Heterogeneity of Single Cell Cytokine Gene Expression in Clonal T Cell Populations

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Summary

T helper type 0 (Th0), Th1, and Th2 $CD4^+$ T cell clones derived from a T cell receptor α/β (TCR-α/β) transgenic mouse were activated by antigen presented on "artificial" antigen-presenting cells that expressed or lacked the costimulatory molecule B7-1, and were analyzed for single cell cytokine mRNA expression by in situ hybridization. There was significant heterogeneity in the frequency of T cells that expressed individual cytokine mRNAs within each clonal population, suggesting that transcriptional control of each of the cytokine genes was not coordinate within an individual cell. The majority of antigen-stimulated Th1 cells expressed mRNA for interferon γ (IFN-γ), but far fewer cells in the same population expressed interleukin 2 (IL-2). Similarly, the frequency of IL-4–expressing cells was greater than that of IL-5– or IL-10–expressing cells in the same Th2 population, but the difference in expression frequencies was more variable between clones. The expression frequencies of each of the cytokines was quite heterogeneous in the antigen-activated Th0 population. The principal effect of increased antigen on the activation of individual cytokine genes in each of the clonal populations was to increase recruitment of mRNA-positive cells, with little or no effect on the level of cytokine mRNA expression in individual positive cells. The effects of B7 costimulation were variable depending on the cytokine gene analyzed. B7 costimulation markedly increased the frequency and the level of IL-2 mRNA expression in individual positive cells in the Th1 and Th0 populations, with less effect on the recruitment and single cell expression level of IFN-γ. IL-4 frequencies were modestly increased by B7 costimulation of the Th2 clones, but there was no detectable increase in single cell IL-4 expression level. The observed patterns of cytokine mRNA expression favor a model of T cell activation in which all-or-none, rather than graded, responses of cytokine genes are dominant.

Beyond the selective events that determine antigen specificity, the developing T cell must differentiate into a particular effector phenotype to generate an appropriate immune response. For $CD4^+$ T cells, this is reflected in the patterns of cytokines that are produced upon antigenic rechallenge (1–3). Th1 cells produce IL-2, IFN-γ, and TNF-β (LT), whereas Th2 cells produced IL-4, IL-5, IL-10, and IL-13. A third subset, termed Th0, has an intermediate phenotype, and produces cytokines characteristic of both the Th1 and Th2 subsets (4). It is not clear whether Th0 cells are precursors of the more functionally committed Th1 and Th2 cells or whether they represent a distinct subset with a unique in vivo functional role (2).

The production of distinct sets of cytokines by these populations of $CD4^+$ T cells is associated with functionally distinct immune responses. Th1 cell cytokines are thought to be the principal regulators of cell-mediated immunity, whereas Th2 cytokines are the principal mediators of humoral immunity (2). There exists a reciprocal, crossregulatory relationship between cytokine products of these subsets such that Th1 or Th2 development tends to be mutually exclusive (2, 5–9). In addition, the costimulatory requirements for clonal expansion of the subsets are distinct. IL-1 is a potent Th2 costimulator but has relatively little effect on Th1 cells (10–12). B7 has been advocated as an important Th1 costimulator, though its effects on Th2 growth are less studied (13).

An implicit, though untested, assumption in most models of the T cell clonal response, it that individual cytokine genes are coordinately regulated in a single cell. Thus a single Th1, Th2, or Th0 cell that is triggered by antigen would be expected to activate all the genes typical of that phenotype, e.g., IL-2, IFN-γ, and TNF-β for Th1 cells; IL-4, IL-10, and IL-5 for Th2 cells; or all for Th0 cells. An implication of this model for cytokine gene regulation is that the factors that positively

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or negatively regulate the promoters of one Th cytokine gene are likely to have similar effects on other Th cytokine genes unique to that phenotype. At present, the data are insufficiently detailed on the regulation of individual cytokine gene promoters to confirm or refute this. Intuitively, this model places restrictions on the range of phenotypes that could exist, and necessitates that regulation of cytokine genes exclusive to Th1 or Th2 cells, such as IFN-γ and IL-4, must be distinct in the Th0 cell, where these genes are thought to be coexpressed.

A limitation of most studies analyzing T cell cytokine production is that the detection of cytokine protein, by either biologic or ELISA assays, measures an integrated quantity of cytokine produced by a population of cells rather than the contribution of individual T cells within the population. Likewise, cytokine mRNA detection by Northern analysis and most reverse transcription–PCR techniques suffer the same limitation of studying only population responses. Limiting dilution analyses have provided data on the frequencies of cells expressing certain cytokines, but these studies have been limited by the availability of sensitive, specific bioassays for cytokine detection (14–17). In this study, we have analyzed the production of cytokine mRNAs by individual cells in cloned T cell populations using sensitive in situ hybridization (ISH)[1] methodologies.

Clones of CD4$^+$ T cells with different cytokine expression phenotypes have been derived from a peptide-specific TCR-α/β transgenic mouse to obtain a homogeneous population for analysis (18, 19). Differences in antigenic responses of this set of clones is thus not due to differences in fine specificity or affinity of the TCR. To provide a homogeneous population of APCs for antigen-specific stimulation of the T cells, and to determine the contribution of the APC costimulatory molecule B7 to the regulation of single cell cytokine gene activation, we have constructed a pair of "artificial" APCs that are constitutively B7-1–positive or –negative (20). T cells activated by these adherent APCs can be easily distinguished for cytokine analysis by ISH. Our results indicate that there is significant heterogeneity of activation of individual cytokine genes within each of the cloned Th1, Th2, and Th0 populations. Further, our data favor a model in which T cell cytokine gene activation tends to be an all-or-none response rather than a graded response.

Materials and Methods

Animals. Mice transgenic for the DO11.10 TCR (18) were selected at age 4–6 wk by staining peripheral blood leukocytes with the anticlonotype mAb KJ1-26 (21). 5–7-wk-old female BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

mAbs and Cytokines. The mAbs KJ1-26 (anti-DO11.10 TCR; [21]), and 11B11 (anti-IL-4; [20]) were purified from hybridoma culture supernatants. Recombinant murine IL-4 and IFN-γ were purchased from Genzyme Corp. (Cambridge, MA). Recombinant murine IL-2 was kindly provided by Dr. O. Kanagawa (Washington University, St. Louis, MO).

cDNA Cloning and Plasmid Construction. A cDNA for the B7-1 coding region was generated by reverse transcription–PCR from LPS-stimulated BALB/c splenocyte total RNA as previously described (Hsieh, C.-S., J. Nasqi, H. D. Shenoi, C. A. Wenner, K. M. Murphy, and C. T. Weaver, manuscript submitted for publication). Briefly, first strand synthesis was generated using a cDNA CYCLE kit (Invitrogen, San Diego, CA) as per the manufacturer's instructions and amplified using *Taq* polymerase (Promega Corp., Madison, WI). Primer sequences were designed to include EcoRI restriction sites for subsequent insertion into pBluescript II SK+ (Stratagene, La Jolla, CA) to generate the plasmid pmB7.cod-BSSK. The B7 cDNA insert was excised from pmB7.cod-BSSK with XhoI and NotI and ligated into the XhoI/NotI-digested expression plasmid pBCMG-neo (23) to generate pmB7.cod-BCMG. Construction of I-A^d-α and I-A^d-β chain genomic coding sequences fused to the class I MHC K^b promoter fragment have been described (24).

Generation of Fibroblast Transfectants. The fibroblast lines RT11-BCMG and RT11-mB7 have been described (Hsieh, et al., manuscript submitted for publication). Briefly, an initial round of transfections was made to generate I-A^d–expressing cells for subsequent transfection with B7-1. 20 μg each of the I-A$^d\alpha$ and β chain constructs together with 5 μg of the hygromycin-resistance plasmid pMON-1118 (Monsanto Co., St. Louis, MO) were introduced into 5×10^6 RT1.1.2 cells (25) by modified calcium phosphate precipitation and resistant clones were expanded in medium supplemented with 200 μg/ml hygromycin (Sigma Chemical Co., St. Louis, MO). Screening for I-A^d expression was done by FACS® analysis (Becton Dickinson & Co., Mountain View, CA) using the mAb MK-D6 (26). Positive clones were subcloned by limiting dilution and rescreened for I-A^d expression. A single I-A^d–positive clone, RT11, was used for transfection with 25 μg of either pmB7.cod-BCMG or the control pBCMG-neo and selected for neomycin resistance (0.5 mg/ml active G418). Dual-resistant clones were expanded and screened for mB7 expression by FACS® analysis using CTLA-4 Ig (27), and B7-1–expressing lines were subcloned and rescreened. A single I-A^d– and B7-1–positive clone, RT11-mB7, and a matched clone expressing equivalent I-A^d but not B7, RT-11-BCMG, were selected for further study.

Generation of Cloned Th1, Th2, and Th0 Lines. CD4$^+$ T cells from transgene-positive mice were purified on a Cellect column (Biotex, Edmonton, Alberta, Canada) as per the manufacturer's instructions. The purified T cells were routinely ~95% CD4$^+$ and 70–80% KJ1-26$^+$ by FACS® analysis. T cells (2.5×10^5/well) were stimulated in 24-well plates with 0.3 μM OVA peptide and irradiated BALB/c splenocytes (2,500 rads, 5×10^6/well) supplemented with nothing (Th0), anti-IL-4 (Th1), or IL-4 (Th2) over three to five sequential passages as described (28). T cells were cloned at limiting dilution in the presence of 20 U/ml recombinant IL-2 and screened for IL-4, IL-2, and IFN-γ production by ELISA after stimulation with OVA peptide presented on BALB/c irradiated splenocytes. Sublines were derived by repeat limiting dilution cloning. A subclone (Th1.C) of the Th1 line not dependent on exogenous IL-2 supplementation for long-term maintenance was derived by progressive removal (three passages) of exogenous IL-2 after limiting dilution cloning. Clones were maintained by passage every 14 d on irradiated BALB/c splenocytes and 0.3 μM OVA peptide, with or without 20 U/ml exogenous IL-2.

T Cell Cultures. T cells were isolated on Ficoll gradients (Pharmacia LKB, Piscataway, NJ) and centrifuged onto a monolayer of fibroblast APCs in 48-well plates (5×10^5 T cells and 10^5 fibroblasts per well). Antigen and/or medium were added to a final

[1] *Abbreviation used in this paper:* ISH, in situ hybridization.

volume of 1 ml/well and the cultures incubated for the indicated times at 37°C. Cells were recovered in EDTA buffer (GIBCO BRL, Gaithersburg, MD).

Cytokine Assays. Quantitation of IL-2, IL-4, and IFN-γ was by capture ELISA as previously described (28). Standard curves were generated using recombinant cytokines.

ISH Analysis. A protocol for the ISH technique is described in detail elsewhere (Karr, L. J., A. Panoskaltsis-Mortori, J. Li, D. Devore-Carter, C. T. Weaver, and R. P. Bucy, manuscript submitted for publication). Briefly, the probes used were anti-sense, single-stranded RNA molecules produced by transcription from a T3 or T7 primer of a cDNA insert cloned into either pBluescript (Stratagene) or pGEM (Promega Corp.). The specific sequences of the individual probes are: IL-2, 237–837; IL-4, 1–393; IL-5, 1–1534; IL-10, 392–937; IFN-γ, 1–270 (nucleotides numbered from the start of translation). For digoxigenin-labeled probes, 1.0 μg of linearized cDNA template was incubated with 50 U of the appropriate RNA polymerase in transcription reaction mix (Tris-$MgCl_2$-spermidine, 10 mM dithiothreitol, 20 U RNasin, 1 mM each of ATP, CTP, GTP, 0.65 mM UTP, and 0.35 mM digoxigenin-labeled UTP) at 37°C for 2 h. The DNA template was removed with RNase-free DNase (1 U/mg DNA) at 37°C for 15 min and the reaction stopped with 1 ml of 0.5 M EDTA (diethylpyrocarbonate [DEPC]-treated). Each probe was titered before use. Digoxigenin-labeled riboprobes were aliquoted and stored at −80°C. For ^{35}S-labeled riboprobes, ^{35}S-labeled UTP (85 μCi, >1,000 Ci/mmol; Amersham Corp., Arlington Heights, IL) was substituted for digoxigenin-UTP in the transcription reaction. Probe dilutions were determined by optimization of signal-to-background in test slides after a 4-d exposure to emulsion.

Recovered cells were cytocentrifuged onto RNase-free glass slides, air dried, fixed in 3% paraformaldehyde, and then washed with 2× SSC. Slides were rinsed with 0.1 M triethanolamine-HCl, pH 8.0, acetylated in 0.1 M triethanolamine, pH 8.0, 0.25% acetic anhydride, for 15 min at room temperature, and rinsed again with the triethanolamine buffer followed by washing in 2× SSC. The slides were prehybridized for 1 h at room temperature (50% formamide, 4× SSC, Denhardt's solution, heat-denatured herring sperm DNA at 500 μg/ml, yeast tRNA at 250 μg/ml and 10% dextran sulfate). After rinsing with 2× SSC, 15 μl of hybridization solution containing heat-denatured RNA probe was applied to each section and the slides were coverslipped and incubated overnight at 50°C in humid chambers. The coverslips were removed, the sections washed in 2× SSC at room temperature, rinsed with sucros-Tris-EDTA (STE) buffer, and incubated with 200 μl RNase A (20 mg/ml in STE) for 30 min at 37°C to remove nonhybridized probe. Sections were then washed sequentially with: 2× SSC with 50% formamide at 50°C; 1× SSC at room temperature; 0.5× SSC at room temperature; and Tris-NaCl, pH 7.5.

Slides hybridized with digoxigenin-labeled probes were blocked with 2% normal horse serum and incubated with antidigoxigenin antibody (alkaline-phosphatase conjugate diluted 1:500 in Tris-NaCl, pH 7.5, 1% herring sperm DNA; Boehringer Mannheim Corp., Indianapolis, IN) in humid chambers at room temperature for 1 h. Unbound antibody was washed away and 500 μl enzyme substrate solution (nitro blue tetrazolium containing levamisole) was applied and incubated overnight in the dark in humid chambers at 4°C. The color reaction was stopped by placing slides in Tris-EDTA, pH 8.0, and coverslips were mounted on aqueous mounting medium. Slides hybridized with ^{35}S-labeled probes were immersed in 70% ethanol and allowed to dry at room temperature for 1 h. After drying, the slides were dipped in autoradiographic emulsion (LM-1; Amersham Corp.) diluted 1:1 in 600 mM ammonium acetate, dried again, and then placed into a light-tight slide box with desiccant. After 4 d of incubation at 4°C, the slides were developed and coverslipped in permount (Fisher Scientific Co., Pittsburgh, PA).

Cells detected with digoxigenin-labeled riboprobes were quantitated with bright-field microscopy by direct counting. All cells in a calibrated ocular grid from sequential fields were counted; fibroblasts were easily excluded on the basis of size and morphology. A minimum of 1,000 cells were counted per condition. Quantitation of grain densities for cells detected with ^{35}S-labeled riboprobes was also by direct counting and bright-field microscopy.

Results

Heterogeneity of Cytokine Gene Expression in T Cell Clones. To study the frequency of T cells expressing individual cytokine mRNAs, we have developed a modified ISH technique that permits detection of low copy number mRNA species in either frozen sections or cytospin preparations of cells manipulated in vitro or in vivo. We have analyzed by ISH, the cytokine mRNA profiles of several cloned Th1, Th2, and Th0 lines that were derived from the DO10 transgenic mouse. T cells were activated by OVA peptide presented on fibroblasts cotransfected with $I\text{-}A^d$ and murine B7-1 (RT11-mB7), and after 4 h, T cells and fibroblasts were recovered. Equivalent numbers of cells were cytocentrifuged onto RNAse-free slides. IL-2, IFN-γ, IL-4, IL-10, and IL-5 mRNA-positive T cells were detected by ISH analysis (Fig. 1 and Table 1).

This analysis revealed significant heterogeneity in patterns of cytokine expression, both between individual cytokine genes within a single cloned population and between different sublines. For example, in the Th1 clones maintained in the absence of exogenous IL-2 (Th1.1 and Th1.2), the frequency of IFN-γ–expressing cells (48–86%) was substantially greater than the IL-2–expressing cells (1.3–2.8%). In a separate Th1 clone maintained without IL-2 supplementation (Th1.C), the frequency of cells positive for IL-2 was ~10-fold greater (24%), but remained a fraction of the total cells that were positive for IFN-γ mRNA expression (68%). Similarly, the frequency of IL-4–expressing Th2 cells was greater than those expressing either IL-5 or IL-10, but varied between clones. The absolute frequencies of cytokine gene expression within these populations has varied between experiments over several months, although the patterns of cytokine expression have been consistent (data below and data not shown). These data indicate that individual T cells can be activated to express one cytokine, but fail to express another characteristic of that population of cloned cells.

Kinetic Analysis of Cytokine mRNA Expression and Protein Production. To exclude the possibility that the heterogeneity of cytokine mRNA expression detected by ISH was due to variable kinetics, we quantitated single cell expression of each of the cytokine mRNAs over a time course (Fig. 2). Replicate slides were prepared from a single culture at each time point and analyzed for the indicated cytokines. For both Th1 and Th2 cells, expression of each of the cytokine mRNAs was detected as early as 2 h after antigenic stimulation. Peak expression of each of the Th1 cytokine mRNAs was seen at 4 h, and steadily decayed thereafter. Th2 cytokine mRNAs

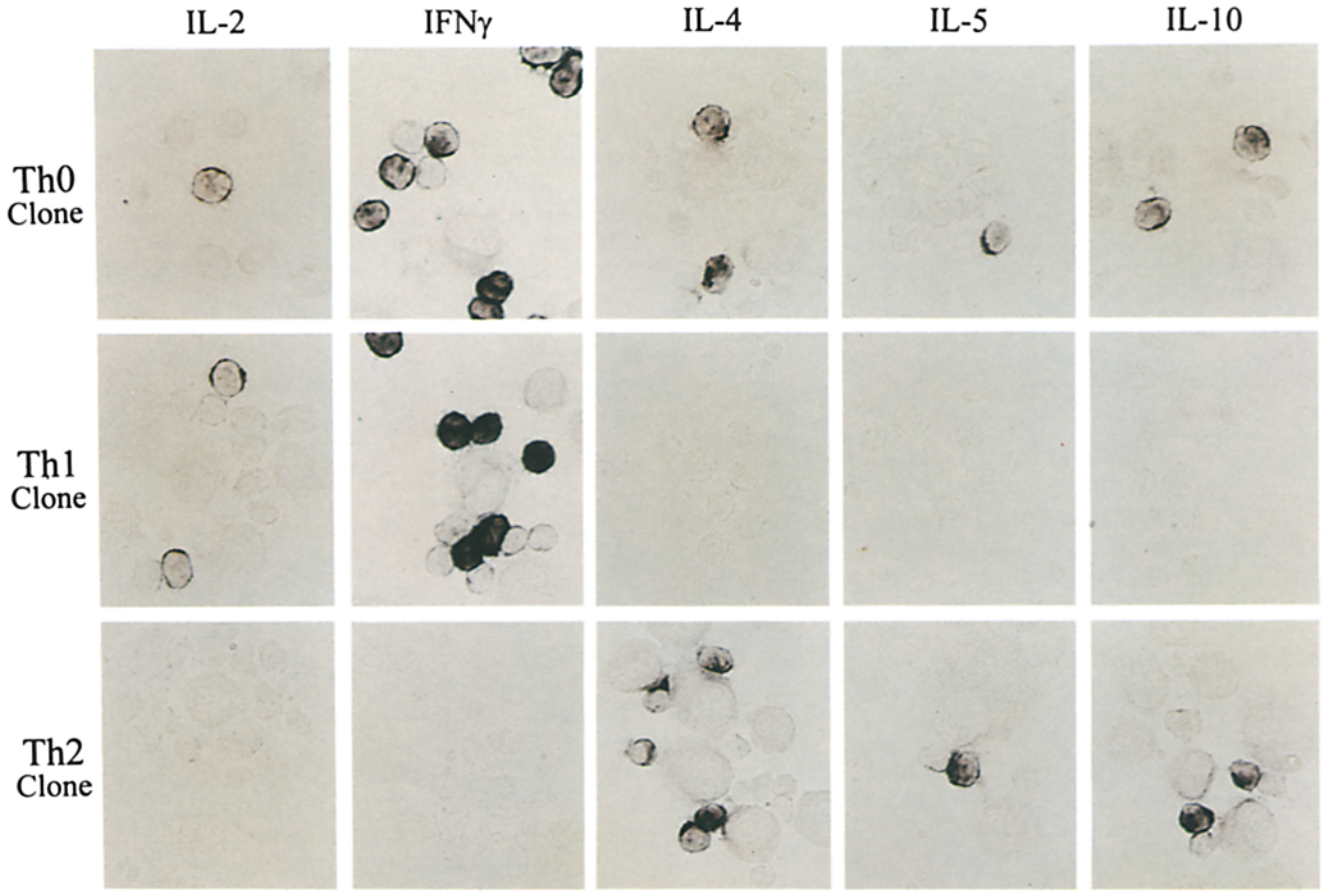

Figure 1. ISH analysis of cytokine mRNA expression in antigen-activated transgenic T cell clones. Th0, Th1.1, and Th2.1 cells were stimulated for 4 h with 3.0 μM OVA peptide presented on RT11-mB7 cells and analyzed by ISH using the indicated digoxigenin-labeled riboprobes. Positive T cells show a characteristic cytoplasmic staining pattern. The large, unstained cells with abundant cytoplasm are RT11-mB7 fibroblast APCs.

showed a more complicated expression pattern, with a small, but reproducible (three experiments) second peak of expression after decay of an initial 4-h peak. At the later time points, the relative frequencies of IL-4 and IL-10 reversed, such that more IL-10–positive cells were present at 24 h. For both Th1 and Th2 clones, discordant expression of the individual cytokine mRNAs could not be attributed to kinetic differences.

At each of the time points in the kinetic analysis, culture supernatants were harvested and the cytokine protein measured by sandwich ELISA assays (Fig. 2 and data below). For each of the cytokines measured, there was good correlation between the percentage of mRNA-positive cells and levels of cytokine protein, although the detection of protein lagged mRNA expression. For both IL-4 and IFN-γ, protein accumulated for 24–48 h, then plateaued and slowly decreased, whereas IL-2 peaked and decayed rapidly. This probably reflects rapid consumption of IL-2 by the activated T cells. In multiple experiments, we have had no case in which cytokine protein has been detected without cells expressing cytokine mRNA being detected by ISH. Instead, very low frequencies of cytokine-positive cells detected by ISH have been undetected by ELISA, indicating that the ISH technique is more sensitive.

Recruitment of Cells into Activation vs. Regulation of Level mRNA Expression. Shown in Figs. 3 and 4 is a representative experiment in which the frequency of mRNA-expressing cells was examined over a range of peptide antigen doses. For these experiments, the cells for ISH analysis were harvested 4 h after activation; the supernatants from replicate cultures were collected at 24 h for ELISA, in accordance with the respective optima determined by kinetic analyses (Fig. 2). The results demonstrate a significant increase in the frequency of cytokine mRNA-expressing cells with increased antigen dose. In contrast, there appeared to be little or no differences in the intensity of staining of individual cells with different antigen doses (Fig. 3 and see below). This same result has been found for all five cytokines examined in multiple experiments. As in Fig. 2, there is good correlation between number of mRNA-positive cells and the quantity of cytokine protein detected by ELISA (Fig. 4). Although the staining intensity of individual cells at the peak of the response was not a function of antigen dose, ISH analysis of the same cytokine at

Table 1. *Frequency of Cytokine mRNA-positive T Cells Detected by ISH*

	Percent positive cells						
	Experiment 1			Experiment 2			
Cytokine	Th1.1	Th2.1	Th0	Th1.2	Th1.C	Th2.2	Th0
IFN-γ	48	0	58	86	68	ND*	48
IL-2	1.3	0.10	4.4	2.8	24	ND	1.3
IL-4	0	24	4.7	0	ND	82	34
IL-5	0	1.1	0.17	0	ND	64	0.25
IL-10	0	14	4.5	0	ND	61	21

* ND, not determined.

5 × 10^5 Th0, Th1, or Th2 clone cells were cultured with 10^5 RT11-mB7 fibroblasts and 3 μM OVA peptide. 4 h after initiation of the cultures, T cells and fibroblasts were recovered and immediately cytocentrifuged onto glass slides for ISH processing as described in Materials and Methods. Data are expressed as the percentage of total T cells positive for the indicated cytokine mRNAs. The lines Th1.1, Th1.2, Th2.1, and Th2.2 were derived from initial limiting dilution clonings by repeat limiting dilution clonings and are maintained by passage with exogenous IL-2. The line Th1.C is maintained in the absence of exogenous IL-2 (see Materials and Methods).

different time points after activation did demonstrate significant differences in staining intensity (not shown), indicating that the absence of significant variability of staining intensity at different antigen concentrations was not due to a limited range of detection of the ISH method.

To confirm this result, we sought to better quantitate the cytokine mRNA expression by individual antigen-activated cells within each population. Because the intensity of staining of individual cells detected by ISH analysis is difficult to quantitate using the digoxigenin-labeled riboprobes, we analyzed Th1 and Th2 cytokine mRNA expression using riboprobes labeled with ^{35}S-labeled UTP and detected by photographic emulsion rather than enzyme-conjugated antidigoxigenin antibody. mRNA expression by individual cells could then be quantitated by grain counts. Importantly, comparison of the frequencies of positive cells in replicate cytospins determined with either digoxigenin- or ^{35}S-labeled riboprobes showed that the two detection methods were quite similar in sensitivity (Fig. 5 and Table 2). Shown in Fig. 6 are the distributions of grains per cell detected with ^{35}S-labeled riboprobes in clones stimulated by the indicated antigen doses on B7-positive APCs. As predicted from the experiments using the digoxigenin-labeled riboprobes (Figs. 3 and 4), the distribution and mean (number in italics) of grain densities, and thus mRNA copies per cell, varied to a much lesser extent than the number of cells in the population recruited to cytokine mRNA expression with increased antigen dose (bold numbers expressed as percentages). At the highest antigen dose, the Th1 cells showed a reproducible, modest increase in IFN-γ grain density. A similar shift in IL-4 grain densities was not seen in the Th2 cells. Similar results were seen in the Th0 population, where IL-4 and IFN-γ retained their distinct expression intensity differences, despite being coexpressed in the same population (not shown).

Notably, the distribution of grain densities at each antigen dose demonstrates that the distinction between a negative (<10 grains/cell) and positive cell is easily determined, suggesting that activation of individual cytokine genes in single cells in the population is largely an all-or-none phenomenon. If graded cytokine responses to antigen were occurring at

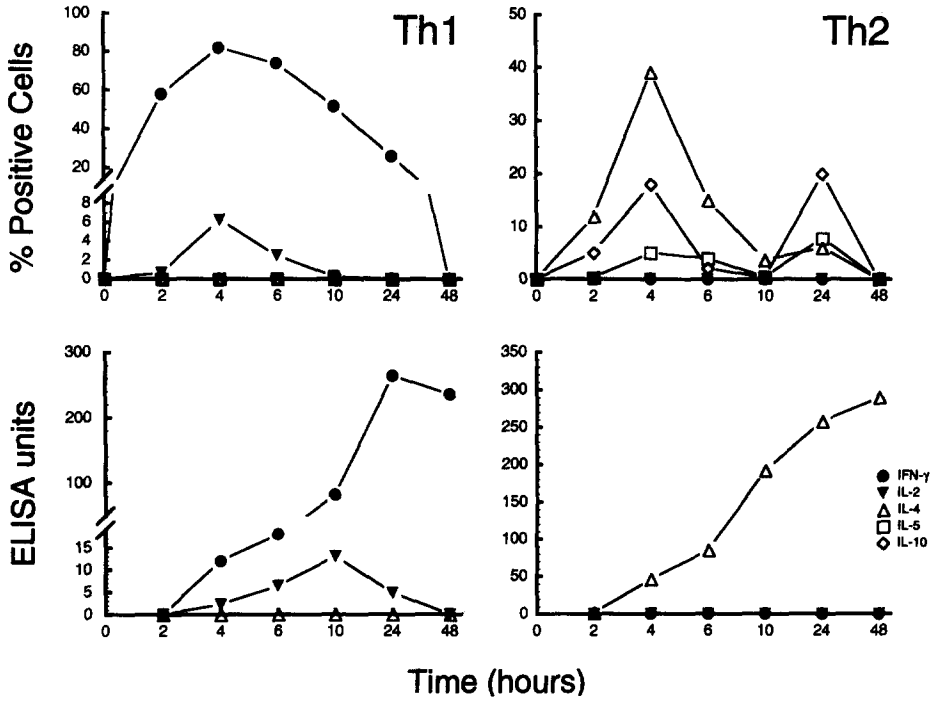

Figure 2. Kinetics of cytokine single-cell mRNA and protein expression. Th1.1 and Th2.1 cells were activated by 3.0 μM OVA peptide presented on B7-positive RT11 cells RT11-mB7. The frequencies of cells positive for IL-2, IFN-γ, IL-4, IL-5, or IL-10 mRNA were determined by ISH analysis using digoxigenin-labeled riboprobes (*top*) and supernatants were assayed by ELISA for IL-2, IFN-γ, and IL-4 cytokine protein content (*bottom*) at the indicated times after culture initiation.

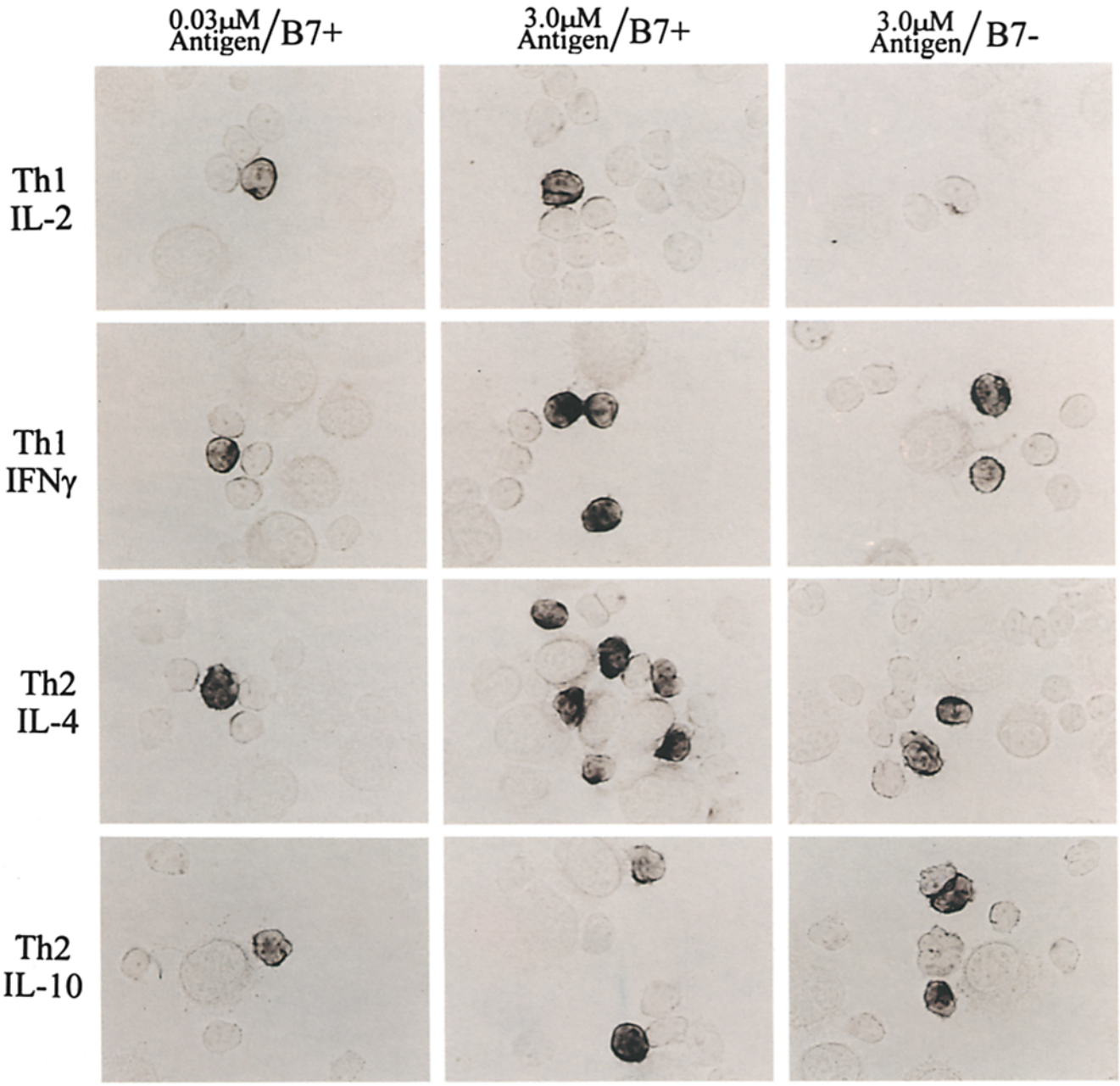

Figure 3. Analysis of antigen dose and B7 costimulation effects on cytokine production by ISH. Th1.1 and Th2.1 clones were activated for 4 or 24 h with the indicated concentrations of OVA peptide presented on B7-positive (RT11-mB7) or B7-negative (RT11-BCMG) fibroblast APCs. Cells were recovered and analyzed for expression of the indicated cytokine mRNAs by ISH using digoxigenin-labeled riboprobes.

the single cell level, one would not expect to see such a clear break between the positive and negative cell populations. Thus, there appears to be a threshold for cytokine gene activation, above which a relatively stereotypical response is initiated. At lower antigen dose, fewer cells are stimulated to respond, but those that do produce similar amounts of cytokine mRNA as individual cells activated at higher antigen dose.

Effects of B7 Costimulation on Cytokine Gene Expression. The effects of B7 costimulation on cytokine mRNA levels in individual cells were determined by stimulating T cell clones with antigen presented on B7-positive and -negative fibroblast transfectants. Single cell cytokine mRNA expression was determined using either digoxigenin-labeled probes (Fig. 3) or ^{35}S-labeled probes (Fig. 7) in independent experiments. At optimal antigen dose, B7 costimulation markedly increased the recruitment of Th1.2 IL-2–positive cells (10-fold, Fig. 7), while having more modest effects on the recruitment of IFN-γ–positive cells in the same population (~1.2-fold). Similar effects were seen with the Th1.C clone, although the IL-2 frequencies were greater. Like IFN-γ in Th1 cells, in-

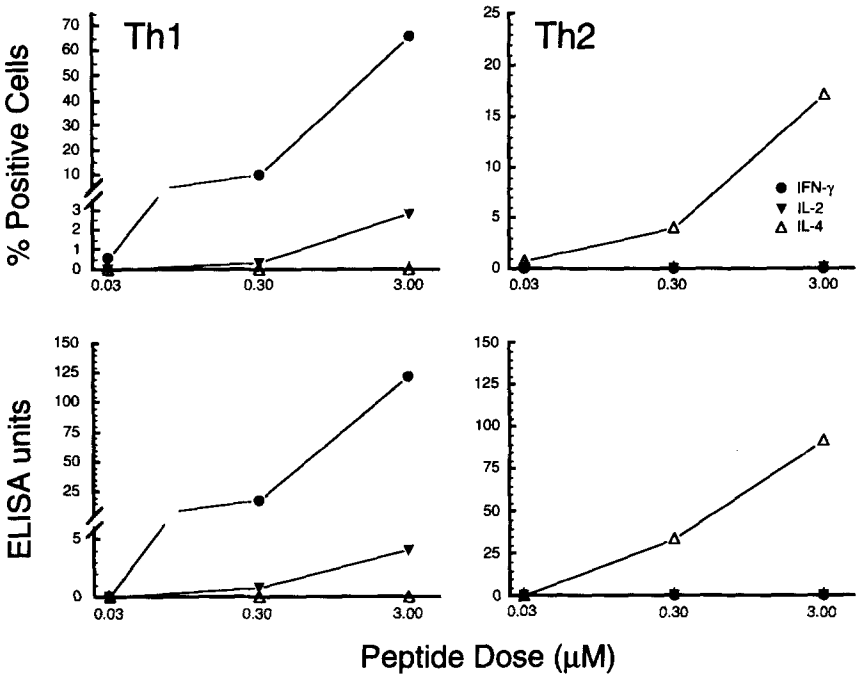

Figure 4. Comparison of ISH and ELISA analysis of Th1 and Th2 clones activated by different doses of antigen. Th1.1 and Th2.1 cells were activated by the indicated concentrations of OVA peptide presented on B7-positive (RT11-mB7) fibroblast APCs. ISH for single-cell mRNA for IL-2, IL-4, IL-5, IL-10, and IFN-γ (*top*) was determined at 4 h using digoxigenin-labeled riboprobes and supernatants were assayed by ELISA for IL-2, IL-4, and IFN-γ cytokine protein content (*bottom*) at 24 h after culture initiation. ELISA values are the mean of duplicate determinations.

creased recruitment of IL-4–positive cells in Th2 cells was significant (~1.6-fold), but much lower than that of IL-2 in Th1 cells. In the Th0 clone, where the frequencies of IFN-γ and IL-4 were lower (26.5 and 14.7%, respectively, with B7 costimulation), B7 effects on recruitment were only slightly greater (1.6-fold or IL-4 and 1.8-fold for IFN-γ), while the effects on IL-2 recruitment were still significantly higher (5.5-fold increase; 13.7 vs. 2.5%). Similarly, at limiting antigen dose, the effects of B7 costimulation on IL-4 and IFN-γ were modest compared to those of IL-2 (data not shown).

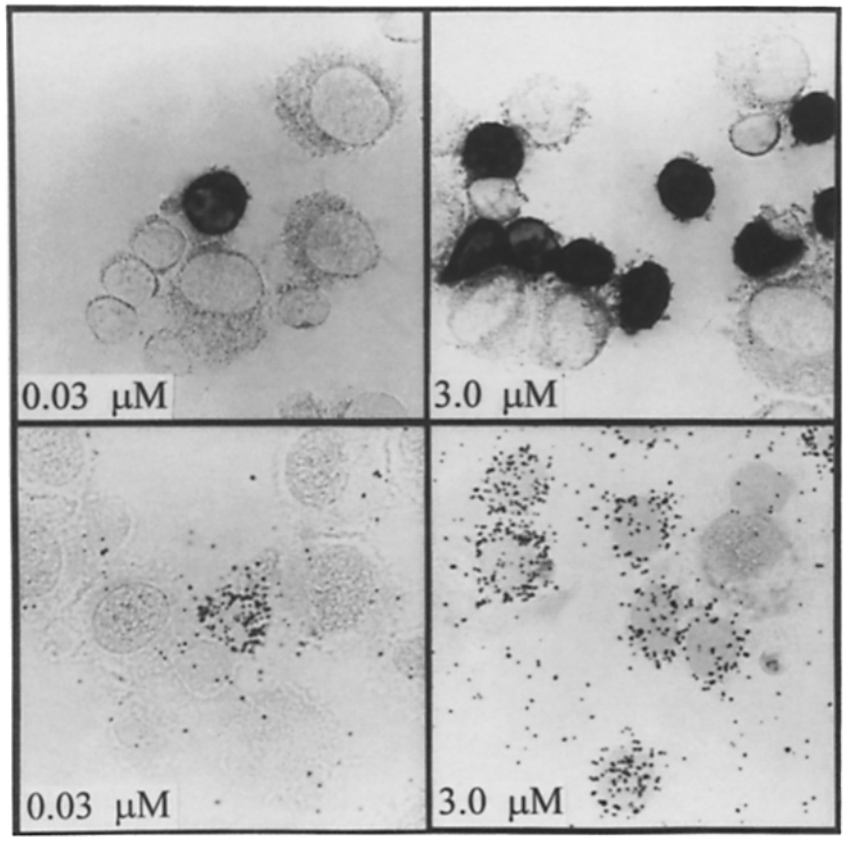

Figure 5. Comparison of staining properties of ^{35}S- and digoxigenin-labeled riboprobe methods. Th1.2 clone cells were cultured with RT11-mB7 fibroblasts and the indicated doses of OVA peptide. 4 h after initiation of the cultures, T cells and fibroblasts were recovered and replicate aliquots were cytocentrifuged onto glass slides for ISH processing. The two sets of slides were developed using a digoxigenin- (*top*) or ^{35}S-labeled (*bottom*) IFN-γ riboprobe, respectively. The ^{35}S-labeled slides were developed for 72 h. Note the similar intensity of probe detection at both antigen doses using both types of probes and the better resolution of staining with the digoxigenin-labeled riboprobes. The Th1.2 cells are the smaller, rounder cells with limited cytoplasm.

Table 2. *Comparison of Sensitivities of ^{35}S- and Digoxigenin-labeled Riboprobes*

Clone	Cytokine	Probe	Percent positive cells		
			0.03 μM OVA	0.3 μM OVA	3.0 μM OVA
Th1.2	IFN-γ	^{35}S	2.7	19	90
		Digoxigenin	4.6	21	95
Th2.2	IL-4	^{35}S	0.74	24	84
		Digoxigenin	1.2	26	86

5 × 10^5 Th1.2 or Th2.2 clone cells were cultured with 10^5 RT11-mB7 fibroblasts and the indicated doses of OVA peptide. 4 h after initiation of the cultures, T cells and fibroblasts were recovered and replicate aliquots immediately cytospun onto glass slides for ISH processing. One set of slides was developed using the ^{35}S-labeled IL-4 or IFN-γ riboprobe, and the replicate set was developed using digoxigenin-labeled riboprobes. The ^{35}S-labeled slides were processed as described in Materials and Methods and developed for 72 h.

The effect of B7 costimulation on the intensity of single cell mRNA expression was also distinct for the individual cytokines. The mean mRNA levels in single cells were slightly increased by B7 costimulation in the case of IFN-γ, and not at all for IL-4 (Figs. 3 and 7). This was in contrast to the effects on single-cell IL-2 mRNA levels, which were significantly increased by B7 costimulation (1.7–2.4-fold increase in three separate experiments). Although the dominant effect on IL-2 expression was increased recruitment of mRNA-positive cells, the intensity of single cell IL-2 mRNA expression was more sensitive to B7 costimulation than that of either IFN-γ or IL-4.

Discussion

In this study, we have examined cytokine gene expression in cloned $CD4^+$ T cell populations by single cell mRNA analysis. Our data indicate that there exists significant heterogeneity of mRNA expression of different cytokine genes within the clonal T cell populations studied. Although there

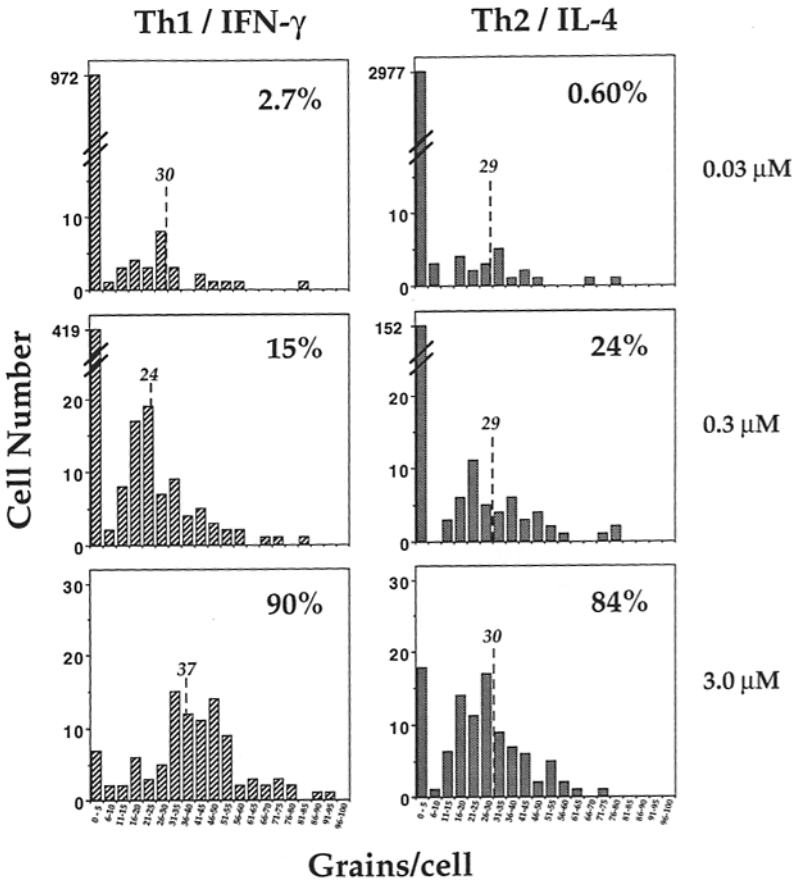

Figure 6. Cytokine mRNA levels in Th1 and Th2 cells quantitated by ^{35}S-ISH. Th1.2 and Th2.2 clone cells were cultured with 10^5 RT11-mB7 fibroblasts and the indicated doses of OVA peptide for 4 h, then analyzed for IFN-γ or IL-4 mRNA expression, respectively, by ISH using ^{35}S-labeled riboprobes. Grain counts per cell were determined by bright-field microscopy. The percentage of positive cells for each determination are shown in the *top right corner*. The mean density of grains for the analyzed populations are denoted in italics (*above the dashed line*).

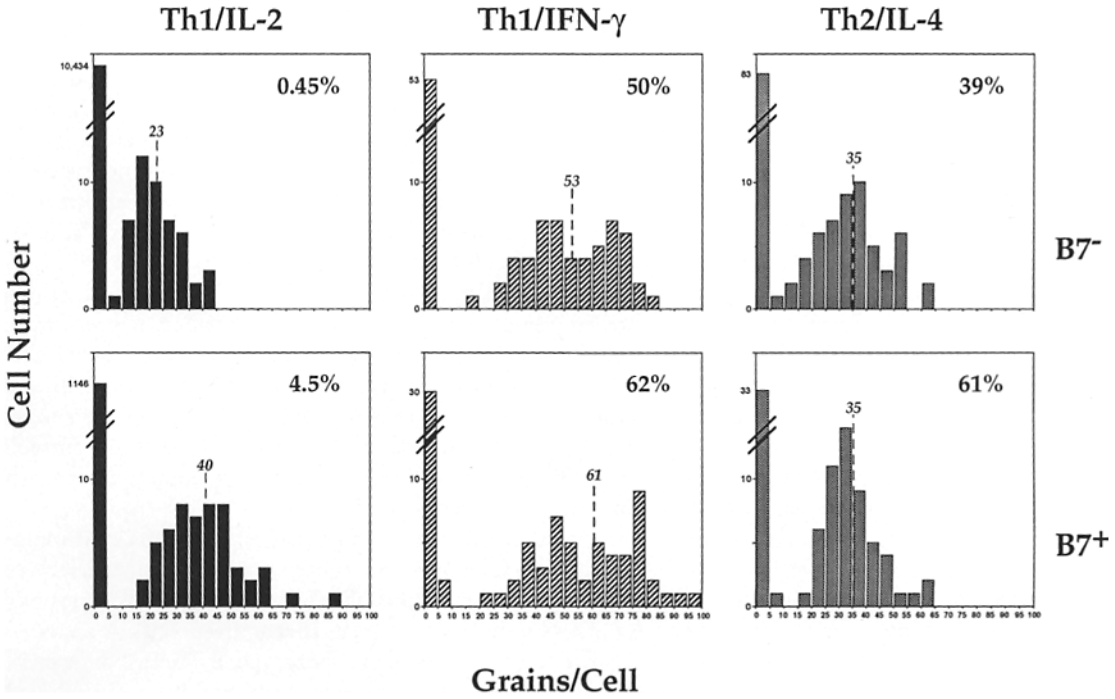

Figure 7. Cytokine mRNA levels in Th1 and Th2 cells activated by antigen presented on B7-negative or B7-positive APCs quantitated by ^{35}S-ISH. Th1.2 and Th2.2 clone cells were cultured with RT11-BCMG or RT11-mB7 fibroblasts with 3.0 μM OVA peptide for 4 h, then analyzed for IL-2, IFN-γ, or IL-4 mRNA expression respectively by ^{35}S-ISH. Grain counts per cell were determined by bright-field microscopy. The percentage of positive cells for each determination are shown in the *top right corner*. The mean density of grains for the analyzed populations are denoted in italics (*above the dashed line*).

is likely to be stochastic heterogeneity even within a clonal population, the relative abundance of cytokine products generated by an activated Th1 or Th2 clonal population has heretofore been thought to reflect the relative activation of each cytokine gene within individual cells in the population. These data suggest that this is not the case. While certain cytokine genes are stably expressed in a clonal population, the activation of individual genes characteristic of the population is not necessarily coordinate within a given T cell, reinforcing the notion that the transcriptional activation and/or mRNA stability of each of the cytokine genes are unique and thus susceptible to independent, if overlapping, control.

An important consideration in the interpretation of these data is the sensitivity of each of the probes used to detect cytokine mRNAs in individual cells. Although a possible explanation for the observed heterogeneity is differential probe sensitivities, several lines of evidence are against this. First, the clarity and intensity of staining for each cytokine mRNA was well above the level of background staining. The least intensely stained cells counted as positive using digoxigenin-labeled riboprobes were distinctly brighter than the cells counted as negative, and the fibroblast cells served as an internal control for negative staining. Second, the grain distributions of cells detected with ^{35}S-labeled probes showed a bimodal distribution for each of the cytokines, with clearly negative and positive populations. In cloned populations with substantially different frequencies of positive cells, the intensity of both positive and background staining was equivalent. Finally, there were no significant differences in directly measured sensitivities of each of the cytokine probes using titered quantities of the cytokine cDNA bound to nitrocellulose filters as targets, arguing that the observed intensity of hybridization was dependent on copy number of the relevant target mRNA and not differential affinities of the probes (Bucy, R. P., unpublished data).

The greatest segregation of cytokine responses within populations in this study was that between Th1 cell IFN-γ and IL-2 expression in clones maintained with exogenous IL-2. In a population in which as many as 90% of T cells were stimulated to activate IFN-γ gene transcription, the frequency of cells that expressed IL-2 mRNA was an order of magnitude or more lower. Thus, under conditions where virtually all of the Th1 cells were sufficiently stimulated to activate IFN-γ transcription, many cells failed to initiate detectable IL-2 transcription. In a Th1 clone maintained without exogenous IL-2, the frequency of IL-2 mRNA-positive cells was increased, but the frequencies of IFN-γ and IL-2 expression were still distinct, with a dominance of IFN-γ. This was not a general property of the expression frequencies of the IL-2 and IFN-γ genes, because in naive transgenic T cells activated by OVA peptide presented by RT11-mB7 fibroblasts or splenic adherent cells, the frequency of IL-2–positive cells was severalfold higher than that of IFN-γ (Bucy, R. P., L. Karr, G.-Q. Huang, J. Li, D. Devore-Carter, K. M. Murphy, and C. T. Weaver, manuscript submitted for publication). In limiting dilution analyses of mitogen or anti-CD3–stimu-

lated primary T cells from other studies, similar frequencies of IL-2–positive cells have been reported (14, 17, 29). Also, in T cells stimulated with PMA and ionomycin instead of antigen, the frequency of IL-2–positive cells exceeded 80%, similar to the expression frequency of IFN-γ, suggesting that the ISH analysis is not failing to detect IL-2–positive cells (our unpublished data).

Like the Th1 clones, Th2 clones that expressed IL-4, IL-5, and IL-10 as a population, had variable frequencies of individual cells that expressed each of these cytokines. Low expression of IL-5 mRNA in one Th2 clone was in contrast to much higher IL-5 expression frequencies in an independent clone, despite similar frequencies of IL-4 expression by both. Also, the relative frequencies of Th2 cells that expressed IL-10 and IL4 was reversed over a time course, with IL-4 tending to dominate early, and IL-10 tending to dominate late. Heterogeneity of cytokine expression frequencies was particularly evident in the Th0 clone, where there was a wide range for the five cytokine genes examined. Similar results were reported by Kelso and Owens (15), who showed that frequencies of IL-3 and GM-CSF measured by limiting dilution bioassays were distinct in a single T cell clone activated by anti-TCR stimulation. Although the mechanism(s) responsible for these divergent expression patterns is unknown, these data suggest that each of the cytokine genes is regulated by sufficiently distinct intracellular signals to allow segregation in individual cells.

The effect of increased antigenic stimulation on enhanced cytokine gene expression was primarily at the level of recruitment, not enhanced single cell cytokine production. The frequency of cytokine mRNA-positive cells in a population of T cells increased with increasing antigenic stimulation, but the single cell production of cytokine mRNA was relatively invariant. Although the intensity of mRNA expression by individual cells within the positive population varied ~10-fold (Figs. 6 and 7), this dynamic range was similar regardless of the antigen dose. This suggests that single cell cytokine gene activation stimulated by T cell receptor engagement is largely an all-or-none phenomenon, rather than a graded response. Once a certain threshold has been reached, a cytokine gene response is initiated that is not significantly enhanced by additional TCR engagement. This is in agreement with a previous report by Fiering et al. (30), wherein a reporter gene (β-galactosidase) driven by a repetitive NFAT enhancer element from the IL-2 promoter showed a bimodal, threshold response to TCR-signaling pathway stimulation. In their study, heterogeneity due to heritable bias in reporter gene responsiveness in the clonal population was excluded and the threshold effect was at the transcriptional rather than translational level. Further, an effect of cell cycle position on transcriptional responsiveness was seen, although this was not absolute. Our study now extends these results to additional cytokine genes driven by their native promoters in Th1, Th2, or Th0 cells.

The effects of B7 costimulation on cytokine gene responses introduced a further level of heterogeneity of cytokine gene responses. The IL-2 gene showed a particularly strict requirement for B7 costimulation, as previously reported (31, 32). In the absence of APC B7 expression, IL-2 gene transcription was virtually undetectable in the Th1 clones. In addition to a large percentage increase in the frequency of cells expressing IL-2 mRNA, B7 costimulation also increased the level of mRNA detected in positive cells, in agreement with its published effects on enhanced IL-2 transcription and decreased IL-2 mRNA degradation (33, 34). This was in contrast to the effects of increased antigenic stimulation, which had minimal effects on the levels of mRNA expression in individual cells. Within the same clonal population of Th1 cells, the frequency of IFN-γ mRNA-expressing cells and single cell levels of IFN-γ mRNA expression were only modestly increased by B7 costimulation, even at low antigen doses. Similarly modest effects of B7 costimulation were seen for the Th2 cytokine IL-4.

In sum, these results argue that although B7 costimulation influenced expression of each of the cytokines irrespective of their association with a Th1, Th2, or Th0 phenotype, its effects could not be generalized. Even within a cloned population of stable cytokine phenotype, B7 exerted differential effects on the individual cytokine genes and there was no clear correlation of B7 costimulatory effects on cytokine gene regulation with T cell phenotype. This further extends the heterogeneity of control of responses of individual cytokine genes within the different cloned T cell populations.

Since the original description of $CD4^+$ T cell subsets defined by unique ensembles of cytokine production (1), the link between T cell cytokine profiles and control of distinct functional responses has both strengthened and broadened. The effector function of differentiated T cell populations is now thought to be virtually synonymous with the mix of cytokines produced subsequent to antigenic stimulation. Because different types of immune response require coordinate expression of certain cytokines for greatest efficiency, the association of cytokine sets with distinct differentiated populations makes biological sense. By extension, it has been assumed that each cell in a clonal T cell population would, when activated by antigen, initiate transcription of each of the cytokine genes characteristic of that population. The data in this study indicate the need for modification of this model, and suggest that while there is clearly a tendency for coordinate regulation of Th1 and Th2 cytokines within clonal populations, this may not be extrapolated to distinct cytokine genes within individual cells in the population. Instead there appears to be more autonomy of regulation of individual cytokine genes than previously implied in most Th1/Th2 models. From this we infer a greater heterogeneity and plasticity in the $CD4^+$ functional repertoire than is encompassed by the current Th1/Th2 paradigm (2, 35, 36). Since we have examined only the effects of antigen dose and B7 costimulation in this study, it is likely that the heterogeneity of individual cytokine gene responses will become even more diverse as additional T cell signaling pathways are examined.

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