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Concurrent Production of Interleukin-2, Interleukin-10, and Gamma Interferon in the Regional Lymph Nodes of Mice with Influenza Pneumonia

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Cytokine production has been assessed at the single-cell level (ELISPOT assay) for freshly isolated mediastinal lymph node cells from C57BL/6 mice with primary, nonfatal influenza pneumonia. The mediastinal lymph node populations were also secondarily stimulated in vitro, and culture supernatants were assayed by enzyme-linked immunosorbent assay. Both approaches showed minimal evidence of protein secretion for interleukin-4 (IL-4), IL-5, and tumor necrosis factor, while IL-2, IL-10, and gamma interferon (IFN-γ) were prominent throughout the response. The numbers of IL-2- and IFN-γ-producing cells were maximal at 7 days after infection, while the total counts for cells secreting IL-10 were fairly constant from day 3 to 7. The cultures that were stimulated with virus in vitro showed an inverse relationship between IL-10 and IFN-γ production, with IL-10 peaking on day 3 and IFN-γ peaking on day 7. Lymphocytes secreting IL-2, IL-10, and/or IFN-γ were present in $CD4^+$ and $CD8^+$ populations separated by fluorescence-activated cell sorting, although the $CD8^+$ T cells produced less cytokine and were at a relatively lower frequency. Addition of recombinant IL-10 to the virus-stimulated cultures decreased the amount of IFN-γ that could be detected, while incorporation of a monoclonal antibody to IL-10 had the opposite effect. A neutralization experiment also indicated that IL-2 was the principal mediator of lymphocyte proliferation. These experiments thus show that the developing T-cell response in the regional lymph nodes of mice with influenza cannot be rigidly categorized on the basis of a TH1 or TH2 phenotype and suggest possible regulatory mechanisms.

Recent experiments with several RNA viruses show clearly that the process of clonal expansion and differentiation leading to the development of a primary $CD8^+$ T-cell response occurs in regional lymphoid tissue (2, 19, 27). The $CD8^+$ cytotoxic T-lymphocyte precursors (CTLp) then leave the lymph nodes and travel to the virus-infected target organ, where they differentiate further to become potent CTL effectors (2, 20). Other lymphocytes, probably progeny of the same clones, apparently go on to constitute the pool of memory CTLp that contributes to the maintenance of long-term protective immunity. Similar processes occur in the $CD4^+$ T-cell and B-lymphocyte populations (1, 9, 30), although less effort has been made to quantify these responses. The humoral response is clearly skewed towards production of immunoglobulin G2a (IgG2a) and IgA (3).

Little has been done to characterize directly the regulatory events that influence the cellular response in regional lymph nodes during the course of a virus infection. The spectrum of potential cytokine production has been assessed by in situ hybridization for both mediastinal lymph node (MLN) and bronchoalveolar lavage (BAL) populations from C57BL/6 mice with nonfatal influenza pneumonia (5). The prevalence of cytokine mRNA-positive cells in both sites was much higher than would be expected from earlier estimates of virus-specific T-cell precursor frequency. Similarly, the frequencies of protein producers found in the BAL by the single-cell ELISPOT assay (14, 39) were, for the majority of the cytokines analyzed, considerably lower than those predicted by the mRNA analysis. Others working with several different models of influenza virus infection have detected a range of cytokines in BAL fluids recovered from the virus-infected respiratory tract (17, 35, 43, 45).

We now extend the ELISPOT analysis to the MLN and further develop the approach by correlating the profiles found for freshly isolated cells with the patterns of secretion following in vitro restimulation. The experiments concentrate on interleukin-2 (IL-2) and gamma interferon (IFN-γ) and on IL-4 and IL-10 as characteristic TH1 and TH2 cytokines, respectively (13, 37). Substantial differences both from the in situ hybridization results and from the spectrum of cytokine production shown for the BAL population (5, 39) are found. The events occurring in the MLN may be much more tightly regulated than those occurring in the lung.

MATERIALS AND METHODS

Mice. The specific-pathogen-free C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and infected with virus at 8 to 10 weeks of age.

Infection and sampling. The A/HKx31 (H3N2) influenza A virus used in these experiments is a recombinant between A/PR8 (H1N1) and A/Aichi with the surface H3N2 glycoproteins of A/Aichi and the internal components of A/PR8 (22). Virus stocks were grown in the allantoic cavity of chicken embryos and shown to be free of bacteria, mycoplasmas, and endotoxins. Mice were infected intranasally with 30 μl of phosphate-buffered saline (PBS) containing 240 hemagglutination units of HKx31, using Avertin (2,2,2-tribromoethanol) anesthesia. The MLNs were excised from 2 to 10 anesthetized virus-infected mice at 3 to 10 days after infection, and single-cell suspensions were prepared (2). Pooled cells from groups of

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mice were used. Each experiment was repeated 3 to 12 times, as specified in the figure legends or in Results.

Restimulation in vitro. Total MLN populations or fluorescence-activated cell sorter (FACS)-separated T cells were resuspended at a final density of 2×10^6 cells per ml in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 1 mM glutamine, and 106 μg of gentamicin per ml. Irradiated (3,000 rads), virus-infected splenocytes were used as stimulators at a final density of 10^6 cells per ml. Supernatants were harvested at 24, 48, and 72 h and stored at −80°C prior to being assayed for cytokines by enzyme-linked immunosorbent assay (ELISA) (see below). When indicated, neutralizing anticytokine antibodies were added to the cultures at a final concentration of 40 μg/ml. The antibodies were anti-IL-2 rabbit polyclonal antibody (Collaborative Biomedical Products, Bedford, Mass.), anti-IL-4 monoclonal antibody (MAb) clone 1D11 (Pharmingen, San Diego, Calif.), anti-IL-10 MAb clone JES5-2A5 (Pharmingen), anti-IL-6 MAb clone MP5-32C11 (Pharmingen), and anti-IFN-γ clone R46A2 (purified from culture supernatant). The following recombinant cytokines were also added to some cultures at 10 or 100 U/ml: human IL-2 (Collaborative Biomedical Products), murine IL-4 (Pharmingen), murine IL-6 (Genzyme, Cambridge, Mass.), murine IL-10 (Pharmingen), and murine IFN-γ (Pharmingen).

Proliferation assay. Proliferation was assessed by [^{3}H]thymidine incorporation. Aliquots (200 μl) from restimulated cultures were distributed in triplicate into 96-well microtiter plates and pulsed for 20 h with 1 μCi of [^{3}H]thymidine (NEN Products, Boston, Mass.) per well. Plates were harvested with a Tomtech plate harvester (Tomtec, Orange, Conn.) onto filter mats (Pharmacia LKB, Piscataway, N.J.), and the radioactivity was counted in an LKB Betaplate liquid scintillation counter, using 10 ml of Betaplate Scint per filter. Results are expressed as stimulation indices, i.e., the mean fold increase in [^{3}H]thymidine incorporation (counts per minute) upon addition of recombinant cytokines or neutralizing MAbs.

Lymphocyte phenotyping and sorting. Pooled MLN populations were analyzed in the three-color mode with Lysis 2 software on a FACScan cytometer (Becton Dickinson, Mountain View, Calif.) equipped with a 488 nm argon laser and linked to a Hewlett-Packard computer. The MAbs used for staining were phycoerythrin conjugated (H57.597 anti-αβ TCR [24], 3A4 anti-NK cell, and GL3 anti-γδ TCR [26]) or fluorescein isothiocyanate conjugated (H129.19 anti-CD4 [36], 53-5-8 anti-Ly3 [25], and RA36B2 anti-B220 [8]). Fluorescein isothiocyanate- or phycoerythrin-conjugated isotype-matched antibodies were used as negative controls; <1% of the cells stained with these reagents. The conjugated H129.9 was supplied by Boehringer Mannheim (Indianapolis, Ind.), and the other MAbs were purchased from Pharmingen. Dead cells were excluded by propidium iodide gating, and erythrocytes and debris were excluded by appropriate forward- and side-scatter gates; 5,000 to 10,000 viable cells were acquired from each preparation. Markers used for the determination of percent positive cells were set on the basis of the fluorescence of unstained cells.

Lymphocyte populations were dual stained with fluorescein isothiocyanate-conjugated RM4-4 (anti-CD4) and phycoerythrin-conjugated 53-6-7 (anti-CD8) as described above, resuspended in PBS containing 1% FCS, and sorted with a FACSTAR-plus (Becton Dickinson) in the two-color mode. The presence of surface-bound antibody appeared to have little effect on function in previous studies (19).

ELISPOT assays. The ELISPOT assays followed established protocols (14, 39). Briefly, 96-well nitrocellulose-based microtiter plates (Millititer HA; Millipore Corporation, Bedford, Mass.) were coated overnight at room temperature with 100 μl of anticytokine MAbs, diluted in PBS, per well. After the plate was washed with PBS, all wells were blocked with RPMI 1640 containing 10% FCS for 1 to 2 h at 37°C. The MLN or sorted lymphocyte populations were then added to the wells (2×10^3 to 1×10^5 per well) in RPMI 1640–10% FCS (100 μl per well) and incubated for 20 h at 37°C in 5% CO_2. After the wells were washed in PBS followed by PBS-Tween, biotinylated anticytokine MAbs were added, diluted in PBS-Tween containing 1% FCS–1% normal mouse serum (100 μl per well), and then incubated overnight at 4°C. Plates were washed in PBS-Tween, and 100 μl of peroxidase-conjugated antibiotin antibody (1/250 dilution in PBS-Tween-FCS; obtained from Jackson Immunoresearch Labs, West Grove, Pa.) per well was added prior to a further overnight incubation at 4°C. Spots representing a single cytokine secreted by individual cells were developed by using the peroxidase substrate 3-amino-9-ethylcarbazole and counted by using an Olympus SZH Stereozoom microscope. All assays were done in triplicate with pooled cell preparations from two to eight animals. Mean numbers of spot-forming cells (SFC) were calculated from the triplicate assays. The results shown are means ± standard deviations for three to eight separate experiments. Similar results were obtained in each experiment.

The following pairs of MAbs were used in the ELISPOT assays: anti-IL-2, 1A17.5 and biotinylated JE56-5H4; anti-IL-4, BVD4-1D11 and biotinylated BVD6,24G2; anti-IL-10, JES-5 and biotinylated SXC.1 (32); anti-IFN-γ, R46A2 (16) and biotinylated XMG1.2 (7); and anti-tumor necrosis factor (anti-TNF), MP6-XT3 and biotinylated MP6-XT22.2. The MAbs were used at a concentration of 2 μg/ml, with the exception of JES-5 and R46A2, which were used at 10 μg/ml. The R46A2 (anti-IFN-γ) hybridoma was obtained from the American Type Culture Collection, and the MAb was purified from culture supernatant by protein G-Sepharose affinity chromatography. All other MAbs were obtained from Pharmingen. The detection limit was set at 0.05% SFC per well (20 spots at the highest cell number per well) to minimize inclusion of artifacts. Biotinylated rat IgG or IgM was used as a negative control detection antibody in the ELISPOT assays; <0.05% SFC were observed in MLN cell preparations with these control antibodies.

Cytokine ELISA. Cytokines in culture supernatants were assayed by sandwich ELISA, using a modification of the method of Mossman and Fong (33) and the method supplied by Pharmingen. Briefly, Dynatech Immulon 4 plates (Dynatech, Chantil, Va.) were coated by incubation overnight at 4°C with anticytokine MAbs diluted in PBS (50 μl per well). Sites that bind protein nonspecifically were blocked with PBS–10% FCS (200 μl per well) for 2 h at room temperature. The plates were then washed extensively with PBS–0.05% Tween 20 after this and each subsequent step, and samples or medium blanks were added (50 μl per well). A standard curve was constructed for each plate by using eight double dilutions of recombinant cytokine, and the plates were again incubated overnight at 4°C prior to addition of biotinylated anticytokine MAbs (50 μl per well). Negative controls using isotype-matched biotinylated control antibodies were also included. After a 45-min incubation at 4°C, 75 μl of streptavidin peroxidase (Jackson Immunoresearch, 2 μg/ml) per well was added, and the plates were incubated at room temperature for 30 min prior to detection with the peroxidase substrate ABTS (Sigma, St. Louis, Mo.). The A_{405} was determined with a Biorad (Richmond, Pa.) 3550 Microplate reader against a reference wavelength of 490 nm. The sensitivity of the assay was taken as 3 standard deviations

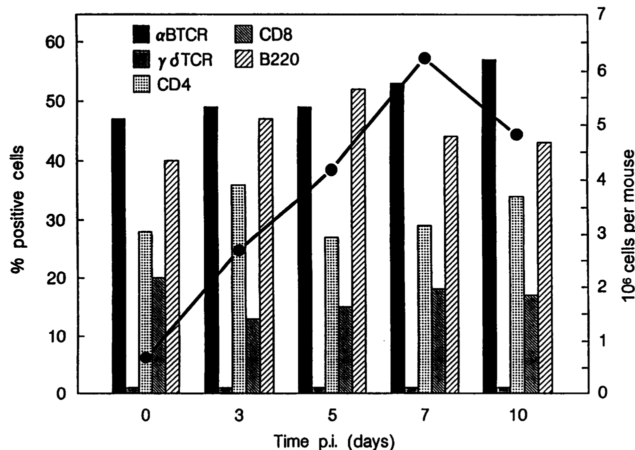

FIG. 1. Cell numbers, expressed as cell counts per mouse (●), and immunophenotypes for the MLNs of mice infected intranasally with the HKx31 influenza A virus 0 to 10 days previously. The day 0 time point represents uninfected mice. The immunophenotypes, expressed as percentages of total leucocytes, were determined by FACS analysis. Results are representative of three separate experiments, p.i., postinfection. The percentage of γδ T cells was not significantly above background (1%) throughout the course of infection. The same result was obtained for NK cells at days 0, 4, 7, and 10 p.i. (data not shown).

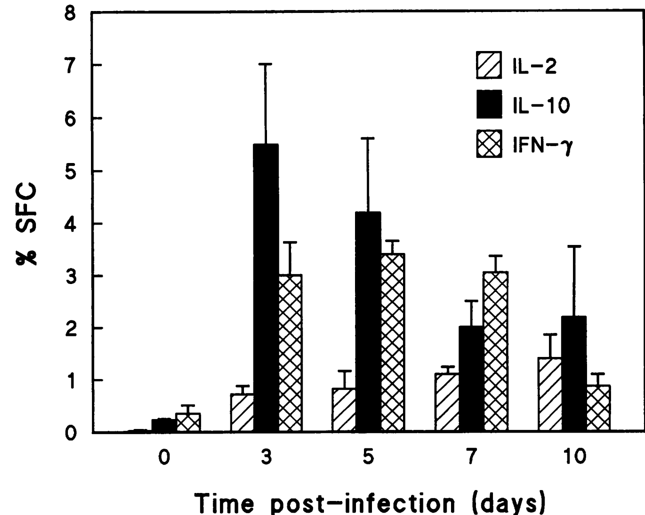

FIG. 2. Frequency of cytokine-producing cells, determined by the ELISPOT assay, for freshly isolated MLN populations taken on day 0 to 10 after intranasal infection with the HKx31 virus. Means ± standard errors of the mean for time points from three to eight separate experiments are shown. p.i., post-infection. No IL-4-, IL-5-, or TNF-producing cells were detected.

above the mean value from eight blank wells. This was below 2 U/ml for TNF and below 0.78 U/ml for the other cytokines. Negative controls also fell below these sensitivity limits. The pairs of anticytokine MAbs described above for the ELISPOT assays were used at the same concentrations. In addition, an ELISA for IL-6 was done with anti-IL-6 MAbs; MP5-20F3 was used for coating the plates, and biotinylated MP5-32C11 was used for detection. Anti-IL-6 MAbs and standard murine recombinant IFN-γ (rIFN-γ) and rIL-4, -5, and -10 were obtained from Pharmingen. Murine rIL-2 was obtained from Collaborative Biomedical Products. Standard rIL-6 and rTNF-α were purchased from Genzyme.

RESULTS

Cellular response in the MLN. The number of cells recovered (Fig. 1) from the MLN increased from 0.5×10^6 per mouse for normal mice to 5×10^6 to 6×10^6 cells per mouse by day 7 after infection with the HKx31 virus. There is no obvious selectivity in this massive increase in cellularity, with the relative proportions of $B220^+$ B lymphocytes and the various T-cell subsets remaining constant. The percentage of γδ T cells was not significantly above the background level (1%) during the course of infection (Fig. 1). Similarly, no significant population of NK cells was detected either in normal controls or at days 4, 7, and 10 postinfection (data not shown). Much of this presumably reflects recruitment rather than antigen-driven proliferation, although this is yet to be rigorously established. The drop in cell counts on day 10 follows the elimination (day 8) of infectious virus from the lung. Evidence of viral replication in the MLN is minimal throughout the course of this response (12).

Freshly isolated MLN populations were analyzed by the ELISPOT assay. Cells secreting IL-4, IL-5, and TNF either were not detected or were found at extremely low frequencies. In marked contrast, from 3 to 5% of the cells present from day 3 to 7 were producing IFN-γ and IL-10, while approximately 1% were $IL-2^+$ (Fig. 2). Comparison of percent SFC (Fig. 2) with the cell counts (Fig. 1) indicates that the total number of $IL-10^+$ cells remained relatively constant during the development of this virus-specific host response, while the values for the $IFN-\gamma^+$ and $IL-2^+$ sets increased steadily from day 3 to 7. Both the percent SFC and the total numbers in the regional MLN (Fig. 1 and 2) declined (days 7 to 10) after the stage at which infectious virus would have been cleared from the lung (2).

The results described above differed from those found for the spectrum of cytokine-producing cells in the BAL by the same technique (39). As would be expected from the mRNA profiles (5, 39), the frequency of cytokine-producing cells peaked later in the BAL. Cells secreting IL-2 and IFN-γ were present in both the BAL and MLN. However, in contrast to results for the MLN, the frequency of BAL cells producing IL-10 was very low, while significant numbers of $IL-4^+$ cells were detected and some TNF^+ inflammatory cells appeared late during the course of infection (39).

In vitro restimulation of MLN cells. The frequency of $IL-10^+$ cells detected by the ELISPOT assay (Fig. 2) was similar to that found previously by in situ hybridization analysis for mRNA (5). However, the relative prevalence of protein-secreting versus mRNA-positive cells was generally lower by a factor of 10 for IL-2 and IFN-γ, and although IL-4 mRNA was found in at least 5% of the MLN cells from day 3 to 7 (5), production of this cytokine was barely detected. Although the two sets of data were not generated concurrently, the discrepancies raise obvious questions.

We thus used an alternative approach to analyze the spectrum of cytokine secretion. The MLN cells were restimulated with virus in vitro, and the level of activity was measured by ELISA for culture supernatants harvested at 24, 48, and 72 h (Fig. 3). Production of IL-2 peaked at 24 h and that of all other cytokines peaked at 48 h, with the maximal values as shown in Fig. 3. Again, IL-10, IFN-γ, and IL-2 were prominent, but there was little evidence of IL-4 secretion. Some IL-6 was also found. The level of IFN-γ secreted increased with time postinfection up to day 7, whereas the level of IL-10 decreased and

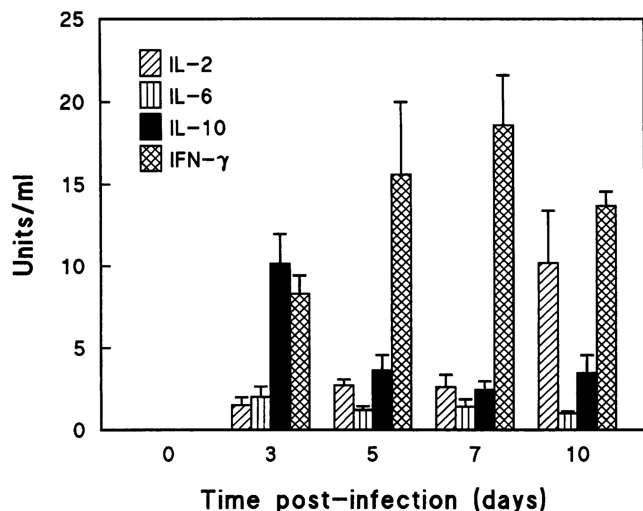

FIG. 3. Levels of cytokine produced for MLN cells (day 0 to 10) restimulated with HKx31-infected, irradiated splenocytes. The results shown are for the maximum level of cytokine that could be detected by ELISA, with the highest levels of IL-2 being present in culture supernatants at 24 h and those of all other cytokines being present at 48 h. No IL-4, IL-5, or TNF was detected. Means ± standard errors of the mean for three to twelve experiments at each time point are shown.

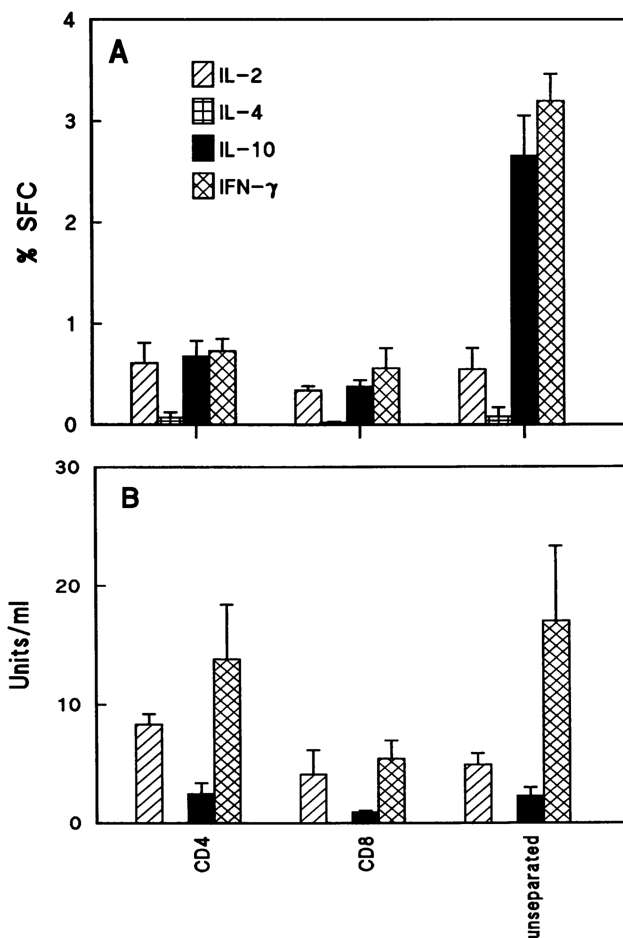

FIG. 4. Frequency of cytokine-producing cells analyzed directly by the ELISPOT assay (A) and levels of cytokines detected by ELISA of culture supernatants (B) following in vitro restimulation (Fig. 3) for FACS-separated CD4$^+$ and CD8$^+$ MLN cells taken on day 7. Other MLN cells were left unseparated. Results are means ± standard errors of the mean for three separate experiments.

IL-2 levels remained relatively constant. To test whether the patterns of cytokine production were clearly distinct at days 3 and 7, the IL-10/IFN-γ, IL-2/IL-10, and IL-2/IFN-γ ratios were compared at days 3 and 7 postinfection by Wilcoxon's exact test. Significant differences were found for the IL-10/IFN-γ (P = 0.001) and IL-10/IL-2 (P = 0.01) ratios between days 3 and 7, while the IL-2/IFN-γ ratio did not show evidence of a statistically significant change. Although the same cytokines were detected by both ELISPOT and ELISA (Fig. 2 and 3), the distribution patterns were not identical. This is hardly surprising, as the ELISPOT assay does not distinguish between individual cells producing high and low levels of cytokine. Also, cytokine consumption is likely to be a factor in vitro, while any nonspecific component of the in vivo response would be minimized in the virus-stimulated cultures. Unstimulated MLN cells or those restimulated with influenza B virus-infected splenocytes failed to produce detectable levels of cytokine (data not shown).

Production by CD4$^+$ and CD8$^+$ T cells. Freshly isolated MLN cells taken on day 7 were separated into CD4$^+$ and CD8$^+$ subsets by the FACS (or left unseparated) and analyzed directly by the ELISPOT assay or stimulated in vitro with virus (Fig. 4). The freshly isolated CD4$^+$ and CD8$^+$ populations both contained lymphocytes producing IL-2, IL-10, or IFN-γ, with the frequencies being generally higher for the CD4$^+$ set (Fig. 4A). A similar pattern of cytokine production was found following in vitro restimulation with virus (Fig. 4B).

Sorting the CD4$^+$ and CD8$^+$ T cells did not (as might be expected) enrich for cytokine-producing cells, indicating that some lymphocytes are lost or lose activity during the prolonged FACS separation. The relative decrease in cytokine-secreting cells seemed greater for IL-10 and IFN-γ than for IL-2. This could also reflect that many of the IL-10- and IFN-γ-producing cells express neither CD4 nor CD8 or that these cells are more dependent on other cell types for costimulatory signals. Cells of the monocyte/macrophage lineage and some B cells make IL-10, while NK cells can secrete IFN-γ (21, 29, 38). However, sorted CD4$^+$ and CD8$^+$ populations produced levels of cytokine comparable to those produced by unsorted MLN cells on restimulation in vitro, so macrophage-derived cytokine was probably not a factor in these cultures. Also, no significant population of NK cells was detected during the course of infection. Furthermore, following depletion in vivo of both T-cell subsets as previously described (39), the only cytokine detected on restimulation of MLN cells in vitro was IL-10 (5.2 and 2.8 U/ml at day 7 postinfection in two separate experiments). Even so, the possible contribution of low numbers of NK cells to IFN-γ production cannot be completely ruled out. Enrichment with the FACS also showed that a few of the T cells were secreting IL-4, although these lymphocytes were detected only as SFC and significant levels of secreted IL-4 were not found in the restimulated cultures (Fig. 4).

Cytokines and lymphocyte proliferation. Freshly isolated MLN populations taken at day 4 or 7 (or from uninfected mice) were stimulated in vitro with virus-infected splenocytes in the presence or absence of various recombinant cytokines (Fig. 5). MLN cells from uninfected mice failed to proliferate after restimulation (125 ± 57 cpm [n = 5]). MLN cells taken from virus-infected mice at day 4 or 7 incorporated 10- to

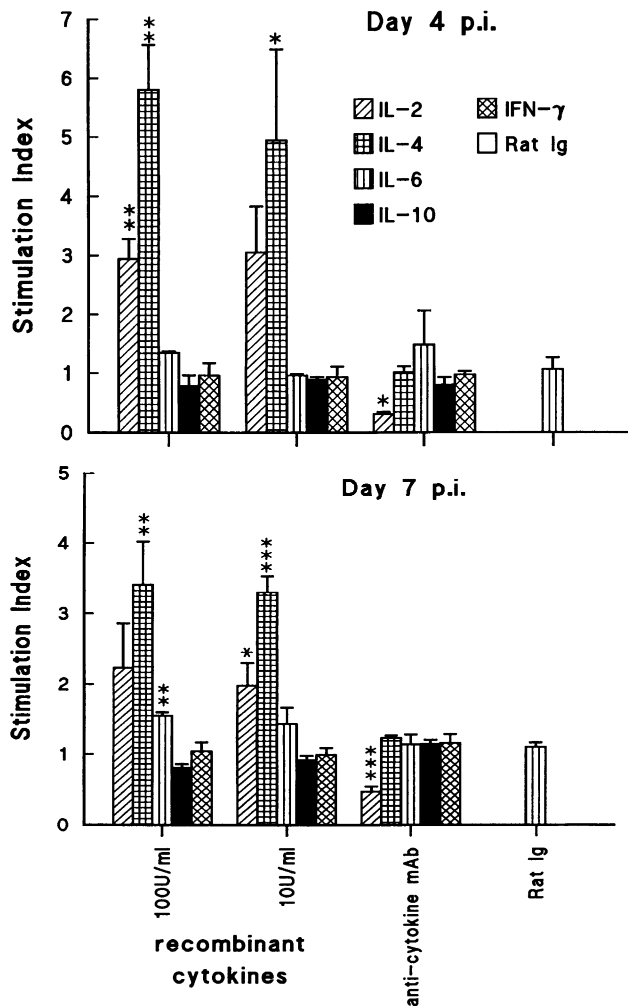

FIG. 5. Effect of adding recombinant cytokines or anticytokine antibodies on proliferation in response to HKx31-infected splenocytes for day 4 and 7 MLN populations. Normal rat Ig was used as a negative control. Mean (± standard error of the mean) stimulation indices comparing test samples with negative controls from three or four separate experiments are shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (Student's t test).

20-fold more [^{3}H]thymidine, with the higher levels generally being found at day 7. Stimulation indices were calculated for cells from infected mice in the presence or absence of recombinant cytokine or MAbs. Addition of rIL-2 or rIL-4 augmented the proliferation of the lymphocytes from the primed mice (Fig. 5) ($P < 0.05$), while minimal replication of the naive MLN cells was seen following exposure only to rIL-4 (314 ± 187 cpm [$n = 3$]). Incorporation of rIL-10 and rIFN-γ had no effect, while rIL-6 caused some increase for the cells at day 7 ($P < 0.01$).

Lymphocytes expressing IL-4 mRNA are prevalent in the MLNs of influenza virus-infected mice, raising the possibility that IL-4 secreted by CD4$^+$ T cells at levels that are generally below the sensitivity of the ELISA and ELISPOT assay is contributing to the proliferative response (Fig. 5). This was tested by using neutralizing MAbs with cultures that did not contain added recombinant cytokines. The only inhibition below background (Fig. 5) was seen with the MAb to IL-2 ($P < 0.001$; day 7 postinfection). The MAb to IL-4 had no effect, although it did block proliferation of the IL-2/4-dependent CTLL cell line in response to 100 U of rIL-4 per ml (data not shown). Apparently the virus-stimulated MLN cells do not secrete significant levels of IL-4, confirming findings derived from alternative approaches that are shown in Fig. 2 to 4.

Cross-regulation with different cytokines. Addition of human rIL-2 (which does not cross-react in the ELISA with murine IL-2) appeared to up-regulate the synthesis of murine IL-2 in MLN cells taken on day 7 ($P < 0.05$) and caused a slight increase in IFN-γ production, but this was not statistically significant (Table 1). Addition of other cytokines to the cultures had no obvious consequences for IL-2, IL-6, or IL-10 production. Neutralizing MAbs removed all evidence of reactivity for the homologous cytokine but did not significantly up-regulate the synthesis of IL-2, -4, -6, or -10. The one definite effect was that IL-10 reduced IFN-γ production when added at either 10 or 100 U/ml ($P < 0.05$), while the anti-IL-10 MAb increased IFN-γ production ($P < 0.05$). A similar situation was seen for MLN cells taken on day 4 (data not shown).

DISCUSSION

Cell populations recovered from the MLNs of influenza virus-infected C57BL/6 mice show a cytokine profile which is neither TH1 nor TH2 in character (34, 42), as IFN-γ and IL-2 (TH1) and IL-10 (TH2) are being produced concurrently. Overall, the response in the present study might be considered to be "TH0," although there is a general underrepresentation of TH2 cytokines (IL-4 and IL-5) other than IL-10. Furthermore, although both CD4$^+$ and CD8$^+$ T-cell populations were shown to produce IL-2, IFN-γ, and IL-10, this does not mean that the same cells are secreting all three cytokines or that they are located in proximity in the lymph node. We are currently trying to develop immunocytochemical approaches so that we can address this point. Possible compartmentalization effects are not likely to be a factor in tissue culture flasks: although IL-10 and IFN-γ were produced concurrently in the MLN, in vitro studies confirmed that IL-10 down-regulates IFN-γ production (Table 1).

Production of IL-10 has been shown for CD4$^+$ T cells, B cells, macrophages, and polyclonally stimulated human CD8$^+$ T cells, and we can now add murine CD8$^+$ T cells responding to an infectious agent to the list (21, 47). Although virus-stimulated production of IL-2 and IFN-γ was completely T-cell dependent in the MLN, in vivo depletion of CD4$^+$ and CD8$^+$ T cells did not completely stop IL-10 production. The fact that IL-10 inhibits IFN-γ secretion and the effector and antigen-presenting functions of macrophages and dendritic cells has led to the generalization that this is a down-regulatory cytokine (13, 21, 32). The early prominence of IL-10-secreting cells in the MLN is at odds with this idea: IL-10 is also known to function as an activation and differentiation factor for B and T lymphocytes (6, 15).

The prominence of IFN-γ in the MLN is reflected in the predominance of IgG2a in the humoral response (3, 41). The antiviral effects of IFN-γ are well known, and the up-regulation of major histocompatibility complex glycoprotein expression (31, 46) and adhesion molecules (11) associated with this cytokine is likely to be an important factor in the development of virus-specific T-cell responses. Also, IFN-γ may have a role in the development of CTL effector function, although not all data support this idea (10). While the CTLp proliferate and differentiate in the MLN, the fact that CTL activity is minimal in the lymph node (2) probably reflects the low level of antigen

TABLE 1. Consequences of adding recombinant cytokines or neutralizing antibodies to cytokines produced by virus-stimulated MLN cells

Cytokine or antibody added[a]	Cytokine production (U/ml)[b]				
	IL-2	IL-4	IL-6	IL-10	IFN-γ
Cytokines (100 U/ml)					
IL-2	6.4 ± 3.5*	0	1.4 ± 1.1	2.0 ± 1.3	21.9 ± 5.4
IL-4	0	44.1 ± 24.3	0.8 ± 0.6	1.0 ± 0.6	15.0 ± 8.1
IL-6	0	0	56.8 ± 27.1	1.5 ± 0.1	24.6 ± 7.2
IL-10	0	0	0.5 ± 0.5	80.4 ± 27.9	9.4 ± 4.9*
IFN-γ	0.8 ± 0.5	0	1.1 ± 0.8	0.8 ± 0.5	45.5 ± 35.3
Cytokines (10 U/ml)					
IL-2	1.8 ± 1.5	0	0.8 ± 0.7	1.3 ± 1.0	25.5 ± 5.6
IL-4	0	2.4 ± 0.2	1.1 ± 0.9	1.1 ± 0.8	18.8 ± 5.9
IL-6	0	0	4.7 ± 1.0	0.8 ± 0.6	20.0 ± 4.3
IL-10	0	0	1.4 ± 0.5	11.4 ± 6.7	12.4 ± 1.4*
IFN-γ	0	0	1.1 ± 0.9	1.5 ± 1.7	21.9 ± 5.2
Anticytokine antibodies					
IL-2	0	0	3.3 ± 3.2	1.8 ± 1.4	17.6 ± 4.0
IL-4	0	0	3.0 ± 2.8	1.5 ± 0.6	19.5 ± 4.2
IL-6	1.0 ± 1.3	0	0	1.6 ± 0.4	22.0 ± 6.3
IL-10	1.7 ± 1.7	0	3.3 ± 3.6	0	30.4 ± 6.1*
IFN-γ	1.1 ± 1.3	0	1.2 ± 0.8	0.8 ± 0.5	0
Controls					
Rat Ig	0.8 ± 0.7	0	1.0 ± 0.7	1.2 ± 0.1	18.1 ± 4.8
No addition	0.8 ± 0.6	0	0.9 ± 0.6	0.9 ± 0.6	18.2 ± 4.0

[a] The recombinant cytokines and neutralizing antibodies are described under "Restimulation in vitro" in Materials and Methods. The antibodies were added at a final concentration of 40 μg/ml, and the MLN cells (day 7) were stimulated with virus-infected, irradiated splenocytes. Normal rat serum was added as a negative control.

[b] Cytokine levels were determined by ELISA. Results are means ± standard deviations for three or four separate experiments. The detection limit was 0.78 U/ml for all five cytokines. *, $P < 0.05$ versus control (Student's t test).

expression rather than any lack of inductive cytokine. Alternatively, IL-4 may be important in this regard (see below).

Both the prevalence of IL-2-secreting cells in the MLN and the in vitro study showing that virus-specific T-cell proliferation is substantially IL-2 dependent are, again, very much in accord with general ideas about the biological roles of this lymphokine (40). It also seems likely that the proliferation and differentiation of B-cell precursors to become IgG producers are, at least in part, driven by IL-2-producing $CD4^+$ T cells. Also, IL-6 (detected in the in vitro cultures) may promote the IgA response that is important for mucosal immunity to this virus (18, 28).

It is surprising that IL-4 production in the MLN is minimal, as in a previous study (5), IL-4 mRNA was found by in situ hybridization to be prevalent in MLN $CD4^+$ T cells. Mice of the same age, sex, and strain infected identically with the HKx31 virus were used in the two sets of experiments (5). It is possible that the culture conditions for the ELISPOT assay and for the in vitro restimulation experiments could have modified the response profile. However, this is not the case for the BAL, as both $IL\text{-}4^+$ SFC and mRNA-positive cells are recovered from the pneumonic lung at about the same frequency as the $IL\text{-}2^+$ set (39). The cytokine profile of this population closely resembled that of a transient population of L-selectin-negative $CD45RB^-$ helper/effector cells which secrete IL-2, IL-4, and IFN-γ, as described by Bradley et al. (4). T cells from the BAL of influenza virus-infected mice are also predominantly (>95%) L-selectin negative (28a). Although IL-4 protein secretion was detected in the latter study, the level of IL-4 produced was not determined. The apparent lack of IL-4 secretion by the MLN T cells could not be modified by adding an excess of (or neutralizing) a range of cytokines in vitro, and there was no evidence that consumed IL-4 was contributing to lymphocyte proliferation. It is possible that the production of IL-4 by mRNA-positive cells is being inhibited in some way in the MLN but not in the BAL.

Perhaps IL-4 production by MLN T cells is blocked by another cytokine, such as transforming growth factor-β, IL-7, or IL-12 (23, 37, 44), which has not been analyzed in the current study. Another possibility is that the level of signal via the clonotypic T-cell receptor may need to be higher for IL-4 production than for IL-2 production: a major difference between the MLN and BAL of mice with influenza is that there are large numbers of productively infected (and thus antigen-presenting) epithelial cells in the lung, while there is little evidence of virus growth in the lymph node (12). This is reflected in the potent effector CTL activity in the BAL, derived from CTLp that exit the MLN before they develop the capacity to be cytotoxic (2). An additional factor is that the various cell populations are likely to be more intimately associated (facilitating short-range cytokine effects) in the grossly swollen lymph node than in the inflamed bronchoalveolar space. Clearly, mRNA and protein production profiles are not always equivalent for activated T cells. Furthermore, the spectrum of cytokine secretion can depend on the particular in vivo microenvironment.

Analysis of freshly isolated regional lymph node populations at the single-cell level thus provides insights that reinforce current ideas about cytokine function derived both from other in vivo experimental systems and from cell culture experiments. However, there are also some surprising findings. The lack of IL-4 production would not have been predicted from the mRNA profiles and reinforces the impression gained from the analysis of the inflammatory BAL populations (39) that while mRNA production is useful in defining the potential for cytokine production, it is essential to look beyond this parameter before making any conclusions about either the levels of cytokine secreted or the biological significance of the particular cytokine. The early prominence of IL-10 production suggests that more attention might be given to the immunity-enhancing properties of this cytokine. Also, the concurrent presence of substantial numbers of $IL\text{-}10^+$ and $IFN\text{-}\gamma^+$ cells underscores

the concept that cytokine responses do not always polarize to TH1 or TH2 types.

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