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Immunology

Enumeration of lymphokine mRNA-containing cells *in vivo* in a murine graft-versus-host reaction using the PCR

(interleukin 3/interleukin 4/interferon γ/limiting dilution analysis/gene expression)

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ABSTRACT **A method of enumerating lymphokine mRNA-containing cells *in vivo* was developed by combining limiting dilution analysis with PCR amplification of cDNA. Single-hit kinetics revealed that the PCR–limiting dilution analysis could detect a single positive cell among >40,000 negative cells. With this method, spleens and lymph nodes of mice undergoing an acute allogeneic graft-versus-host reaction were found to contain lymphokine mRNA-expressing cells at frequencies of 3% for interferon γ, 0.05% for granulocyte/macrophage colony-stimulating factor, 0.002% for interleukin 3, and 0.03% for interleukin 4; these frequencies were 20- to 175-fold higher than in lymphoid tissues of normal mice. In contrast to their low frequencies of lymphokine mRNA-containing cells *in vivo*, graft-versus-host reaction populations restimulated *in vitro* for 24 hr with anti-CD3 antibody yielded frequencies ranging from 3% for interleukin 4 to nearly 70% for interferon γ. Furthermore, lymphokine transcripts were also detected in single micromanipulated cells from these populations. Because frequencies of anti-CD3-inducible lymphokine mRNA-containing cells in normal mice were only 0.03–1%, it was concluded that lymphoid tissues of graft-versus-host reaction mice contained high frequencies of cells that had been primed for lymphokine synthesis. Only a small fraction of these cells, however, expressed lymphokine mRNAs at a given time point *in vivo*.**

T-cell-derived lymphokines exert potent positive or negative effects on the growth, differentiation, and effector functions of many cells of the immune system. Although selective production of certain lymphokines has been found to influence the immune response to some antigens and infectious agents (1, 2), the mechanisms underlying this differential production are not well understood. Mosmann and others (3, 4) have shown that long-term murine T-cell clones can be divided into two subsets based on the mutually exclusive production of certain lymphokines: Th1, which express interleukin 2 (IL-2), interferon γ (IFN-γ), and tumor necrosis factor β; and Th2, which express IL-4, IL-5, IL-6, and IL-10. However, short-term clones frequently coproduce Th1 and Th2 lymphokines (5–8). Because it is not yet known whether individual T cells *in vivo* exhibit restricted patterns of lymphokine production, the physiological significance of these proposed subsets remains unclear.

In vivo identification of subsets of lymphokine-producing T cells will require the analysis of individual normal lymphocytes. We previously described the detection of IL-3 and granulocyte/macrophage colony-stimulating factor (GM-CSF) secretion by single lymphoid cells from mice undergoing a graft-versus-host reaction (GVHR) designed to activate T cells at high frequency *in vivo* (9). However, only certain lymphokine assays were sensitive enough to detect lymphokine production by single cells, even in this potent T-cell response, and this approach did not allow assessment of lymphokine synthesis without *in vitro* culture. Because of its exquisite sensitivity, PCR amplification of cDNA is being widely used to detect low-abundance transcripts, including lymphokines (2, 10, 11). Therefore, we have combined an adaptation of the PCR with the technique of limiting dilution analysis (LDA) to analyze the frequency of lymphokine mRNA-containing cells in an acute GVHR *in vivo*.

MATERIALS AND METHODS

GVHR. Specific pathogen-free mice were used at 6–12 wk of age. DBA/2 (H-2^d *Mls*^{a}) mice were irradiated with 900 rads (1 rad = 0.01 Gy) and injected i.p. with 5×10^7 C57 (H-2^b Mls^b) spleen cells. Lymph nodes and spleens were harvested 5 days later.

Bulk Lymphocyte Culture. Single-cell suspensions of spleens and lymph nodes were cultured as described (9) at 10^6 cells per ml in tissue culture plates coated with anti-CD3 antibody at 5 μg/ml. After 24 hr, the cells were harvested, and RNA was prepared for analysis of lymphokine mRNA.

LDA of Lymphokine-Secreting Cells. Pooled spleen and lymph node cells were added in graded numbers to wells of Terasaki plates in the presence or absence of purified human recombinant IL-2 from *Escherichia coli* at 20 units per ml (Cetus) and solid-phase anti-CD3 antibody. After 24 hr, two 5-μl aliquots of supernatants were removed and assayed for secreted lymphokine by incubation as described (9) with factor-dependent cell lines FDC-P1, which responds maximally to GM-CSF or IL-3 and submaximally to IFN-γ or IL-4, and 32D, which responds to IL-3.

PCR–LDA of Lymphokine mRNA-Containing Cells. Freshly prepared single-cell suspensions of pooled spleens and lymph nodes or cells that had been cultured for 24 hr with anti-CD3 as described above were added in graded numbers (10 samples per cell number) or by micromanipulation to 1.5-ml tubes (Sarstedt). RNA preparation, cDNA synthesis, PCR, and first-round PCR oligonucleotide primers have been described (12). The first-round PCR products were diluted 1:1000, and 2 μl of each dilution was used in second-round PCR reactions. Second-round PCR oligonucleotide primers were as follows: IL-3: 5′ primer, GCAGCTCTATTGTCAAGGAG, and 3′ primer, GCAGAGTCATTCGCAGATGTAG; IL-4: 5′ primer, CACTTGAGAGAGATCATCGG, and 3′ primer, GGCTTTCCAGGAAGTCTTTCA; GM-CSF: 5′ primer, CATTGTGGTCTACAGCCTCT, and 3′ primer, CAGTCTGAGAAGCTGGATTC; IFN-γ: 5′ primer, CCTCAGACTCTTTGAAGTCT, and 3′ primer, CAGCGACTCCTTTTCCGCTT.

Each primer pair spanned at least one intron in the respective genomic sequence, allowing unambiguous discrimina-

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Abbreviations: GM-CSF, granulocyte/macrophage colony-stimulating factor; GVHR, graft-versus-host reaction; IFN-γ, interferon γ; IL, interleukin; LDA, limiting dilution analysis.

tion between cDNA and genomic amplification products. All four pairs of the appropriate cytokine primers were included in each reaction. Five microliters of each second-round PCR reaction was electrophoresed through a 1.8% agarose gel and transferred to Zeta-Probe membrane by alkaline blotting according to the manufacturer's instructions (Bio-Rad). Membranes were hybridized with radiolabeled cDNA probes as described (12). Each membrane was sequentially hybridized with all five cDNA probes by multiple rounds of hybridization and stripping according to the manufacturer's instructions. A minimum of seven negative control samples were included at each step in the procedure. *E. coli* tRNA and HeLa cell total RNA were included during cDNA synthesis, and reactions containing no cDNA were included in each round of PCR. Occasional false positives (≤6%) were detected for IL-3 and IL-4, but these were calculated to lead to, at most, a 2- to 3-fold alteration in frequency estimates (data not shown).

Frequency Estimations. Based on the Poisson probability distribution, frequency estimates were obtained from the slope of the line of best fit determined by the maximum likelihood method for the relationship between the number of cells per sample and the logarithm of the percentage of negative samples (13, 14) or by direct enumeration of responding micromanipulated single cells.

RESULTS

T Lymphocytes Activated *in Vivo* Require Restimulation for High-Frequency Lymphokine Secretion. Lymphoid populations containing a high proportion of *in vivo*-activated T cells were generated by establishing a GVHR in lethally irradiated DBA mice by injection of C57 spleen cells. Spleen and lymph node cells were harvested after 5 days when >90% of cells were of donor origin and their *in vitro* lymphokine production was maximal. Cells were cultured at limiting dilution for 24 hr with or without solid-phase anti-CD3 and IL-2; culture supernatants were then assayed for their ability to support the survival and proliferation of FDC-P1 cells (which respond maximally to GM-CSF and IL-3 and submaximally to IL-4 and IFN-γ) and 32D cells (which respond to IL-3). Estimates of the frequencies of constitutive and anti-CD3/IL-2-inducible lymphokine-secreting cells were obtained by statistical analysis of these data (Fig. 1). As reported (9), the frequency of lymphokine-secreting cells in unstimulated populations from GVHR mice was <0.1%, whereas LDA of GVHR cells stimulated with anti-CD3 and IL-2 yielded frequencies of 22% in FDC-P1 assays and 12% in 32D assays. Constitutive lymphokine secretion was not detected in lymphoid cells from normal C57 mice, and <0.1% exhibited inducible lymphokine secretion. The multi-hit curves generated by unstimulated GVHR cells and anti-CD3-stimulated normal lymphoid cells suggest that activation in these cultures depended on cell interactions or that detection of lymphokine activity required the presence of several producing cells (13).

Estimation of the Frequency of Lymphokine mRNA-Containing Cells *in Vivo* and *in Vitro*. The low frequency of constitutive lymphokine-secreting cells in lymphoid populations from GVHR mice suggested that the number of T cells engaged in lymphokine production at any given time *in vivo* may be very small or that the assays used were too insensitive to detect low levels of lymphokine secretion per cell. To address these possibilities and to extend the range of lymphokines assayed, PCR–LDA was conducted of lymphokine mRNA-containing cells in freshly extracted spleens and lymph nodes from the same experimental animals analyzed for lymphokine secretion in Fig. 1. Total RNA was prepared from graded numbers of lymphoid cells from GVHR and normal C57 mice, either immediately after harvest or after

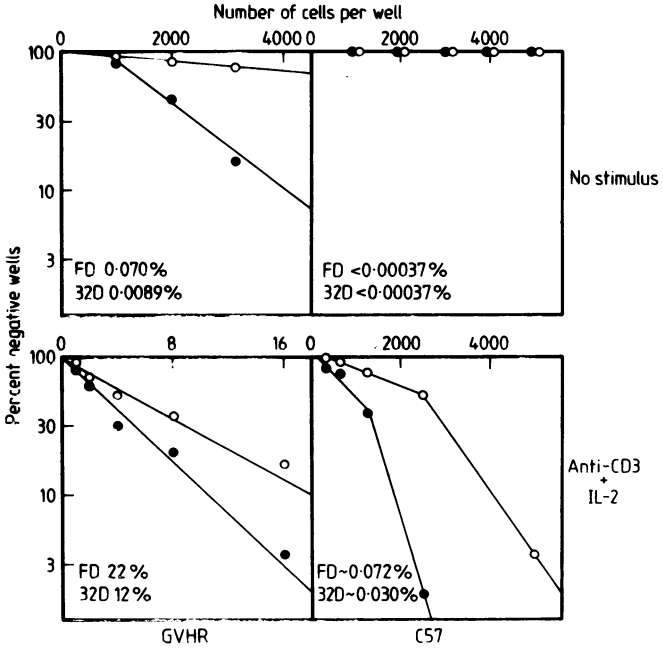

FIG. 1. LDA of lymphokine-secreting cells. Pooled spleen and lymph node cells from GVHR or normal C57 mice were cultured at the indicated numbers with or without anti-CD3 antibody and IL-2. After 24 hr, supernatants were assayed for lymphokines by incubation with FDC-P1 (FD) (●) and 32D (○) cells.

culture for 24 hr with anti-CD3 antibody. cDNA was synthesized using this template and subjected to two rounds of PCR by using lymphokine-specific oligonucleotide primers. The resultant PCR products were assessed for lymphokine cDNA by Southern blot hybridization (Fig. 2). The expression of β-actin mRNA was analyzed as a positive control and was detected in all samples except that indicated. As shown in Fig. 3 for freshly harvested GVHR cells, these data conformed to the zero term of the Poisson distribution, suggesting that a positive result depended only on the presence of a single mRNA-containing cell. All LDA samples from *in vitro*-stimulated GVHR cells contained mRNA for IFN-γ and GM-CSF, so frequencies for these lymphokines were determined by analysis of individual micromanipulated cells (Fig. 4).

Table 1 summarizes all frequency estimates obtained in this experiment. Frequencies of GVHR cells expressing lymphokine mRNA *in vivo* varied widely for the four lymphokines and were increased 20- to 2000-fold after *in vitro* restimulation. Although frequencies of mRNA-containing cells detected in normal lymphoid populations were also increased 10- to >500-fold by *in vitro* restimulation, all frequency estimates for normal cells were 20- to 175-fold lower than the equivalent estimate for GVHR cells. Furthermore, estimates for IL-3 and GM-CSF mRNA-containing cells *in vitro* and *in vivo* corresponded to those obtained for secretion of activities stimulating 32D cells (IL-3) and FDC-P1 (mainly IL-3 and GM-CSF) with and without restimulation *in vitro* (Fig. 1).

DISCUSSION

Here we describe application of the PCR to measure the frequency of cells containing lymphokine mRNA in lymphoid tissues of mice during an acute GVHR. This approach has an important advantage over other methods of analyzing lymphokine synthesis in that it allows measurement of the frequency of cells expressing multiple lymphokine genes in a single cell preparation. In other studies we have used a single round of PCR for simultaneously analyzing eight mRNAs

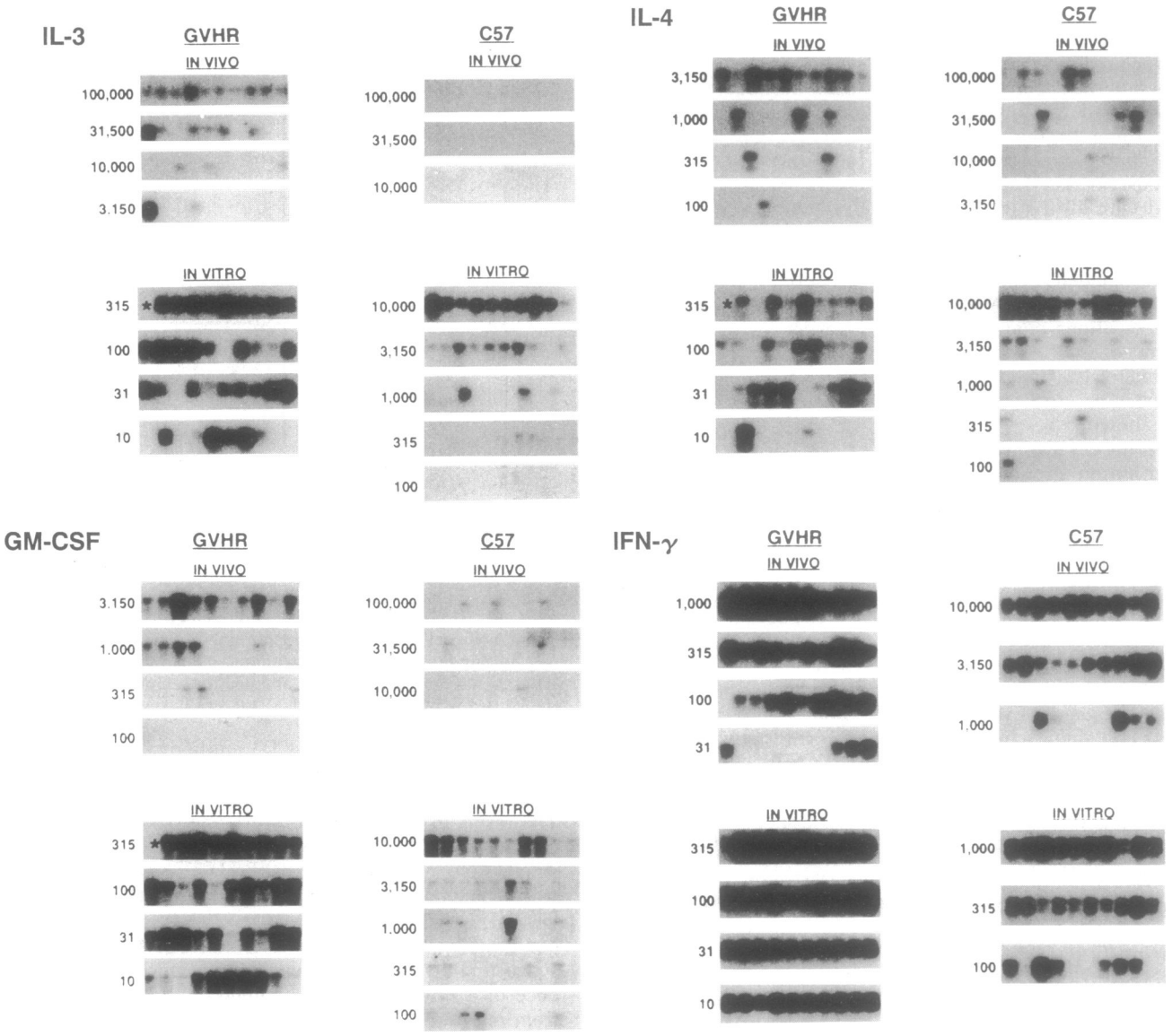

FIG. 2. LDA of lymphokine mRNA-containing cells. Single-cell suspensions were prepared from pooled spleen and lymph node cells from GVHR and normal C57 mice. RNA was prepared from 10 aliquots of each of the indicated cell numbers, either immediately (*in vivo*) or after 24-hr culture at 10^6 cells per ml with anti-CD3 antibody (*in vitro*). The indicated sample (*) was negative for all probes, including β-actin. All autoradiographs were derived from a single set of samples.

(12), and there is no theoretical maximum for the number of different transcripts that can be assayed in a single sample. Experiments done by using cloned lymphokine cDNAs showed that inclusion of a second round of PCR increased sensitivity 10- to 1000-fold and that the detection limit in this system was <30 molecules (unpublished observations). Although the variability introduced by inclusion of a second round of PCR has yet to be fully analyzed, three independent studies of IFN-γ, GM-CSF, and IL-3 expression *in vivo* in the GVHR conducted using a single round of PCR gave the same relative ranking of frequencies as reported here, and frequency estimates obtained in these three experiments differed by no more than 3- to 6-fold (unpublished observations).

PCR–LDA of freshly harvested lymphoid tissues yielded linear plots (e.g., Fig. 3) that intercepted the *y* axis near the origin, indicating that a positive result depended only on the presence of an individual mRNA-containing cell. Similarly, for *in vitro*-restimulated GVHR cells that contained high frequencies of positive cells, transcripts were detected even when samples contained an average of only 10 cells (Fig. 2) or a single micromanipulated cell (Fig. 4). Therefore, at least some *in vivo*-activated cells and many *in vitro*-restimulated cells contained sufficient transcripts to yield the minimum of 30 cDNA molecules detectable after PCR amplification. As for other mRNA detection methods, it is not yet clear how this threshold of detection relates to the production of physiologically active lymphokine levels. However, for both IL-3 and GM-CSF, frequency estimates for cells positive for mRNA were similar to those for cells that secreted biologically detectable protein (minimum 10^6–10^7 molecules per cell per 24 hr). Together with evidence that lymphokine synthesis is controlled mainly at the level of mRNA accumulation (8, 15, 16), this result suggests that numbers of lymphokine mRNA-containing cells detected by PCR–LDA may, indeed, provide a valid estimate of numbers of cells secreting the corresponding protein.

An acute GVHR in lethally irradiated, allogeneic mice was used as a model system for analysis by PCR–LDA with the expectation that lymphoid tissues in these animals would contain high frequencies of activated lymphokine-producing cells. As shown here and elsewhere (9), GVHR spleens and lymph nodes did contain high frequencies of activated T cells by the criterion that these cells could transcribe and secrete

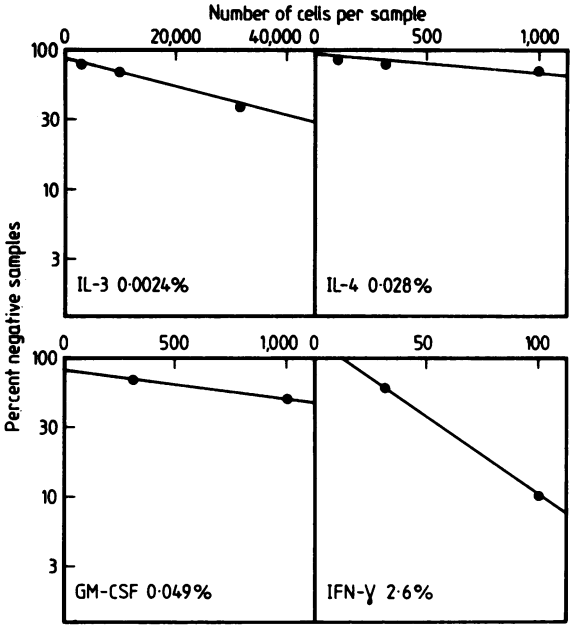

FIG. 3. Estimation of frequencies of lymphokine mRNA-containing cells *in vivo* in the GVHR. Data are derived from Fig. 2.

lymphokines when restimulated with anti-CD3 antibody and IL-2; comparison of frequencies of anti-CD3-inducible cells in GVHR and normal mice suggests that the ability to produce lymphokines in these culture conditions is a property of previously activated T cells. Given the high frequency of cells primed for lymphokine synthesis in GVHR populations, the frequencies containing lymphokine transcripts in freshly harvested tissues were unexpectedly low, ranging from 0.002% for IL-3 to 3% for IFN-γ. These estimates were consistent with the low levels of lymphokine mRNA detected in freshly extracted bulk spleen compared with anti-CD3-restimulated spleen cells (8) and, for IL-3 and GM-CSF, with frequencies of cells that secreted these lymphokines *in vitro* without restimulation. GVHR populations, nevertheless, contained at least 20-fold higher frequencies of lymphokine mRNA-containing cells than normal lymphoid tissues.

We conclude that only a small proportion of the primed cells in lymphoid tissues of GVHR mice contained detectable lymphokine mRNA, although tissues were harvested at the peak of this acute T-cell response. There are several possible explanations for these low frequencies. (*i*) Mean transcript levels per positive cell may have been lower in freshly harvested cells than after *in vitro* restimulation, so that fewer cells reached the threshold of detection. In this case, however, LDA would be expected to yield multi-hit curves (14). (*ii*) A second possibility is that mRNA accumulation was inhibited *in vivo* by unknown regulatory mechanisms that

Table 1. Frequencies of lymphokine mRNA-containing cells in pooled spleens and lymph nodes of GVHR and normal mice

	Positive cells, %			
	GVHR mice		C57 mice	
Lymphokine	*In vivo*	*In vitro*	*In vivo*	*In vitro*
IFN-γ	2.6	67	0.069	0.92
GM-CSF	0.049	33	0.00028	0.032
IL-3	0.0024	6.3	<0.0001	0.057
IL-4	0.028	3.3	0.00032	0.034

Frequency estimates were derived from data in Figs. 2 and 4.

were overcome when cells were restimulated *in vitro*. (*iii*) Although explanation *ii* may, indeed, occur, we favor a third possibility, that the low frequency of lymphokine mRNA-containing cells reflects two phenomena: a low frequency of interaction with specific antigen major histocompatibility complex in the tissues studied and the transience of the lymphokine synthetic response so induced. *In vitro*, lymphokine mRNA accumulation and protein synthesis by activated T cells depend on ligand binding to the antigen receptor or a limited number of other receptors; synthesis is then short-lived—due, in part, to cessation of stimulation and to the short half-lives of lymphokine mRNAs (15, 17). If this is also true *in vivo*, and the frequency and duration of T-cell encounters with antigen-presenting cells are low, only a small fraction of primed cells would be engaged in lymphokine synthesis at a given moment. It is not known whether other sites of antigen exposure in GVHR mice, such as the skin and gut, contain higher frequencies of lymphokine mRNA-containing cells.

PCR–LDA of freshly harvested GVHR tissues revealed marked differences in the frequencies of cells containing mRNA for IFN-γ (3%), GM-CSF (0.05%), IL-4 (0.03%), and IL-3 (0.002%). IFN-γ mRNA was expressed at by far the highest frequency, consistent with the notion that this lymphokine plays an important role in activating effectors of the GVHR, such as cytolytic T cells and macrophages (18). It should be noted that, although most lymphokine-secreting cells detected after anti-CD3 restimulation were $CD4^+$ and $CD8^+$ T cells (9), the phenotypes of positive cells detected *in vivo* have not been determined but might be expected to include natural killer and mast cells as well as T cells. The 100-fold excess of IFN-γ mRNA producers over IL-4 mRNA producers is also interesting in light of the antagonistic effects of these two lymphokines on many cellular responses (19). Because of the excess of IFN-γ producers, it was not possible to determine the frequency of coexpression of IFN-γ and IL-4 *in vivo* in the GVHR. However, an important future application of the PCR–LDA will be the direct enumeration of single and double producers of these major regulatory lymphokines in other immune responses *in vivo*.

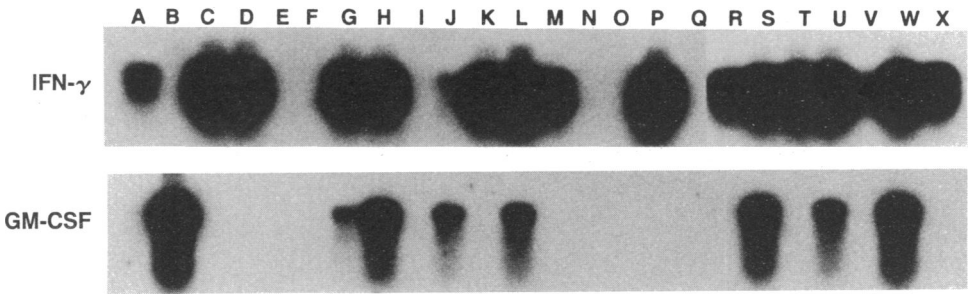

FIG. 4. Analysis of lymphokine mRNA in single cells. RNA was prepared from individual micromanipulated cells from GVHR mice after 24-hr culture at 10^6 cells per ml with anti-CD3 antibody. The same samples were sequentially analyzed for hybridization with GM-CSF and IFN-γ probes.

In conclusion, the PCR–LDA allowed quantitation of cells producing multiple mRNA species over a wide frequency range. Analysis of an acute GVHR by this approach produced the unexpected finding that only a small fraction of cells in peripheral lymphoid tissues contained detectable IFN-γ, GM-CSF, IL-3, or IL-4 transcripts, even though the majority could produce one or more of these lymphokines when restimulated *in vitro*. This result provides direct quantitative support for the view that lymphokine synthesis is tightly regulated under physiological conditions. In addition, the finding of marked disparities in the frequencies of cells containing different lymphokine mRNAs *in vivo* is intriguing. Application of the PCR–LDA to other populations of activated T cells, particularly at the single-cell level, should reveal whether differences in lymphokine levels in different immune responses are due to activation of cells with distinct lymphokine production patterns.

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