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Induction of Cytokines in Mice with Parainfluenza Pneumonia

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The possible involvement of cytokines in the acute viral pneumonia induced by the murine parainfluenza type 1 virus, Sendai virus, was studied. Cytokine profiles for both the respiratory tract and the draining mediastinal lymph node (MLN) of virus-infected C57BL/6J mice were quantified by using the single-cell cytokine (ELISPOT) assay with freshly isolated cell populations and enzyme-linked immunosorbent assay for lung lavage fluids and culture supernatants. Maximal levels of interleukin 2 (IL-2), gamma interferon (IFN-γ), tumor necrosis factor, IL-6, and IL-10 were detected at the inflammatory site 7 to 10 days after infection, about the time that virus is cleared from the lung. The frequencies of cells producing IL-2, IL-4, IL-6, IL-10, IFN-γ, and tumor necrosis factor were much higher for the bronchoalveolar lavage (BAL) cell population than for the MLN cell population. Cytokine production after in vitro restimulation of MLN cells was dominated by IL-2 and IFN-γ, with low levels of IL-10 and IL-6 also being present. Most of the cytokine was produced by the $CD4^+$ cells, although the $CD8^+$ subset was also involved. No IL-4 was found in the BAL fluid or in culture supernatants from restimulated BAL or MLN cells, although a high frequency of IL-4-producing cells was demonstrated in the BAL population by ELISPOT analysis.

Human parainfluenza virus type 1, a paramyxovirus, is a major cause of acute lower respiratory disease in infants and children (1, 13). The development of effective preventive or therapeutic stratagems has been hampered by a lack of information concerning pathogenesis and the immune response. The intriguing feature of these infections is that people are reinfected with the same viruses, year after year.

Antiviral immunity can be both beneficial and detrimental to the host. Immunopathology mediated by $CD8^+$ cytotoxic T lymphocytes has been shown for various RNA virus infections (8, 12, 14). The spectrum of cytokines produced by $CD4^+$ T_H cells is known to influence the pathology of the disease process caused by another paramyxovirus, respiratory syncytial virus (3, 4, 11). Infection with vaccinia virus expressing the respiratory syncytial virus G protein causes BALB/c mice to produce T_H2 cytokines, such as interleukin 4 (IL-4) and IL-5 (which promotes eosinophilia), while a vaccinia construct incorporating the respiratory syncytial virus F and NP genes induced T_H1 cytokines (gamma interferon [IFN-γ] and IL-2) (5).

The murine parainfluenza type 1 virus (Sendai virus) induces acute bronchiolitis and interstitial pneumonia in mice. Mice infected intranasally with a nonfatal dose of Sendai virus show maximal inflammation 7 to 9 days later and clear the virus completely by day 10 to 14 postinfection (16, 25, 27). Virus-immune $CD4^+$ or $CD8^+$ T cells are indispensable for complete clearance (16, 18, 19, 22). The T-cell response is initiated in regional lymphoid tissue (15, 24), with the $CD8^+$ cytotoxic T lymphocyte precursors then migrating to the lung, where they further differentiate into potent effector cytotoxic T lymphocytes (2, 15). Little has been done to characterize the cytokines induced by parainfluenza infection or to assess the relevance of particular cytokines to the regulation of inflammation and host immunity.

A broad spectrum of cytokine $mRNA^+$ (6, 7) and cytokine-producing cells (21) has been detected in bronchoalveolar lavage (BAL) cells from mice with acute influenza pneumonia. Early studies with Sendai virus showed that the virus is an effective inducer of type 1 IFN both in vivo and in vitro (9, 30) and that tumor necrosis factor alpha (TNF-α) may be present in the infected lung (17). The present analysis defines production profiles for cytokines that are considered to be characteristic of T_H1 or T_H2 responses, comparing the situation for the regional lymph nodes and the pneumonic lung.

C57BL/6J (B6) female mice were purchased from Jackson Laboratory (Bar Harbor, Maine) and held under specific-pathogen-free conditions prior to intranasal infection with the Enders strain of Sendai virus (26) at 8 to 10 weeks of age. The BAL cell populations were obtained from anesthetized, infected mice (2), and the supernatant fluid (BAL fluid) from all areas of the lungs was taken after centrifugation of the first lavage sample (3,000 × *g* for 10 min). The BAL fluid was then concentrated 5- to 10-fold by centrifugation by using Centriprep-10 concentrators (Amicon, Beverly, Mass.). For in vitro restimulation experiments, BAL cells were incubated in petri dishes in RPMI 1640–10% fetal calf serum for 90 min to remove adherent cells. The mediastinal lymph nodes (MLN) were excised and disrupted with tissue grinders to give single-cell suspensions. Total cell populations from BAL fluid and MLN were used for subsequent ELISPOT analysis (10, 27, 28), for restimulations to produce cytokines (29), or for immunophenotyping (15).

Mice infected with a 200 50% egg infective dose of Sendai virus developed focal areas of inflammation in the lungs (data not shown), with evidence of cellular infiltration being apparent by day 3 (Fig. 1A). The inflammatory process was dominated by $CD8^+$ T cells, with substantial numbers of $CD4^+$ T lymphocytes, a few B cells (Fig. 1A), and <2% $\gamma\delta TCR^+$ cells or NK cells. The virus was substantially cleared (16) from the lung by day 10, with no evidence of infection remaining on day 14 (data not shown). The predominant lymphocytes in the greatly enlarged MLN were $B220^+$ B cells, $CD4^+$ T cells, and $CD8^+$ T cells. In contrast to the situation for influenza infection (29), phenotyping showed that the ratio of T lymphocytes to B lymphocytes (T/B) decreased dramatically ($P < 0.025$, Student's *t* test), from 1.84 ± 0.52 on day 3 to 0.71 ± 0.19 on day 7, and eventually increased to 1.80 ± 0.50 by day 14 ($P <$

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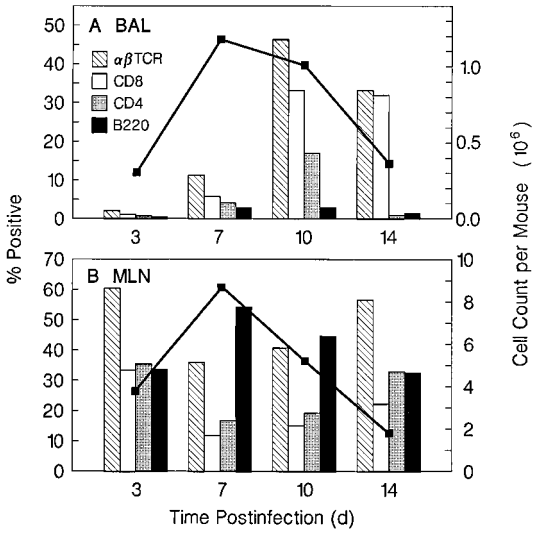

FIG. 1. Characteristics of the BAL (A) and MLN (B) cell populations are shown at different time points after intranasal infection of B6 mice with a sublethal dose (200 50% egg infective dose) of Sendai virus. BAL was performed as described previously (2). Briefly, a cannula with a syringe was inserted through an incision immediately posterior to the larynx. The respiratory tract was then washed out by infusing and withdrawing 1.0 ml of phosphate-buffered saline–0.8% bovine serum albumin three times. Since all lobes of the lung were inflated while washing, BAL fluid and cells were obtained from all areas of the lung. Also, the collected MLN cells were processed to give a single cell suspension before phenotyping. Lymphocytes were phenotyped by FACS, as described previously (6, 15). All the fluorescein isothiocyanate- or phycoerythrin-conjugated monoclonal antibodies were purchased from Pharmingen (San Diego, Calif.). The detection limits were less than 0.5%, on the basis of staining with fluorescein isothiocyanate- or phycoerythrin-conjugated isotype-matched negative controls. The cell counts per mouse are presented by solid lines, and the phenotypes are presented by bar histograms. The data are means of three to four separate experiments. Lavage of normal, specific-pathogen-free B6 mouse lungs leads to recovery of less than 1.1×10^5 cells per mouse.

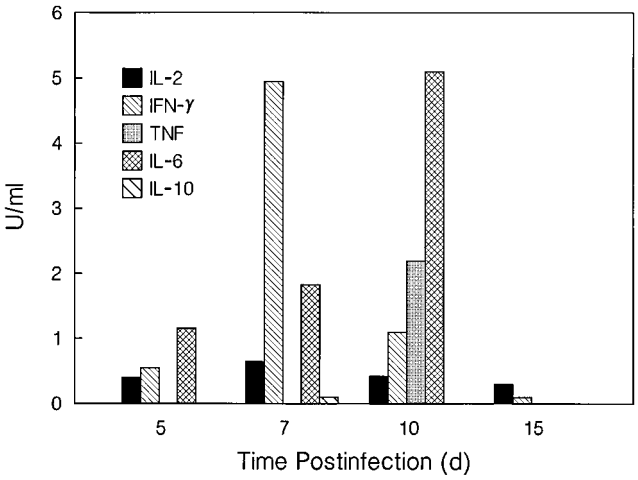

FIG. 2. Levels of cytokine are shown for fluid lavaged (2) from the lungs of mice with Sendai virus pneumonia. Cell-free BAL fluid was collected from groups of three to six B6 mice at intervals, following intranasal infection. BAL fluid from three to eight separate experiments at each time point was pooled, filtered through a 0.45-μm pore-size filter, and then concentrated 5- to 10-fold (see text). Cytokine activity was determined by sandwich ELISA (29). All the anticytokine monoclonal antibodies (MAbs) and biotinylated anticytokine MAbs were purchased from Pharmingen (San Diego), except clone R46A2 hybridoma (anti-IFN-γ) MAb was obtained from the American Type Culture Collection (28). The data are expressed as mean levels in 1 ml of pooled BAL fluid (without concentration) for two separate pools of BAL fluid, obtained as described above, which gave comparable results. The baseline for each assay was threefold of the standard deviation of eight negative-control samples, which was <0.78 U/ml (the lowest concentration of the standard curve) for every cytokine.

0.05, Student's *t* test) (Fig. 1B). This may reflect that Sendai virus is mitogenic for B lymphocytes (23). Low numbers of $\gamma\delta TCR^+$ T cells and NK cells (1 to 2%; data not shown) were present. The cellularity of the MLN declined rapidly following virus clearance.

Pooled, concentrated, cell-free BAL fluid contained significant levels of IL-2, IFN-γ, TNF, IL-6, and IL-10 (but not IL-4): the data in Fig. 2 have been calculated to reflect units per milliliter of BAL fluid prior to concentration. Minimal levels of IL-2, IL-6, and IFN-γ were detected on day 3 (<1 U/ml; data not shown). Kinetic studies indicated that IL-2 remained at a constantly low level of less than 1 U/ml throughout. Small amounts of IFN-γ were found from day 5 to day 15 (with the peak on day 7). IL-6 was first apparent on day 5, reached peak titers on day 10, and disappeared by day 15. Some TNF was found on day 10 (the assay measures both TNF-α and TNF-β), while IL-10 was also present on day 7. Thus, the highest levels of secreted cytokines were found between day 7 and day 10, declining with virus clearance (data not shown) and the resolution of the inflammatory process (Fig. 1A). The concentrations of IFN-γ, TNF, IL-6, and IL-2 in BAL fluid were such that these would be expected to have biological effects. Furthermore, it is important to recognize that the levels of secreted cytokines we are measuring represent the excess after consumption: the amounts produced are likely to be much greater.

A better index of cytokine production may be the numbers of cytokine-producing cells in the BAL population and MLN, as measured by the ELISPOT assay (28, 29). The situation for the BAL cells is described first, since the relative prevalence of spot-forming cells (SFC) was much higher for the inflammatory population (Fig. 3). IL-2-producing cells appeared on day 3 and were at peak frequency on day 7 (Fig. 3), although maximal numbers of T cells were present at later time points

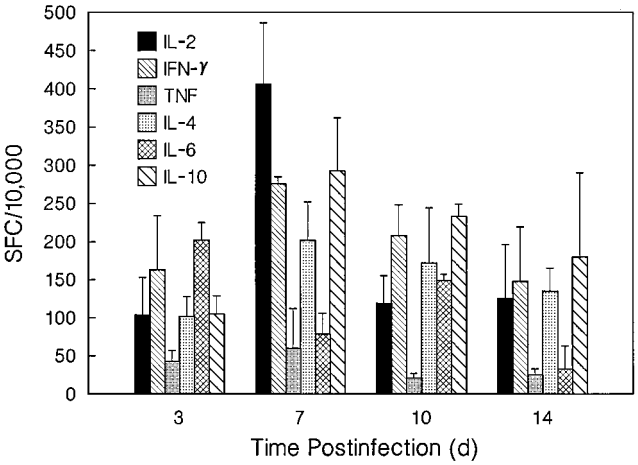

FIG. 3. Frequencies of cytokine-producing cells are shown for pooled BAL cells from groups of six virus-infected mice. The cells were plated out at 4×10^3 to 5×10^5 per well for the ELISPOT assay (10, 27, 28). The monoclonal antibodies used were the same as those used in the cytokine ELISA described in the legend to Fig. 2. The detection limit was set at 20 spots per well for the highest concentration, to avoid the inclusion of artifacts. Isotype-matched negative controls for the biotinylated antibodies in this assay gave numbers of SFC below the detection limit. The frequencies of cytokine-producing cells are expressed as mean (+ standard errors of the mean) SFC counts per 10,000 cells from three to four separate experiments, except for day 14 when only two experiments were performed.

(Fig. 1A). This early prominence may reflect the well-established role of IL-2 in promoting T-cell proliferation. The kinetics of detection for IFN-γ SFCs (Fig. 3) were in accord with the findings for IFN-γ levels in BAL fluid (Fig. 2), while TNF SFCs were at a very low frequency throughout. Cells producing IL-4 and IL-10 were present as early as day 3, with the highest frequencies being apparent on day 7. The prevalence of IL-6 SFCs (when corrected for cell numbers) increased steadily between days 3 and 7 and then fell (Fig. 3; Fig. 1A). The SFC counts were, for most cytokines, unexpectedly high on day 3, considering the low percentage of T cells present at this time point (Fig. 1A). Therefore, either a high proportion of the T cells infiltrating the lung early in the immune response are cytokine producers, or other cell types are involved. This early population may help to initiate the inflammatory process, though massive cellular extravasation into the BAL fluid tends to correlate with the time (day 7 to 8) that virus-specific $CD8^+$ T cells are first found in the BAL population (15).

The SFC profiles do not necessarily reflect the results of the enzyme-linked immunosorbent assay (ELISA) analysis for soluble cytokines in BAL fluid (Fig. 2), in which IFN-γ and IL-6 were dominant. In particular, numerous IL-4-producing cells were detected in the BAL population (Fig. 3), whereas no secreted IL-4 was found in the BAL fluid (Fig. 2). This divergence is not unexpected, as the various detection methods reflect different aspects of cytokine production. Clearly, the ELISPOT assay does not measure levels of cytokine secretion by individual cells. Previous studies have shown that the cells producing levels of cytokine below the detection limit of ELISA are positive with the ELISPOT technique (20). Furthermore, the cytokine levels in the BAL fluid represent the excess after consumption or degradation, whereas in the ELISPOT assay, cytokine is likely to be captured by plate-bound antibody prior to utilization or loss.

The fact that the frequencies of cytokine-producing cells were much lower in the MLN than in the BAL population may simply reflect that the antigen-responsive cells are diluted in a much larger pool of lymphocytes. Very few IL-2-, IL-4-, IL-6-, and IFN-γ-producing cells were detected (2 to 20 SFC per 10,000 cells), whereas IL-10-secreting cells were more prevalent. Frequencies of 100 to 140 IL-10 SFC per 10,000 cells were found on day 3, decreasing steadily to 50 to 70 SFC per 10,000 cells at day 10: these data (not shown) were from two to three independent experiments at each time point, using pooled MLN cell populations from groups of five mice per experiment. The overall, lower frequency of cytokine-producing cells in the MLN may reflect that, although the T-cell response develops in this site, there is little (if any) evidence of productive infection.

The MLN cells do, however, have the capacity to produce significant amounts of cytokine following in vitro restimulation. In general, high levels of IFN-γ and IL-2 were detected in culture supernatants, together with smaller amounts of IL-10 and IL-6 (Fig. 4). Very little cytokine production was induced in the MLN from day 3 postinfection, perhaps indicating the low frequencies of virus-specific cells at this time or a requirement for further in vivo priming. High levels of IL-2 and IFN-γ were found for restimulated MLN samples on days 7 and 10 (Fig. 4). Smaller amounts of IL-10 and IL-6 were detected, but only on day 7 (Fig. 4) or day 10 (occasionally). The in vitro response was virus specific, as no cytokine production was shown following restimulation of MLN cells from naive mice (day 0) with Sendai virus-infected stimulators or when MLN cells from Sendai virus-infected mice were restimulated with influenza virus (strain AHK/X31)-infected splenocytes (data not shown). We also found no IL-4 in the culture supernatants,

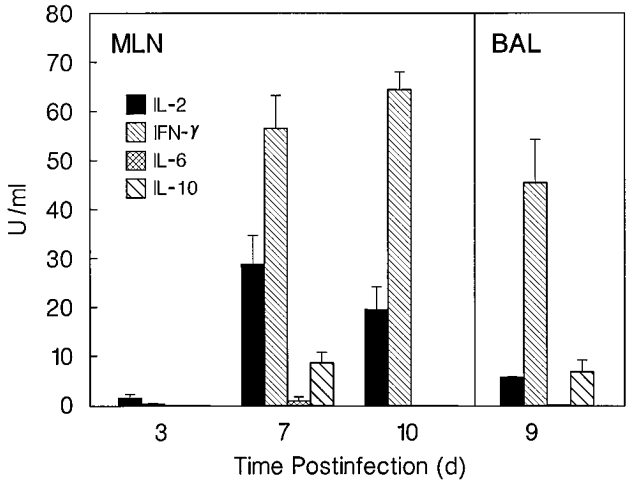

FIG. 4. Profiles of cytokine production following in vitro restimulation with Sendai virus are shown for MLN samples from groups of five to six B6 mice or BAL cells from groups of 12 mice. Lymphocytes from the BAL or MLN cell population were incubated with virus-infected stimulators as described previously (29). Culture supernatants were collected for cytokine ELISAs by centrifugation at 5,600 × *g* for 5 min. The data are means + standard errors of the means for two to five separate experiments. No IL-4 was detected (data not shown). Maximal values are presented: as found previously with the influenza model (29), levels of IL-2 peaked at 24 h after restimulation, decreasing rapidly thereafter, whereas concentrations of all other cytokines were maximal at 48 h and remained constant for the next 24 h.

even though low numbers of cells producing this cytokine were detected in the MLN samples by the ELISPOT technique.

The spectrum of cytokines obtained after restimulation of BAL cells at day 9 was similar to that obtained for the MLN (Fig. 4). Low levels of IL-6 production (~0.8 U/ml) were occasionally seen. Again, no IL-4 was detected in culture supernatants, despite the presence of numerous $IL\text{-}4^+$ SFCs in the freshly isolated BAL cell population. The alternatives are that the levels of IL-4 production are low, that all the IL-4 is consumed, or that the IL-4 response is not virus specific and thus not restimulated in culture.

The relative roles of MLN $CD4^+$ or $CD8^+$ T cells for fluorescence-activated cell sorter (FACS)-separated populations taken on day 7 after infection were analyzed. As expected, most of the secreted cytokine was produced by the $CD4^+$ T cell subset (Fig. 5). The $CD8^+$ T cells produced the same range of cytokines but at lower levels (Fig. 5). Such sorted lymphocytes performed poorly in ELISPOT assays (data not shown).

Cytokines characteristic of both T_H1 and T_H2 responses are thus generated in this murine parainfluenza pneumonia. There are similarities and differences between the cytokine profiles documented in the present study of Sendai virus-infected B6 mice and those previously reported for influenza pneumonia in the same mouse strain (28, 29). The same range of cytokine-producing cells is found for BAL cell populations in both respiratory infections. However, relatively fewer cells were found to produce IL-10 in the influenza model. Furthermore, numerous IFN-γ SFCs were detected in the MLN of influenza-infected mice by the ELISPOT technique, whereas only minimal numbers were found in Sendai virus-infected mice. Even so, Sendai-primed MLN cells were sensitized for IFN-γ production and produced this cytokine on restimulation. The difference could reflect divergence in the activation status of MLN cells for these two viral infections, although MLN cells responsive to influenza virus and Sendai virus produced the same range of cytokines following in vitro restimulation. Also,

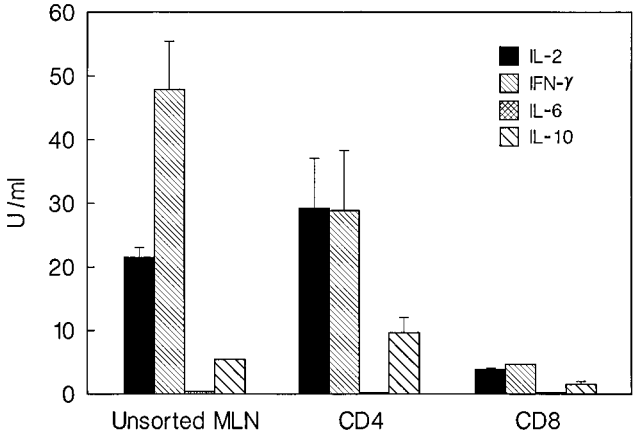

FIG. 5. Patterns of cytokine production are shown for FACS-separated $CD4^+$ and $CD8^+$ T lymphocytes after restimulation. The MLN cells were pooled from 10 to 12 virus-infected mice on day 7 after infection and prepared for FACS separation as previously described (29). Sorted populations were >99% pure on reanalysis. The unsorted MLN, $CD4^+$, or $CD8^+$ cell populations were restimulated with Sendai virus-infected irradiated syngeneic splenocytes for 24 to 48 h. The presence of surface-bound antibody appeared to have little effect on the function of these lymphocytes (15, 29). The results shown are mean values for three separate experiments + standard errors of the means.

both $CD4^+$ and $CD8^+$ T cells contributed to cytokine production in these two respiratory infections.

In conclusion, we have now defined the patterns of production for T_H1 and T_H2 type cytokines in normal B6 mice infected with Sendai virus. The results provide a basis for further studies using genetically disrupted mice or in vivo neutralization to determine the biological roles of these molecules in this viral pneumonia.

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