Activation of Cytokine Genes in T Cells during Primary and Secondary Murine Influenza Pneumonia

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Summary

The patterns of cytokine mRNA expression in mice with primary or secondary influenza pneumonia have been assessed by in situ hybridization analysis of cells from both the mediastinal lymph node (MLN) and the virus-infected lung. Evidence of substantial transcriptional activity was found in all lymphocyte subsets recovered from both anatomical sites. The kinetics of cytokine mRNA expression after primary infection with an H3N2 virus were in accord with the idea that the initial response occurs in regional lymphoid tissue, with the effector T cells later moving to the lung. This temporal separation was much less apparent for the more rapid secondary response resulting from challenge of H3N2-primed mice with an H1N1 virus. Among the T cell receptor α/β^+ subsets, transcripts for interferon (IFN) γ and tumor necrosis factor β were most commonly found in the $CD8^+$ population whereas mRNA for interleukin (IL) 4 and IL-10 was much more prevalent in $CD4^+$ T cells. The γ/δ T cells expressed mRNA for all cytokines tested, with IL-2, IL-4, and IFN-γ predominating among those recovered from the inflammatory exudate. At particular time points, especially early in the MLN and late in the infected lung, the frequency of $mRNA^+$ lymphocytes was much higher than would be expected from current understanding of the prevalence of virus-specific precursors and effectors. If this response is typical, induction of cytokine gene expression for T cells that are not responding directly to the invading pathogen may be a prominent feature of acute virus infections.

Cell-mediated immunity (CMI)[1] reflects a complex interplay of events involving NK cells, APCs, T lymphocytes, and activated macrophages. Communication between these diverse cell types operates via surface ligands (such as the clonotypic TCR) that promote cell–cell interaction and by a spectrum of secreted factors, the cytokines, that may variously induce activation, proliferation, or downregulation (1–4). Rigorous analysis of the role of different cytokines in the in vivo situation is likely to provide key insights into the pathogenesis of disease processes, and to suggest rational measures for control and therapy. The now classical example is that a response skewed towards IL-4 production leads to a progressive, fatal disease in BALB/c mice infected with *Leishmania tropica*, whereas C3H mice emphasize secretion of IFN-γ and clear the parasite (5).

The murine influenza pneumonia model is one of the better defined experimental systems for dissecting the complexities of CMI in virus infections (2, 6). It is clear that $CD8^+$ T cells are normally the key mediators of virus clearance, though $CD4^+$ effectors may also function with varying success depending on the virulence of the challenge virus (7–9). Little is known about the roles of secreted mediators in this viral pneumonia. Levels of various cytokines have been measured in the cell-free bronchoalveolar lavage (BAL) fluid from BALB/c mice dying within 6 d of intranasal (i.n.) infection with the A/PR8/34 (H1N1) influenza A virus, the surprising finding being that there was little evidence for the production of the lymphokines that are normally associated with T lymphocyte activation (10). Perhaps this reflects the inability of cytokine bioassays to detect low levels of the product, or consumption of these cytokines by the various cellular elements involved in the host response. The difficulties associated with assaying cytokines directly from the in vivo situation are avoided in the present experiments by using in situ hybrid-

[1] *Abbreviations used in this paper:* BAL, bronchoalveolar lavage; CMI, cell-mediated immunity; H, influenza virus hemagglutinin molecule; HAU, hemagglutinating units; MACS, magnetic activated cell sorter; MLN, mediastinal lymph node; N, influenza virus neuraminidase molecule; NA, nonadherent (to plastic); i.n., intranasal; HSSC, high side, or 90°, scatter on the FACS®; LSSC, low side scatter.

ization (11, 12) to assess the prevalence of cytokine mRNA$^+$ BAL and regional mediastinal lymph nodes (MLN) cells during the course of both primary and secondary, nonfatal influenza pneumonia in C57BL/6 mice (2, 6, 7). This unique analysis of a disease process defines the potential for cytokine secretion by freshly isolated T cells, though the question of the levels of production of these mediators is left to subsequent studies.

Materials and Methods

Mice. The C57BL/J (B6) female mice used in these experiments were purchased from the Jackson Laboratory (Bar Harbor, ME) and infected initially with virus at 8–10 wk of age.

Virus Infection. The A/HKx31 (H3N2) influenza A virus is a recombinant between A/PR8 (H1N1) and A/Aichi with the surface H3N2 proteins of A/Aichi and many of the internal components of A/PR8 (13). Virus stocks were grown in the allantoic cavity of chick embryos and shown to be free of bacteria, including mycoplasma, and endotoxin. Mice were infected i.n. under Avertin (2,2,2 tribromoethanol) anesthesia with 30 μl of PBS containing 240 hemagglutinating units (HAU) of HKx31 or 60 HAU of PR8, which cause a severe (but generally nonlethal) infection in B6 mice (7).

Sampling the Mice. Pooled BAL cells were obtained from the lungs of two to eight anesthetized, virus-infected mice (7). The MLN were then removed and disrupted using tissue grinders, and the BAL population was adhered on plastic for 90 min at 37°C to remove macrophages. The nonadherent (NA) BAL lymphocytes and MLN cells were then used to prepare paraformaldehyde-fixed centrifuge preparations for the in situ hybridization studies (11, 12), with or without prior separation into different lymphocyte subsets (7, 14, 15).

Hybridization Probes. All probes used for in situ hybridization were cDNA fragments, consisting of sequence complementary to coding sequence only, inserted into the polylinker site of the pGEM-3 or -4 plasmids (Promega Corp., Madison, WI) using standard procedures. The IL-2, IL-4, and IFN-γ probes have been described previously (11, 12). The IL-6 probe, provided by S. Townsend (Genetics Institute, Cambridge, MA) was a 612-bp PstI-BglII fragment corresponding to a full-length cDNA clone. The IL-10 probe, generously provided by K. Moore (DNAX, Palo Alto, CA), consisted of a 554-bp PstI-SacI fragment corresponding to a full-length cDNA clone (16). The TNF-β probe, provided by P. Gray (Genentech Inc., San Francisco, CA), was a 592-bp PvuII-KpnI fragment corresponding to the 1,543–2,135 bp region of the gene (17). pGEM/cytokine cDNA constructs were linearized and sense and antisense ^{35}S-labeled RNA transcripts were synthesized using SP6 or T7 RNA polymerase according to the supplier's recommendations (Promega Corp.).

In Situ Hybridization. Cytocentrifuge preparations of mononuclear cells isolated from the MLN and BAL of virus-infected mice were processed for hybridization in situ as previously described (11, 12). Coded slides were hybridized with sense and antisense probes in triplicate, and the number of silver grains overlaying samples hybridized with sense probes was used to determine the level of nonspecific hybridization (routinely between 1 and 3 grains per cell) and to identify "cytokine mRNA$^+$" cells in samples hybridized with antisense probes. From each coded slide, over 1,000 positive cells from at least 10 different fields were examined to provide the frequency of positive cells per slide.

Lymphocyte Separation and Phenotyping. Pooled lymphocyte populations were sorted on a FACStar Plus® (Becton Dickinson & Co., Mountain View, CA) in two-color mode (14, 15). The mAbs used for staining were the H57.597 anti-α/β-TCR (18), GL3 anti-γ/δ-TCR (19), 53.6.7 anti-CD8 (20), and RM-4-5 anti-CD4 (21). Streptavidin RED 613 (Gibco BRL, Gaithersburg, MD) was used as a second step reagent. Biotinylated mAbs and H57.597-PE, 53.6.7-FITC, and RM-4-5-FITC were purchased from Pharmingen (San Diego, CA).

The magnetic activated cell sorter (MACS) was used with biotinylated magnetic beads as described previously (14), according to the protocol provided with the instrument by the manufacturer (Miltenyi Biotec, Sunnyvale, CA). Lymphocytes were separated by plastic adherence, then incubated with the H129.19 mAb to CD4, or the 53.6.72 mAb to CD8 (20). This was followed by a biotinylated goat anti-rat Ig (Southern Biotechnology Associates, Birmingham, AL), then streptavidin-FITC (Amersham International, Amersham, Bucks, UK) and the biotinylated magnetic beads (Miltenyi Biotec).

Results

The patterns of cellular accumulation in the MLN and NA BAL for naive mice infected i.n. with the HKx31 (H3N2) virus, and for recovered HKx31-primed mice that were later challenged with the PR8 (H1N1) virus are shown as solid lines in Figs. 1 and 2. The secondary kinetics apparent for the BAL from the H1N1-H3N2 group on day 5 (compare Figs. 1 *B* and 2 *B*) reflects that many of the peptide epitopes recognized by both CD4$^+$ and CD8$^+$ T cells are derived from internal proteins common to these two viruses (22–24). The consequence is that the virus is eliminated from the lung on day 5 or 6, rather than on day 7 or 8 (24). Previous studies have shown that the predominant surface TCR$^+$ BAL lym-

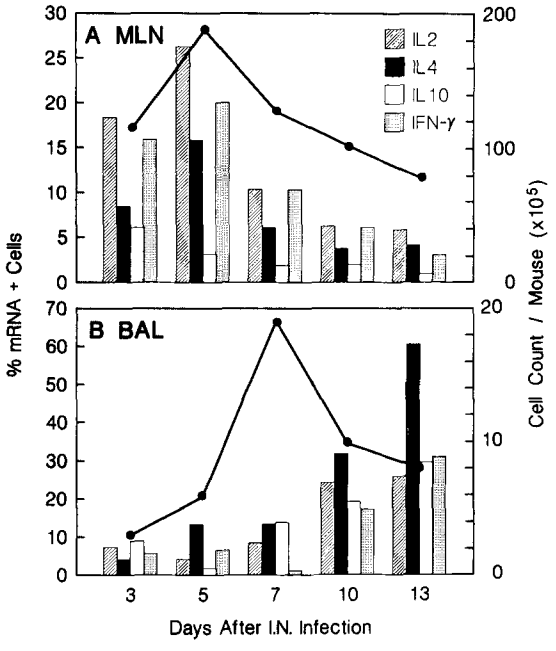

Figure 1. Cell counts per mouse (× 10^5, *solid lines*) and cytokine mRNA profiles (%, as histograms) are shown for MLN (*A*) and NA BAL (*B*) populations after primary infection of B6 mice with 640 HAU of the HKx31 influenza A virus.

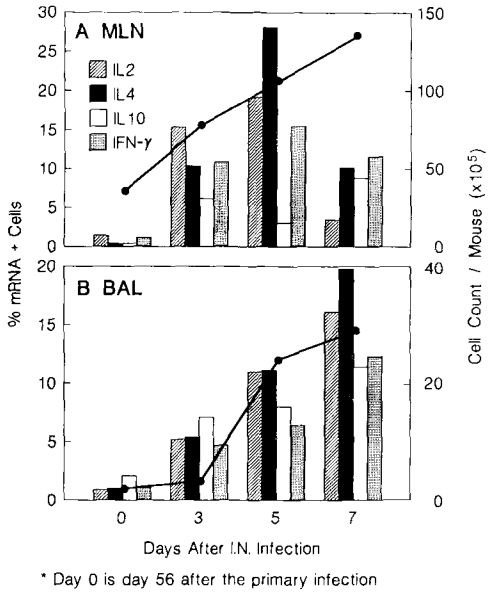

Figure 2. Cell counts per mouse (× 10^5, *solid lines*) and cytokine mRNA profiles (%, as histograms) are shown for MLN (*A*) and NA BAL (*B*) populations from HKx31 (H3N2)-primed mice that were challenged i.n. 8 wk later with 60 HAU of the PR8 (H1N1) virus.

Table 1. *Distribution of mRNA in FACS®-separated α/β and γ/δ TCR⁺ BAL Lymphocytes*

	Primary		Secondary	
	Day 10 H3N2		Day 7 H1N1→H3N2	
mRNA	α/β⁺	γ/δ	α/β⁺	γ/δ⁺
IL-2	24*	45	31	12
IL-4	16	32	13	29
IL-6	11	18	17	6
IL-10	9	27	6	15
IFN-γ	21	49	21	39
TNF-β	10	27	18	24
TCR Cα	71	11	67	NT
TCR Cδ	9	85	6	57

The cells were separated in two-color mode using H57-597-PE and biotinylated GL3 plus streptavidin-FITC.
* Results are expressed as percent mRNA cells for nonadherent BAL populations.

phocytes in both the primary and secondary responses are CD8⁺ α/β TCR⁺ (50–60%), CD4⁺ α/β-TCR⁺ (20–30%), and CD4⁻8⁻ γ/δ-TCR⁺ (5–10%), whereas there are very few B cells. The numbers of CD4⁺ and CD8⁺ T cells in the MLN are approximately equivalent, with <5% γ/δ T cells and large numbers of B lymphocytes (7, 8, 15).

Kinetics of Cytokine Gene Expression in the Primary and Secondary Response. The first experiments concentrated on mRNA for cytokines characteristic of CD8⁺ and CD4⁺ TH_1 (IL-2 and IFN-γ) and the CD4⁺ TH_2 (IL-4 and IL-10) subsets. The patterns of cytokine production in MLN and NA BAL populations for primary infection with the HKx31 virus (Fig. 1, *A* and *B*) are very much in accord with the idea that the initial events in this virus-specific immune response are occurring in the regional MLN, where there is little evidence of virus replication (25), rather than in the target organ (the lung) that supports maximal virus growth. Profiles of cytokine mRNA expression similar to that shown for the MLN might, of course, be found in the bronchus-associated lymphoid tissue, but this has not been analyzed in the present experiments. The divergence between the BAL and MLN is much less apparent for the more rapid secondary response (Fig. 2, *A* and *B*).

Expression of mRNA for IL-2, IL-4, and IFN-γ is in no sense mutually exclusive, at least at the cell population level, with transcripts for all three cytokines being prominent as early as day 3 in the primary response (Fig. 1 *A*). The frequency of cells with IL-10 mRNA is generally lower in the MLN than in the BAL, and increases with time in the BAL (Figs. 1 *B* and 2 *B*). The greater prevalence of all mRNA⁺ cells in the BAL on days 10 and 13 after primary infection (Fig. 1 *B*) could reflect ongoing processes after the virus is cleared (day 7 or 8), or simply the retention of activated, cytokine mRNA⁺ lymphocytes in the inflammatory exudate during the period when cell numbers decline (Fig. 1 *B*). There is, however, an increase in both the total numbers and percentage of cytokine mRNA⁺ BAL cells between days 5 and 7 of the secondary response (Fig. 2 *B*).

Evidence of substantial transcriptional activity is, therefore, found for all the cytokines tested. The numbers of mRNA⁺ cells in the regional LN are maximal at the early stages after challenge, and then decline as the inflammatory process develops in the lung. This difference is most apparent for the slower, primary response.

The γ/δ-TCR⁺ Lymphocytes. The NA BAL population was separated using the FACS® into α/β-TCR⁺ and γ/δ-TCR⁺ subsets (Table 1). The percentage of cells expressing mRNA for IL-4, IL-10, IFN-γ, and TNF-β was two to three-fold higher for the γ/δ-TCR⁺ population than that observed for the α/β-TCR⁺ population, both on day 10 after primary infection and on day 7 after secondary challenge. Substantial percentages of IL-2 (45%), IFN-γ (49%), and IL-6 (18%) mRNA⁺ γ/δ T cells were also found in the BAL on day 10 of the primary response. In addition, the mRNA profiles for FACS®-selected γ/δ T cells from the MLN were analyzed on days 5 and 7 after i.n. exposure of naive mice to the HKx31 virus: from 15 to 20% of the γ/δ T cells obtained on day 7 expressed mRNA for IL-2, IL-4, or IFN-γ (γ/δ-TCR⁺, Fig. 3). The relative frequency of IL-2, IL-4, and IFN-γ mRNA⁺ lymphocytes was highest for the α/β-TCR⁺ population on day 5 and for the γ/δ-TCR⁺ set on day 7 (Fig. 3).

This analysis establishes that there is substantial activation of cytokine genes in γ/δ T cells recovered from both the BAL

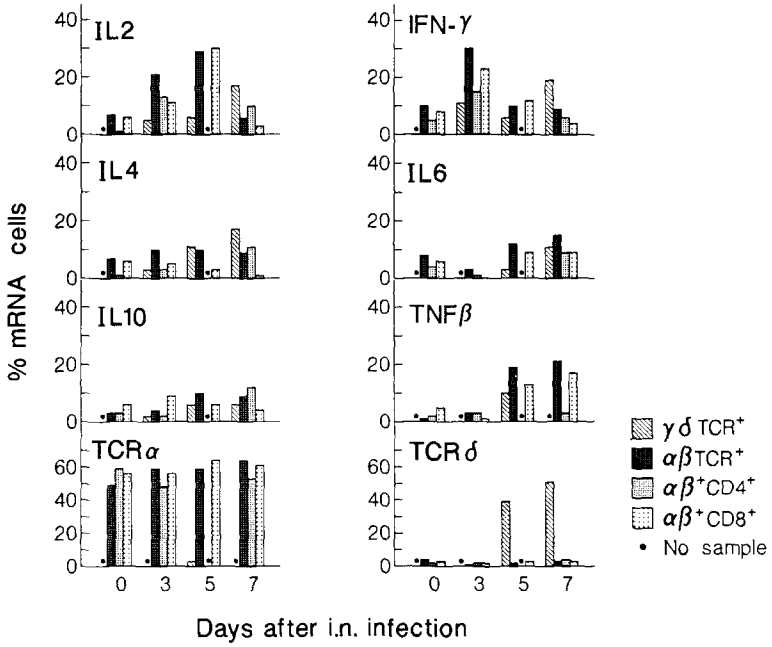

Figure 3. The prevalence of cytokine mRNA expression in different lymphocyte subsets is shown for the first 7 d of the primary response in the MLN to the HKx31 virus. The cells were separated in two-color mode using PE-conjugated anti-TCR-β versus FITC-conjugated anti-TCR-δ, CD8, or CD4. There were insufficient γ/δ T cells to analyze on day 0, or for some cytokines on day 3. The CD4+ T cells from day 5 were lost because of a technical error.

and the MLN, with the percentage of mRNA+ cells often exceeding that found for the α/β-TCR+ population. However, by FACS® phenotyping, the CD4−8− γ/δ T cells are much less prevalent in both sites than the conventional CD4+ and CD8+ subsets (15). The majority of the T lymphocytes expressing lymphokine mRNA in both the MLN and the BAL are thus α/β T cells.

The CD4+ and CD8+ α/β T Cells. The mRNA profiles for FACS®-separated CD8+ α/β-TCR+ and CD4+ α/β-TCR+ MLN cells from the primary response are presented in Fig. 3. The frequency of IFN-γ mRNA+ MLN cells was lower on day 7 (compared with day 3) for both the CD4+ and CD8+ subsets, whereas the percentage of CD4+ lymphocytes with IL-4 and IL-10 transcripts had increased (Fig. 3). This could reflect the emergence of a TH_2 response. The percentage of CD8+ T cells with TNF-β transcripts increased with time (Fig. 3).

The NA BAL cells were analyzed using both FACS® and the MACS separation procedures (Fig. 4, Table 2). Previous studies (15) have shown that some activated T cells are normally gated-out (because of autofluorescence and nonspecific staining) in a high 90° scatter, or side scatter (HSSC), set

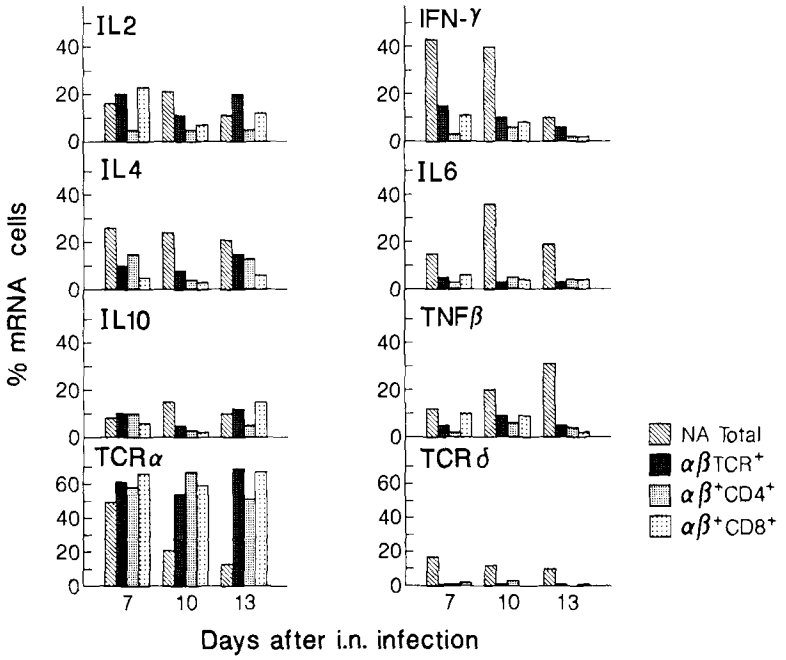

Figure 4. The cytokine mRNA profiles in the NA BAL are shown on days 7–13 of the primary response, for the same experiment as in Fig. 3.

Table 2. *Expression of mRNA in CD4⁺ and CD8⁺ T Cells Enriched from Nonadherent BAL Populations Using the MACS*

	Primary		Secondary H1N1→H3N2			
	Day 10 H3N2		Day 3		Day 5	
mRNA	CD4⁺	CD8⁺	CD4⁺	CD8⁺	CD4⁺	CD8⁺
IL-2	32*	49	13	19	39	41
IL-4	26	7	24	6	42	10
IL-6	10	3	11	21	20	11
IL-10	24	2	21	8	13	8
IFN-γ	12	10	3	25	11	69
TNF-β	3	19	14	17	15	46
TCR-α	73	69	72	79	84	71
TCR-δ	11	16	12	15	11	13

* Results are expressed as percent mRNA⁺ cells.

that constitutes a minority of the NA BAL cells from day 5 of the secondary response. Determining cytokine mRNA profiles for secondary NA BAL cells sorted on the basis of low SSC (LSSC) or HSSC showed that the HSSC set contained many cells with the morphology of activated (or effete) macrophages (15), which might be expected (26, 27) to produce TNF and IL-6. In addition to the expression of the genes encoding these cytokines, there were also substantial numbers of HSSC cells expressing mRNA for IL-2, IL-4, and IFN-γ (Fig. 5). In the analysis of cytokine production by T cell subsets for FACS®-sorted BAL populations, therefore, it is likely that some large, responding α/β and γ/δ TCR⁺ lymphocytes were excluded. On the other hand, the MACS separation procedure is probably less precise.

Despite the various technical limitations and quantitative differences, the FACS® and MACS-sorting experiments with these highly activated BAL populations generally showed the same trends (Fig. 4, Table 2). Evidence of substantial IL-4 and IL-10 transcription was found mainly for the CD4⁺ subset. Cells with mRNA for IFN-γ and TNF-β are more commonly found in the CD8⁺ population. The CD4⁺ T cell response might seem to be skewed towards a TH_2 profile (1), if it were not for the fact that many of the CD4⁺ T cells isolated on the MACS expressed IL-2 mRNA (Table 2). The percentage of IFN-γ mRNA⁺ CD8⁺ and IL-4 mRNA⁺ CD4⁺ BAL T cells was very high at the peak of the secondary response on day 5 (Fig. 2, Table 2). It seems very unlikely that the majority of these lymphocytes could be responding specifically to the virus (2). Perhaps this reflects that the cytokine-rich milieu of the inflammatory exudate induces transcriptional activity in a broader spectrum of T cells.

Discussion

Analysis of cytokine mRNA profiles, often determined by semiquantitative approaches using the PCR, is a feasible method for assessing the involvement of these mediators in inflammatory processes (28, 29). However, PCR analysis of tissue samples does not localize the distribution of cytokine production within populations of cells, and gives no insight into the relative proportion that are expressing the message. The in situ hybridization approach overcomes these limitations of PCR analysis (11, 12), and has the advantage that the patterns determined are not subject to modification by

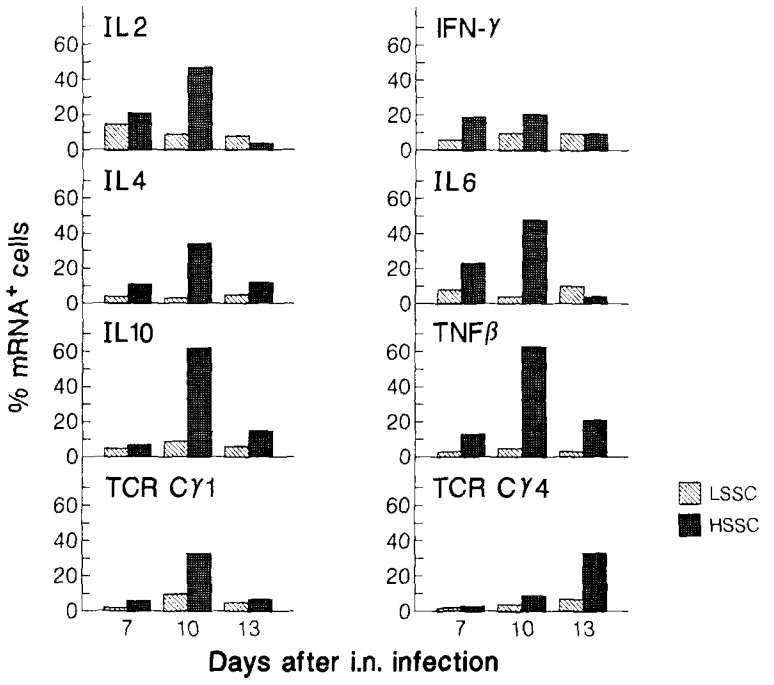

Figure 5. The NA BAL populations from secondarily-stimulated mice were separated into high and low side-scatter populations with the FACS® (13). The LSSC set is characteristically used for FACS® phenotyping and sorting lymphocyte subsets. The percentage of cells in these two populations on days 3, 5, and 7 were: LSSC, 30, 49, and 59; HSSC, 31, 14, and 13. The highly granular NK cells would be prominent in the HSSC set during the early phases of the response.

cytokine consumption or degradation, either in the tissue or during the process of sampling.

The correlation between cytokine gene expression and cytokine release is complex. Detection of cytokine mRNA in freshly isolated cells is obviously a very sensitive measure of lymphocyte activation in vivo. However, the presence of message is not a measure of the synthesis and secretion of active protein. Given that individual mRNA$^+$ cells are likely to produce more or less of a particular cytokine, we cannot say how well the mRNA profiles are likely to correlate with the level of functional activity. Even so, the utility of in situ hybridization for analyzing the kinetics of an in vivo immune response to a pathogen is clearly demonstrated by the present lymphocyte subset analysis.

The first obvious change in the primary response to an influenza A virus given i.n. is the massive increase in the cellularity of the regional MLN. This occurs in the absence of significant virus replication in this site (25). At least 15% of these LN cells are expressing IL-2 and/or IFN-γ mRNA within 3 d of the infection. Transcripts are found in both CD4$^+$ and CD8$^+$ α/β T cells, though the CD4$^+$ set seems to assume more of a TH_2 phenotype later in the response. It seems very unlikely that one in seven of the cells in the MLN at day 3 is responding specifically to the virus. Some of this IFN-γ mRNA may be expressed in NK cells, which are prominent in the early stages of the host response to most viruses and may be induced by cytokines produced by virus-infected cells. Draining lymph from the virus-infected lung is, for example, likely to contain IFN-α/β which has been shown (in other experimental systems) to cause early LN enlargement (30, 31).

The divergence in the prevalence of cytokine mRNA$^+$ cells for the MLN (early) and BAL (late) seen for the primary response is less obvious after secondary challenge. This could simply reflect that the kinetics of the secondary response are much more rapid. However, it might also indicate that virus-immune memory T cells, which are more readily triggered to effector function (32), are less dependent on the LN environment for stimulation. Recent studies (33) indicate that trafficking through lymphoid tissue is characteristic of naive rather than memory T cells.

Transcriptional activity for IL-10, the cytokine thought to be involved with downregulation (4, 34) of many aspects of immunity, is clearly prominent in the later stages of the inflammatory process during the primary response, reaching maximal prevalence on day 10, 2–3 d after infectious virus is cleared from the lung (7). Expression of IL-10 mRNA was mostly, though not exclusively, associated with the CD4$^+$ and γ/δ-TCR$^+$ subsets. However, IL-10 mRNA$^+$ cells were also very evident in the early stages of the secondary pneumonia, which may indicate that memory CD4$^+$ TH_2 cells are being preferentially induced.

These experiments further indicate that the γ/δ T cells are an active component of influenza pneumonia (14, 35). This lymphocyte subset showed, at the different time points tested, evidence of transcription for all cytokines analyzed. Perhaps the role of the γ/δ T cells in this virus-induced inflammatory process is to act as cytokine factories which may, for instance, help to regulate macrophage activation and other essentially nonspecific effector mechanisms (4, 35, 36).

The present in situ hybridization analysis provides a spectrum of insights that will greatly facilitate the further dissection of this experimental system. For instance, there is no lack of transcriptional activity for the conventional T cell lymphokines in this nonfatal pneumonia, though experiments measuring cytokine levels in a lethal influenza infection did not indicate significant T cell involvement (10). The two situations need to be compared using the same technical approaches, to see if differences in lymphokine profiles are influencing the clinical outcome. Questions are raised concerning the relative significance of the TH_1 and TH_2 CD4$^+$ subsets. Separated CD4$^+$ T cells in both the primary and secondary response showed mRNA profiles more characteristic of TH_2 cells, though many of the CD4$^+$ T cell clones recovered from influenza virus-infected mice have the TH_1 cytokine phenotype (T. J. Braciale, personal communication; 37).

It is also apparent that we need to consider activation mechanisms that do not depend on the engagement of the antigen-specific TCR, particularly in sites of inflammatory pathology. Many more T cells show evidence of transcriptional activity than would be expected to be responding specifically to the virus (2, 6, 7). Even so, the mRNA expression profiles described here are generally in accord with current thinking concerning the cellular origins and biological role of particular cytokines (1, 3, 4).

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