each egg was sampled and assayed for the presence of hemagglutinating activity with 0.5% chicken erythrocytes. Virus titers were expressed as the dilutions at which virus was detected in 50% of eggs (EID_{50}) (9). The frequency of cells that contained virus was expressed as the inverse of the number of cells which allowed virus growth in 50% of eggs.

Isolation of APC populations. Mice infected 2 days before were anesthetized, bled out from their axillae, and bronchoalveolar lavage (BAL) cells were collected as previously described (1). BAL cells were used as a source of bronchoalveolar macrophages. Lungs and MLN were then removed, cut into small pieces, and digested for 90 min in complete medium (RPMI 1640 [Life Technologies] plus 10% FCS, 2 mM glutamine, 100 U of penicillin G per ml, and 100 μg of streptomycin [Life Technologies] per ml) containing 150 U of collagenase (Worthington Biochemicals, Freehold, N.J.) per ml and 30 U of DNase I (Sigma, St. Louis, Mo.) per ml. The digested tissue was then pressed through 200-μm mesh (Netwells; Costar, Cambridge, Mass.) to obtain a single-cell suspension. Lung and MLN cells were washed in complete medium, treated with buffered ammonium sulfate to lyse erythrocytes, and washed again. Cells were counted, and viability was measured by trypan blue exclusion. The lung cell suspension was incubated at room temperature for a few minutes to allow particulate matter to settle. This fraction was subsequently used as a source of alveolar macrophages (27). Lung cells were separated into fractions on a discontinuous Percoll (Pharmacia, Piscataway, N.J.) gradient. Cells were suspended in the highest-density Percoll, overlaid with lowering densities, and centrifuged at 400 × *g* for 20 min. Two pooled fractions, a low-density fraction (<1.075 g/ml) and a high-density fraction (>1.075 g/ml), were obtained. Cells from each fraction were washed and counted, and viability was checked by trypan blue exclusion. Subsequently, cells were resuspended in complete medium at 3×10^6 to 5×10^6/ml and incubated on tissue culture plates (Falcon, Franklin Lakes, N.J.) overnight at 37°C. Nonadherent, high-density (>1.075 g of Percoll per ml) cells were collected, washed, and used as enriched lung B cells. During overnight incubation, some of the adherent, low-density (<1.075 g of Percoll per ml) cells spontaneously detached from the tissue culture plates. These cells were collected, washed, and used as enriched lung dendritic cells.

MLN cells were resuspended in complete medium at 3×10^6 to 5×10^6/ml and incubated overnight at 37°C on tissue culture plates (Falcon). Nonadherent MLN cells were removed, washed, and resuspended at 5×10^6/ml in RPMI 1640 containing 15% FCS, and 3 to 8 ml was layered over a metrizamide gradient (Nycodenz; 14.5 g/100 ml [Life Technologies]). The gradient was spun at 400 × *g* for 20 min. Cells at the interphase were harvested, washed, and used as enriched MLN dendritic cells. Cells in the pellet were resuspended, washed, and used as enriched MLN B cells.

Cytotoxicity assay. MLN cells from three to four mice were pooled for analysis of CTL specific for influenza virus. Initially, lymph node cells were stimulated in vitro with syngeneic, X-31-infected splenocytes for 5 days. Influenza virus-specific effectors were then assayed on ^{51}Cr-labeled, X-31-infected MC57G fibroblasts (7). Target cells were labeled with 0.1 mCi of ^{51}Cr (Na^{51}Cr-chromate; Amersham, Arlington Heights, Ill.)/10^7 cells. Specific cytotoxicity was calculated in relation to the level of ^{51}Cr release in medium alone (34).

Mixed leukocyte culture. B6 APC populations were used to stimulate B/c T cells on 96-well flat-bottom tissue culture plates (Costar). Each potential APC population described above (BAL macrophages, lung B and dendritic cells, and MLN B and dendritic cells) as well as enriched B cells and splenocytes from B6 mice was used as stimulators. Enriched B cells were obtained by immunodepletion rather than gradient centrifugation. MLN were removed, homogenized, and passed through nylon mesh to obtain a single-cell suspension. Cells were plated at a concentration of 5×10^6/ml in complete medium and incubated overnight at 37°C. Subsequently, cells were harvested, washed, and resuspended at 10^7/ml in a 15-ml conical tube (Falcon). Following a 20-min incubation on ice with previously determined optimal concentrations of 53-6.72 and H129.19 MAbs, cells were incubated with sheep anti-rat immunoglobulin G dynabeads (Dynal, Great Neck, N.Y.) for 45 min at 4°C. Cell-antibody-bead complexes were removed by placing the tube against a magnet and collecting the cells in the supernate. Each stimulator population was washed, counted, and stained with the appropriate lineage-specific MAb to determine the percentage of that cell type in the population. Stimulators were then plated at appropriate concentrations to result in 10^5, 10^4, or 10^3 cells of a particular lineage in each well. Stimulator cells were then irradiated (3,000 rads). The responding T-cell population was derived from B/c spleens that were depleted of B cells by using RA3-6B2 and sheep anti-rat immunoglobulin dynabeads. T cells (350,000) were added to each well, and cultures were incubated for 4 days at 37°C. Eight hours before harvest, each culture received 1 μCi of [^{3}H]thymidine (Amersham), and the amount of radioactivity was determined from triplicate cultures with a Betaplate counter (LKB Wallac, Turku, Finland).

Stimulation of virus-specific T-cell hybridomas and interleukin-2 assay. Potential APC populations were placed on 96-well round-bottom tissue culture plates (Costar) at various cell concentrations. The plates were centrifuged at 400 × *g* for 5 min, and the supernatant was removed. Twenty microliters of PBS containing 1 μg (final volume) of either the influenza virus NP peptide or the Sendai virus NP peptide per ml or 20 μl of PBS alone was added to the appropriate wells. Cells were incubated for 1 h at 37°C. One hundred microliters of S minimal essential medium containing 10% FCS and supplements was added to each well, and plates were irradiated (2,000 rads). An equal volume containing 2×10^4 virus-specific T-cell hybridomas was then added to the appropriate wells. Cultures were incubated for 20 to 24 h at 37°C. Supernatants were harvested and tested for the presence of interleukin-2 with the CTLL indicator cell line (11). CTLL cells were cultured in triplicate samples of supernatants, and proliferation was measured by the incorporation of [^{3}H]thymidine (Amersham) during the last 6 h of 24-h culture.

RESULTS

Early presence of antigen and virus-specific CTL in the MLN. Mice were infected i.n. with X-31 influenza A virus, and at various times postinfection, virus titers in the MLN were determined. Although virus was evident in the MLN on day 1 postinfection (log_{10} EID_{50}, 1.5), the highest titers were seen on day 2 (log_{10} EID_{50}, 3.3). One day later, virus-specific cytotoxic $CD8^+$ T cells were cultured from bulk populations of MLN cells which had been stimulated in vitro with virus-infected splenocytes (Fig. 1).

Isolation and characterization of APC populations from the lungs and MLN of influenza virus-infected mice. Potential APC populations from the lungs and MLN of virus-infected mice were enriched to evaluate which cell population(s) is capable of antigen presentation during influenza virus infection. Two days after infection, when optimal virus is present (see above), the lungs and MLN were removed and processed as described in Materials and Methods. The percentages of various cell lineages within each enriched APC population were obtained by flow cytometry (Fig. 2 and 3). These results are representative of multiple experiments and reflect the usual enrichment obtained. Forward and side scatter properties, as well as positive staining with M1/70.15 antibody (specific for CD11b [Mac-1]), were used to distinguish macrophages in the BAL and lung populations (Fig. 2). The percentages of macrophages in the BAL and lung populations were 90 and 30%, respectively. The majority of the remaining cells in the lung preparation were lymphocytes, as depicted by the forward and side scatter profile.

Enriched B-lymphocyte populations from the lungs and MLN, which were assessed by staining for B220, contained 39 and 58% B cells, respectively (Fig. 3A). Forward and side scatter properties and staining with anti-Mac-1 and anti-CD11c MAbs demonstrated that enriched B-cell populations

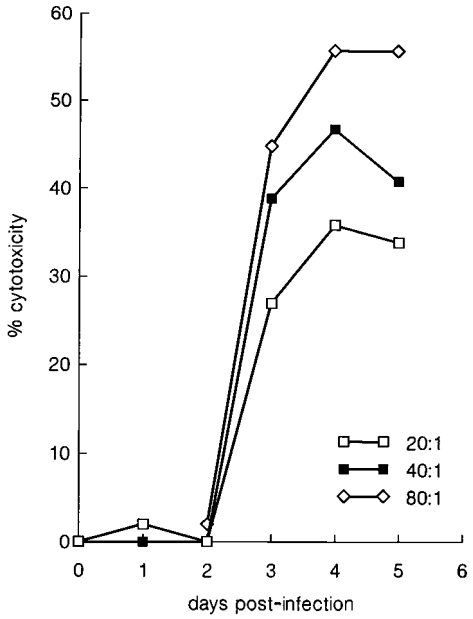

FIG. 1. Cytotoxic activities in the MLN of influenza virus-infected mice. At various times after i.n. infection with X-31, MLN were removed and single-cell suspensions were stimulated with γ-irradiated, virus-infected, syngeneic splenocytes. Cultures were assayed for cytotoxic activities on day 5 with virus-infected MC57G targets. The results shown are representative of three independent experiments.

from either tissue were not contaminated with significant numbers of macrophages or dendritic cells. The remaining cells in the enriched B-lymphocyte population were T lymphocytes, as demonstrated by positive staining with anti-Thy1.2 MAb (data not shown).

The percentages of dendritic cells in the enriched lung and MLN populations were determined with the anti-CD11c MAb N418 (Fig. 3B). The enriched population from the lungs contained 23% dendritic cells, while 73% of the enriched MLN population were dendritic cells. Dendritic cells express CD11b (24), thereby accounting for positive staining with the M1/70.15 MAb. Forward and side scatter profiles showed some lymphocyte contamination of the enriched dendritic cell populations from both the lungs and MLN. However, the numbers of B lymphocytes in these populations were low (approximately 3 and 13% of the total population from the lungs and MLN, respectively). With some experimental variations, these results are representative of the composition of each enriched APC population used in this study.

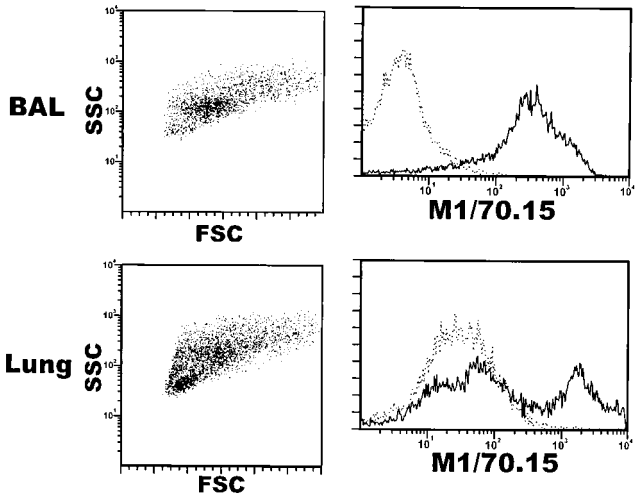

FIG. 2. Flow cytometric analysis of macrophage populations from the BAL and lungs of virus-infected mice. BAL cells and enriched alveolar macrophages (see Materials and Methods) were stained with either an isotype-matched control (dotted line) or anti-Mac-1 (CD11b) MAb (solid line). The forward and side scatter (FSC and SSC, respectively) properties of both populations as well as histograms of positive staining with the anti-Mac-1 MAb are depicted. Mac-1$^+$ cells represent 90% of the BAL preparation and 30% of the lung preparation. The results depicted are representative of multiple experiments.

Since it is known that dendritic cells are potent stimulators of the mixed leukocyte reaction (32, 33), the stimulatory activity of each APC population was tested by mixed leukocyte culture. These results (Table 1) indicated that the enriched APC populations used in this study have the expected functional properties. As few as 10^3 MLN or 10^4 lung dendritic cells from B6 mice were sufficient to elicit significant stimulation of B/c T cells. The enriched B-cell populations from the MLN and lungs did not stimulate significant proliferative responses. These responses were similar to that obtained with B cells enriched by T-cell depletion of homogenized MLN or with whole-splenocyte populations (Table 1).

All potential APC populations from lungs and MLN contain virus. As an indication of a population's ability to present antigen during an influenza virus infection, the number of cells containing virus within each population was determined by the titration of cells in embryonated chicken eggs. As expected, the highest frequencies of cells that had virus were those for unseparated BAL cells, sorted macrophages from BAL cells, and unseparated lung cells (Table 2). The frequencies of enriched dendritic cells from the lungs and MLN containing virus were also high. The macrophage population in the MLN is too small to allow complete separation and thus was not included in this study. Both lung and MLN enriched B- and T-cell populations did not appear to contain significant amounts of virus, as evidenced by low frequencies. In fact, the virus present in these enriched populations may be a consequence of contaminating dendritic cells. This was evident in early experiments with a slightly different protocol for the preparation of APC populations (Table 2, footnote *a*).

To circumvent such contamination, APC populations were sorted by flow cytometry and titrated in eggs to reflect more accurately the frequency of cells that contained influenza virus. The frequencies for virus-positive B and T cells sorted from the lungs were similar, with values much higher than those for both populations sorted from the MLN (Table 3). The frequency of sorted lung dendritic cells that had virus associated with them was lower than that obtained for enriched lung dendritic cells (Table 2), possibly because of other contaminating cell types (perhaps epithelial cells) in the enriched dendritic cell population or experimental variation. Sorted B lymphocytes from the MLN were not shown to contain virus when as many as 10^5 cells were injected into eggs (Table 3). Virus was detected in B lymphocytes from the MLN only when greater than 10^6 sorted B cells were injected into eggs (data not shown).

All potential lung APC stimulate a virus-specific T-cell hybridoma. To answer which APC can present influenza virus antigens to CD8$^+$ T cells, we used an influenza virus-specific T-cell hybridoma to provide a reproducible, sensitive readout. Cells of the LIE 13.14 line were cultured in the presence of various concentrations of influenza virus NP peptide for 1 h, with the subsequent addition of a panel of D^b-restricted T-cell hybridomas specific for the immunodominant NP epitope. Supernatants were harvested 24 h later and assayed for interleu-

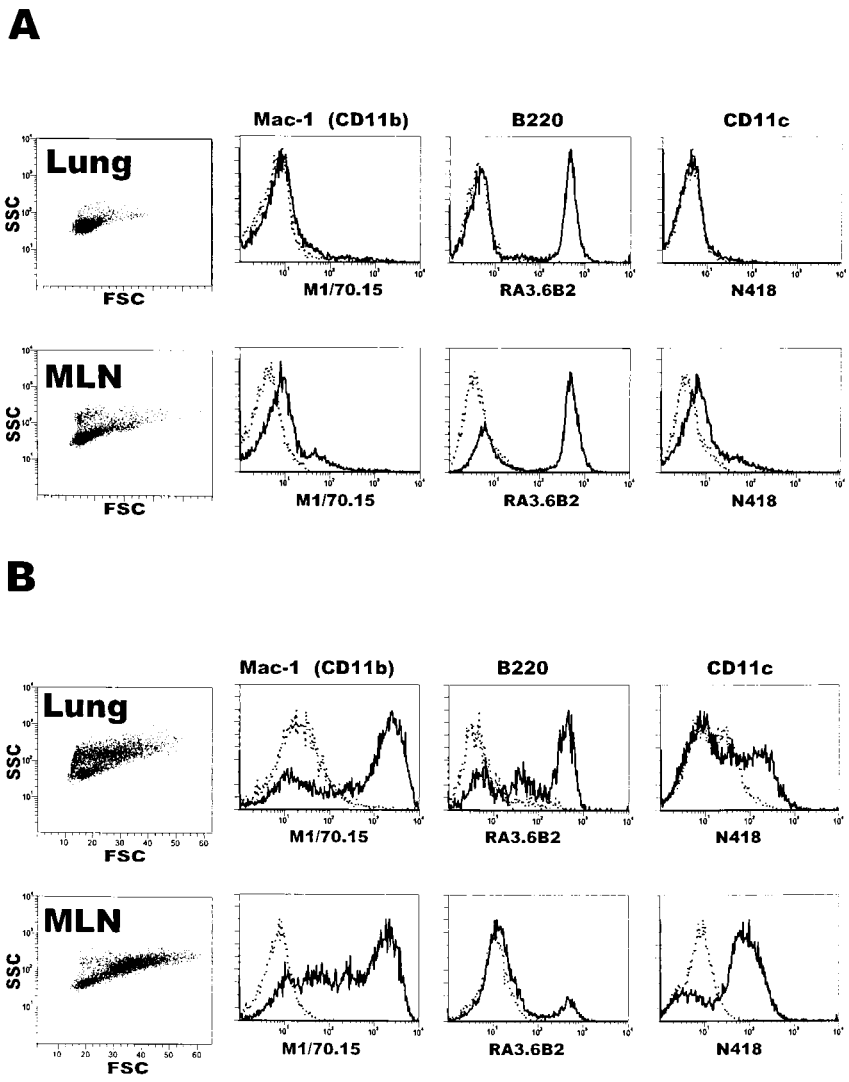

FIG. 3. Flow cytometric analyses of enriched lymphocyte (A) and dendritic cell (B) populations from the lungs and MLN of virus-infected mice. Single-cell suspensions of enriched lymphocyte and dendritic cell populations from the lungs and MLN of infected mice were stained with anti-Mac-1, anti-B220, and anti-CD11c to assess the cell lineage composition of each population. The isotype-matched control is represented as a dotted line in each histogram. The forward and side scatter (FSC and SSC, respectively) properties of each population are depicted. The relative contamination of each population with other cell lineages can be ascertained from the histogram for each MAb. For B220, only staining of the lymphocyte population (based on FSC and SSC) is shown. The enriched lymphocyte population from the lungs contained 6% $CD11b^+$, 4% $CD11c^+$, and 39% $B220^+$ cells. The enriched lymphocyte population from the MLN contained 14% $CD11b^+$, 17% $CD11c^+$, and 58% $B220^+$ cells. The enriched dendritic cell population from the lungs contained 59% $CD11b^+$ and 23% $CD11c^+$ cells. Of the total population, 25% were lymphocytes, of which 12% were $B220^+$. The enriched dendritic cell population from the MLN contained 79% $CD11b^+$, 73% $CD11c^+$, and 13% $B220^+$ cells. Macrophages were eliminated from these populations by adherence to tissue culture plates. The results shown are representative of multiple independent experiments.

kin-2. The 11.2.2 and 12.91 hybridomas did not respond as well as 12.64 and E19.3 did (Fig. 4). The 12.64 hybridoma gave a measurable response at an NP peptide concentration of 0.1 μg/ml and thus was used in subsequent experiments.

Mice were infected with X-31 virus, BAL cells and lungs were removed on day 2 postinfection, and populations of enriched macrophages, dendritic cells, and B lymphocytes were obtained. Each of these was used to stimulate the 12.64 NP-specific T-cell hybridoma. The macrophage cell line LIE 13.14 was treated with NP peptide and used as a positive control. Enriched BAL macrophages, as well as alveolar macrophages, dendritic cells, and B cells isolated from infected lungs, were capable of stimulating the 12.64 hybridoma (Fig. 5 and 6). Macrophages and dendritic cells were more potent stimulators than lung B cells were, as evidenced by the cell numbers required to obtain a virus-specific response. Although both 10^5 and 10^4 APC were used as stimulators, as few as 10^4 macrophages or dendritic cells per well were sufficient to generate a response, whereas 10^5 B cells were required. However, equivalent responses were obtained when the same number of cells were pulsed with peptide, indicating that when the MHC class I molecule is "loaded," each APC population is an effective stimulator of the 12.64 hybridoma.

Dendritic cells from the MLN induce a virus-specific response. Stimulation of the influenza virus-specific T-cell hybridoma was used as a measure of the capacities of B cells and dendritic cells from the MLN of infected mice to function as APC. Populations of enriched dendritic cells or B lymphocytes were obtained from the MLN of mice 2 days after infection with X-31. These potential APC were cocultured with the 12.64

TABLE 1. Presentation of alloantigen by potential APC populations[a]

Cell type	Mean [^{3}H]thymidine incorporation ± SD (cpm) for APC/well of[b]:		
	10^5	10^4	10^3
BAL macrophage	2,638 ± 462	1,031 ± 296	599 ± 136
Lung B cell	ND	841 ± 93	802 ± 209
Lung dendritic cell	ND	7,201 ± 1,666	976 ± 70
MLN B cell	4,650 ± 803	1,710 ± 210	888 ± 63
MLN dendritic cell	ND	165,629 ± 20,383	35,607 ± 5,778
Purified MLN B cell	ND	2,692 ± 380	842 ± 233
Splenocyte	8,835 ± 307	1,202 ± 144	987 ± 256

[a] B6 mice were inoculated i.n. with 240 HAU of X-31 virus. Two days later, BAL, lungs, and MLN were removed and enriched cell fractions were obtained as described in Materials and Methods. Purified B cells from homogenized MLN of infected B6 mice (2 days postinfection) were obtained by the depletion of $CD8^+$ and $CD4^+$ T cells as described above. Splenocytes were obtained from B6 mice. The effector population was splenocytes obtained from B/c mice that were depleted of B cells as described above to obtain enriched T cells.

[b] Irradiated stimulators were cultured at the cell numbers shown with 3.5×10^5 B/c T cells for 4 days. Eight hours prior to harvest, [^{3}H]thymidine was added to cultures. Data are for triplicate cultures. The result with effector cells alone was 734 ± 173 cpm, while the results with irradiated APC populations were less than 350 cpm. ND, not done.

TABLE 2. Prevalence of virus-infected cells in enriched cell populations[a]

Tissue source	Enriched population	Frequency of virus-positive cells[b]	Composition of cell population (%)		
			Dendritic cells	B cells	T cells
BAL	Unseparated	>1/391[c]	ND[d]	ND	ND
	Macrophage	>1/391	<1	<1	<1
LUNG	Unseparated	>1/391	ND	ND	ND
	Dendritic cell	1/8,850	70	8	8
	B cell	1/119,048	24	37	24
	T cell	1/100,000	17	4	82
MLN	Unseparated	<1/200,000[e]	ND	ND	ND
	Dendritic cell	1/83,333	53	5	5
	B cell	1/142,857	35	55	10
	T cell	<1/200,000	25	9	56

[a] B6 mice were inoculated i.n. with 240 HAU of X-31 virus; two days after infection, BAL, lungs, and MLN were removed. In the preliminary experiment, the enriched cell fractions were obtained as described in Materials and Methods with the following additional steps. Macrophages were sorted from BAL cells according to their forward and side scatter properties. Single-cell suspensions of the lungs were separated into fractions on a discontinuous Percoll gradient, and cells from each fraction (≤1.052 or ≥1.063 g of Percoll per ml) were incubated on tissue culture plates at 37°C. After 1.5 to 2 h, nonadherent cells from the low-density fraction were removed, and fresh medium was added to the plate. Some dendritic cells which contaminated the high-density fraction by this method were not adequately removed by this short incubation. After overnight incubation, the cells in suspension were recovered from both sets of plates. Nonadherent cells recovered from the low-density fraction were used as lung dendritic cells, while those from the high-density fraction were used as lung B cells. For enriched dendritic cell populations, T cells, macrophages, and B cells were depleted by using lineage-specific MAbs and dynabeads. Enriched B-cell and T-cell populations were obtained in a similar manner by depleting the appropriate cell types with the corresponding MAbs and dynabeads. Single-cell suspensions of the MLN were incubated for 1.5 to 2 h at 37°C. Nonadherent cells were removed and placed on new tissue culture plates. Fresh medium was then added to the original plates. Both nonadherent cells (lymphocyte fraction) and adherent cells (dendritic cell fraction) were incubated overnight. Instead of a metrizamide gradient, enriched MLN B-cell populations were obtained by the depletion of T cells and macrophages with appropriate MAbs. Single-cell suspensions were stained with a dendritic cell-specific MAb, a B-cell-specific MAb, or a T-cell-specific MAb to determine the composition of each enriched population of cells. Subsequent staining of these populations showed that the initial short incubation did not remove all dendritic cells from the lymphocyte fraction. The antibodies used for depletion were different than those used for staining.

[b] Serial dilutions of cell suspensions were titrated in eggs for the presence of virus. Each frequency is the cell number at which virus was detected in 50% of eggs.

[c] The smallest number of cells injected into eggs still contained virus; therefore, the frequency is >1/391 cells.

[d] ND, not done.

[e] The largest number of cells injected into eggs did not contain virus; therefore, the frequency is <1/200,000 cells.

influenza virus NP-specific T-cell hybridoma for 20 to 24 h, and supernatants were harvested and subsequently analyzed by the standard CTLL assay. Cultured at 10^5 cells per well, enriched B lymphocytes from virus-infected mice were unable to stimulate a significant virus-specific T-cell response (Fig. 7A). In contrast, although a significant response was seen with 10^5 MLN dendritic cells per well (data not shown), as few as 10^4 dendritic cells per well stimulated a virus-specific response (Fig. 7B). Both dendritic and B cells from the MLN of influenza virus-infected mice elicited responses when they were pulsed with influenza virus NP peptide prior to coculture with the T-cell hybridoma (Fig. 7). The MHC class I-restricted Sendai virus-specific T-cell hybridoma M3.4H3 was not stimulated by dendritic cells from influenza virus-infected mice, thereby indicating that the response of the influenza virus-specific T-cell hybridoma to dendritic cells was antigen specific.

DISCUSSION

The establishment of protective immunity to a viral infection is dependent on initiating events in a primary immune response to the virus. The APC and molecular interactions which are required to activate a naive $CD8^+$ T cell and generate a virus-specific CTL are not well understood. Although B cells, macrophages, and dendritic cells are all considered professional APC, dendritic cells have been shown to have the capacity to generate an antigen-specific response by naive T cells (20, 33). It is therefore likely that dendritic cells are the APC that initiate a CTL response to virus infection in vivo. Current knowledge suggests that for antigens entering via mucosal surfaces, $CD8^+$ T cells are primed in the lymph nodes that drain the site of infection (19). The results of this study indicate that influenza virus can be detected in the draining MLN as early as 1 day after infection, with maximal amounts on day 2 postinfection. Consequently, on day 3 postinfection, virus-specific CTL were cultured from this site. The dendritic cell population from the MLN contained virus and was able to stimulate an MHC class I-restricted virus-specific T-cell response. The titration of enriched dendritic cells in eggs suggested that 1 in 84,000 cells contained virus. However, consistent stimulation was obtained even with 10^4 dendritic cells per well, indicating that the frequency of cells containing viral antigen which can be presented to $CD8^+$ T lymphocytes must be greater than that estimated by titration in eggs. This may be due to a loss of virus infectivity during sample preparation or to experimental variations. Previous studies of rats have demonstrated that 24 to 48 h after inhalation of aerosolized antigens, there are increased numbers of dendritic cells in the lungs (22, 29). Forty-eight hours after inhalation, the number of dendritic cells within the draining lymph nodes is also increased (22). Hence, differences between the frequency of dendritic cells found to contain influenza virus and the number of dendritic cells required to stimulate a virus-specific T-cell response may be due to differences in the exact time postinfection when APC populations were isolated from infected mice.

Our results clearly demonstrate that many cell types contain virus particles during infection with high doses of influenza virus. This is not surprising, since sialic acid, which is the

TABLE 3. Frequencies of virus-infected cells in FACScan-sorted populations[a]

Tissue source	Cell population	% Purity	Frequency of virus-positive cells[b]
Lung	Macrophage	>50	1/329
	$B220^+$	>99	1/12,500
	$Thy1.2^+$	>99	1/14,663
	$N418^+$	>97	1/57,143
MLN	$B220^+$	>99	<1/100,000
	$Thy1.2^+$	>99	<1/100,000

[a] B6 mice were inoculated i.n. with 240 HAU of X-31 virus. After 2 days, the lungs and MLN were removed and various cell populations were obtained. Single-cell suspensions of lungs were left at room temperature for 2 to 5 min to allow alveolar macrophages to settle out of the solution. Other cell populations were sorted on the basis of cell surface marker expression.

[b] See Table 2, footnote *b*.

receptor for influenza virus, is present on all cells. However, it is generally believed that the only cells able to produce infectious virions are type 2 epithelial cells, presumably because they express the trypsin-like enzyme required for cleavage of the HA_0 molecule, which in turn permits the viral envelope to fuse with the lysosomal membrane during entry. Virus production in macrophages is nonproductive, i.e., the virions released are not infectious (36). Similarly, B and T lymphocytes, as well as dendritic cells, may be nonproductively infected or simply may have virus particles attached to their cell surfaces. Although $CD8^+$ T lymphocytes usually respond to antigens which are synthesized intracellularly, it is possible that surface-attached influenza virus virions may enter a nonclassical MHC class I processing pathway similar to those reported for Sendai virus (18), leading to the generation of CTL responses.

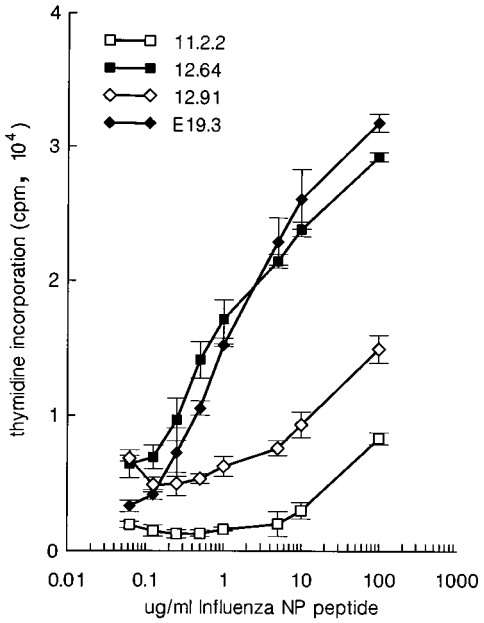

FIG. 4. Sensitivities of influenza virus-specific T-cell hybridomas to various concentrations of influenza virus NP peptide. LIE 13.14 cells (10,000), a macrophage cell line, were pulsed with various concentrations of influenza virus NP peptide for 1 h and then cultured with equal numbers of each virus-specific T-cell hybridoma. After 20 to 24 h, supernatants were harvested and analyzed by the standard CTLL assay. Data are the means ± standard deviations of [^{3}H]thymidine incorporation from triplicate cultures. The means of thymidine incorporation for triplicate cultures of these hybridomas in medium alone were as follows (in counts per minute): 12.64, 822; 11.2.2, 1,121; E19.3, 1,408; 12.91, 7,939.

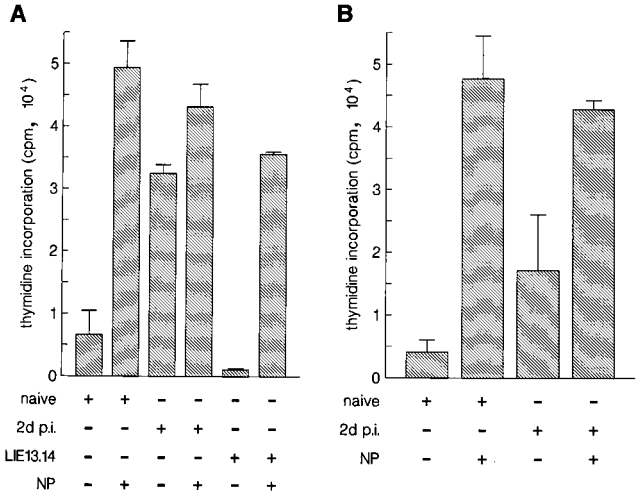

FIG. 5. Functional analyses of BAL cells (A) and lung alveolar macrophages (B). These potential APC populations were isolated from naive mice and virus-infected mice (2 days postinfection [2d p.i.]) and cultured at 10^4 cells per well with 2×10^4 12.64 influenza virus-specific T-cell hybridomas. After 20 to 24 h, supernatants were harvested and analyzed by the standard CTLL assay. Data are the means ± standard deviations of [^{3}H]thymidine incorporation from triplicate cultures. Each potential APC population was pulsed with influenza virus NP peptide and used as a positive control (NP). In addition, the macrophage cell line LIE 13.14 was used as a positive control APC.

Clearly, B lymphocytes can act as APC to primed T cells (23). However, the ability of B cells to stimulate or activate naive T cells is controversial (2, 10, 25, 28). In this study, B lymphocytes from the lungs of influenza virus-infected mice were capable of eliciting a virus-specific T-cell response, which correlates with the ability of B cells to present antigens to primed T cells. The inability of B cells from the MLN to stimulate the 12.64 hybridoma corresponds with the small number of cells which contain virus at this location. Perhaps if more cells were used in this assay, a response would be generated.

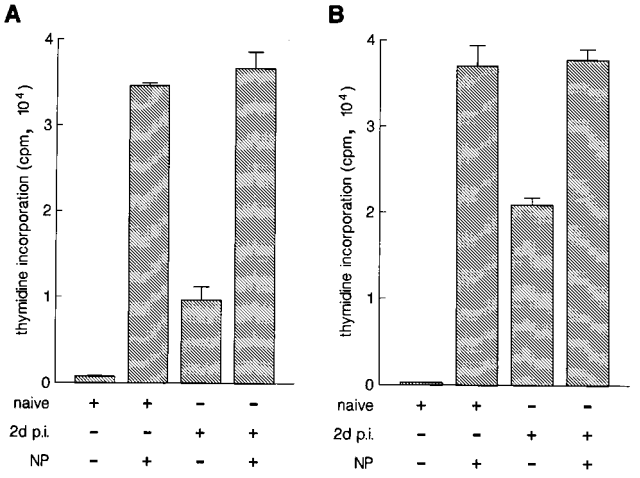

FIG. 6. Functional analyses of B cells (A) and dendritic cells (B) isolated from the lungs of virus-infected animals. Enriched B-cell and dendritic cell populations were obtained from naive mice and infected mice (2 days postinfection [2d p.i.]) and cultured with 2×10^4 12.64 T-cell hybridomas. B cells were used at a concentration of 10^5 cells per well, whereas dendritic cells were cultured at 10^4 cells per well. Data are the means ± standard deviations of [^{3}H]thymidine incorporation from triplicate cultures.

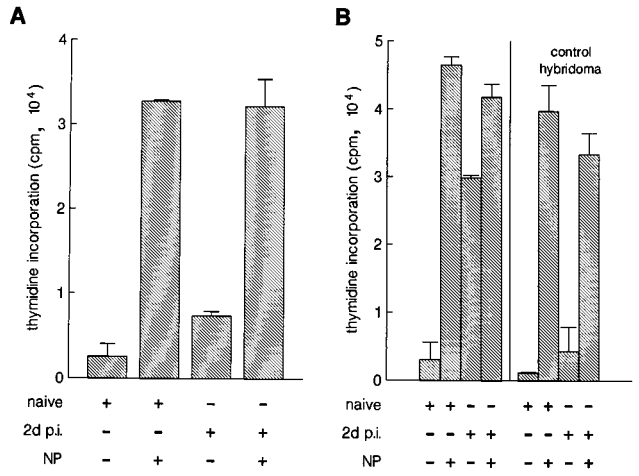

FIG. 7. Functional analyses of B cells (A) and dendritic cells (B) isolated from the MLN of virus-infected mice. Enriched B-cell and dendritic cell populations were obtained from naive mice and infected mice (2 days postinfection [2d p.i.]) and cultured with the 12.64 T-cell hybridoma as described in the legend to Fig. 6. Data are the means ± standard deviations of [^{3}H]thymidine incorporation from triplicate cultures. A Sendai virus-specific T-cell hybridoma was used as a negative control (control hybridoma). The NP peptide used to stimulate the control hybridoma was the Sendai virus NP peptide (residues 324 to 332).

Although this study was not designed to indicate the most potent APC population, the results suggest that macrophages and dendritic cells from the lungs and dendritic cells from the MLN were more effective APC than were B cells from either tissue. This is reflected in the fact that 10^4 macrophages or dendritic cells were sufficient to stimulate the virus-specific T-cell hybridoma, whereas 10^5 B cells were required to generate a response. In addition, the weak alloantigen response to B cells from the MLN and the lack of a response to B cells from the lungs indicate that neither population was contaminated with dendritic cells.

Our results suggest that it is feasible to generate antigen-specific responses to infections of the respiratory tract in the lungs themselves. All professional APC populations within the lungs contained virus and were able to stimulate virus-specific MHC class I-restricted T-cell responses, although macrophages and dendritic cells appeared to be the most effective APC. The abilities of both lung and MLN dendritic cells to stimulate virus-specific T-cell hybridomas are reflective of responses obtained in other systems (20, 33). It is possible that there is sufficient opportunity for an APC in bronchus-associated lymphoid tissue to stimulate a naive T cell. However, in vivo, the alveolar macrophage has been shown to down regulate the ability of dendritic cells to stimulate T lymphocytes (12). Hence, the MLN may be the only site at which the immune response is efficiently initiated. Our current experiments address this issue.

Future experiments to determine whether dendritic cells from the MLN or lungs or macrophages from the lungs are able to stimulate naive T cells will allow us to assess which population is more effective at initiating the immune response. It is thought that dendritic cells, because of increased expression of costimulatory molecules (16), will prove to be the most competent.

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