TWO TYPES OF MOUSE HELPER T CELL CLONE

III. Further Differences in Lymphokine Synthesis between Th1 and Th2 Clones Revealed by RNA Hybridization, Functionally Monospecific Bioassays, and Monoclonal Antibodies

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The diversity of lymphocytes was first identified in 1968 (1), with the realization that B cells, responsible for antibody production, could be separated from T cells, which are responsible for delayed-type hypersensitivity (DTH), cytotoxicity, and regulation of immune functions. Subset-specific surface antigens subsequently allowed the division of T cells into two classes: T cells bearing Lyt-1 (2) and L3T4 (3) antigens are responsible for helper and DTH functions, whereas cytotoxicity and suppression are predominantly mediated by T cells bearing the Lyt-2 antigen. Several studies have since indicated that the L3T4+(CD4+) helper/DTH T cell population could be further subdivided into at least two subtypes (4-11), based on functional criteria. Recently, we and others have shown that CD4+ T cell clones grown in vitro can be divided into at least two groups, based on the type of help provided to B cells (12) or the pattern of lymphokines secreted after antigen or Con A stimulation (13). The lymphokines secreted only by the first type of clone, T helper 1 (Th1) were IL-2 and IFN-γ, whereas only Th2 clones secreted IL-4 (13). Recently, we have also shown that only the Th1 clones, producing IL-2 and IFN-γ, are capable of producing an antigen-specific swelling reaction with the characteristics of DTH (14). A similar division may exist in the rat, since the CD4+,OX22+ population is responsible for most IL-2 production, whereas the CD4+,OX22− population is responsible for the majority of B cell help (15).

The bioassays used to detect most, if not all lymphokines, are now known to be very complex. Assay cell lines typically respond to more than one lymphokine, and there are often positive or negative interactions between the activities of different lymphokines. In addition, many lymphokines have effects on more than one cell type. These complexities mean that rigorously monospecific bioassays

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Abbreviations used in this paper: DTH, delayed-type hypersensitivity; GM-CSF, granulocyte/macrophage colony-stimulating factor; LT, lymphotoxin; MTT, 3-(4,5-dimethylazol-2-yl)-2,5-diphenyltetrazolium bromide; PBST, PBS containing Tween; ppENK, preproenkephalin; Th1, T helper cell type 1; Th2, T helper cell type 2.

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for lymphokines are difficult to establish, and for several T cell products we do not yet have sufficient information to produce monospecific functional assays.

To further analyze the production of various induced proteins of Th1 and Th2 clones, we have turned to the analysis of mRNA by cytoplasmic dot hybridization (16) as an alternative method to evaluate differences between the T cell clones. We have also established monospecific assays for four lymphokines in T cell supernatants, using mAbs in bioassays to increase specificity, and a new monoclonal anti-IFN-γ antibody in an ELISA. Within a panel of 19 clones, our results indicate that the differences between Th1 and Th2 clones are even more extensive than originally realized, and also show that for the four lymphokines for which both RNA and protein or activity data were compared, a good correlation exists between the two evaluation methods.

Materials and Methods

Cell Lines. The HT2 mouse T cell line (17) was obtained from S. Strober (Stanford University, Stanford, CA), the MC/9 mouse mast cell line (18) and D9 T cell line (19) from G. Nabel and H. Cantor (Harvard University, Boston, MA), the D10.G4.1 T cell clone (20) from C. Janeway (Yale University, New Haven, CT), and the CDC-25 and CDC-35 mouse T cell clones (21) from D. Parker (University of Massachusetts, Worcester, MA). The production and maintenance of our antigen-specific mouse T cell clones have been described elsewhere (22). The P3X63Ag myeloma cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD).

Antibodies. The S4B6 anti-IL-2 hybridoma has been described previously (13). Purified 11B11 anti-IL-4 mAb (23) was a kind gift of R. Coffman, DNAX. mAbs recognizing IFN-γ were derived from a Lewis rat after immunizing on eight sequential d with soluble recombinant mouse IFN-γ (170 µg/injection). This rat was obtained from M. Kehry (DNAX), and later boosted with IFN-γ (100 µg) in CFA (Gibco Laboratories, Grand Island, NY). After 21 d, the rat was boosted with 100 µg IFN-γ without adjuvant. 3 d later, the spleen cells were fused with P3X63Ag myeloma cells using 50% polyethylene glycol (Sigma Chemical Co., St. Louis, MO) (24). Antibodies directed against IFN-γ were identified by ELISA testing of supernatants. Nine of these were able to block the biological activity of IFN-γ in two different bioassays: the effect of IFN-γ on proliferation of the NFS60 cell line (13) and the inhibition by IFN-γ of the enhancement of IgE synthesis by IL-4 (25). XMG1.2 is a rat IgG1 mAb that is effective in both ELISA and blocking assays. Affinity-purified rabbit anti-IFN-γ polyclonal antibodies were obtained by passing serum from rabbits immunized with recombinant mouse IFN-γ over a solid-phase immunoadsorbent column containing IFN-γ and eluting the anti-IFN-γ-specific antibodies.

Labeled DNA Probes. The coordinates of the DNA probes used for hybridization were as follows: IL-2, nucleotides 498–570 (26); IL-3, 469–528 (27); IL-4, 445–498 (28); IL-5, 404–461 (29); granulocyte/macrophage colony-stimulating factor (GM-CSF), 549–639 (30); IFN-γ, 900–989 (31); lymphotoxin (LT), 517–618 (32); TNF, 765–865 (33); preproenkephalin (ppENK), 642–730 (34); TY5, 630–732 (Zurawski, G., personal communication); P600, 91–691 (Brown, K. D., unpublished observations).

With the exception of IL-5 and P600, radioactive probes were prepared by fill-in polymerization essentially following the procedure of Drouin (35). To label a given probe, 0.4 µg each of a pair of synthetic oligonucleotides were mixed. These oligonucleotides (ranging in length from 30 to 59 nucleotides) were synthesized on a DNA synthesizer (model 380A; Applied Biosystems, Inc., Foster City, CA). The 3’ terminal regions of these synthetic strands were complementary for 6–15 bp. Fill-in synthesis was carried out for 60 min at 37°C using 5 U of DNA polymerase (Klenow fragment; IBI, Miami, FL) in the presence of 200 µCi each of α-[32P]dCTP and α-[32P]dTTP (6,000 Ci/mmol, Amer sham Corp., Arlington Heights, IL) and 0.01 mM unlabeled dATP and 0.1 mM unlabeled dGTP and dTTP in a total volume of 70 µl Klenow reaction buffer (35). The IL-5 probe
was end-labeled essentially by the kinase procedure described by Maniatis et al. (36). A synthetic 55-residue oligonucleotide (0.414 g) was incubated for 60 min at 37°C with 5 U of T4 polynucleotide kinase (IBI) in the presence of 100 μCi of γ-[32P]ATP (6,000 Ci/mmol, Amersham Corp.) and kinase buffer in a volume of 70 μl. The P600 DNA fragment (a 600-bp PstI restriction fragment of the parent cDNA clone) was purified from a PstI restriction digest by PAGE, and recovered from the gel by the method of Maxam and Gilbert (37). Radioactively labeled P600 was prepared using the hexanucleotide-primed labeling kit (RPN-1601; Amersham Corp.). The reaction mixture contained 200 μCi of [32P]CTP (6,000 Ci/mmol, Amersham Corp.) and 200 ng of P600 DNA. It was incubated for 5 h at room temperature.

After all labeling reactions were complete, 20 μg of carrier tRNA (Escherichia coli; Sigma Chemical Co.) were added and unincorporated nucleotides were removed by two passages on a spin column (1.5 ml of Sephadex G50), as described by Maniatis et al. (36). Probes labeled by the fill-in or kinase methods had a specific activity of ~100 μCi/μg DNA, while probes labeled by hexanucleotide-primed synthesis had a specific activity of ~200 μCi/μg DNA.

**Cytoplasmic Dot Hybridization Analysis.** Cytoplasmic messenger RNA fractions of T cell clones were prepared using the method of White and Bancroft (16). T cell clones were harvested by centrifugation and resuspended at 2 × 10⁶ cells/ml in RPMI 1640 (M. A. Bioproducts, Walkersville, MD) plus 10% FCS (J. R. Scientific, Woodland, CA) containing 5 μg/ml Con A (Calbiochem-Behring Corp., La Jolla, CA) or medium without Con A as a negative control. Cells were pelleted after 6 h of incubation by centrifugation (1,500 g, 5 min), resuspended in 1 ml PBS, and repelleted in a sterile, 1.5-ml tube. Cells were lysed by resuspending in lysis buffer (0.14 M NaCl, 1.5 mM MgCl₂, 10 mM Tris-HCl, pH 8.6, 0.5% NP-40) at 4°C at a final concentration of 10⁶ cells/ml. The suspension was vortexed for 10 s and the nuclei were pelleted (13,000 g, 2.5 min). The resulting cytoplasmic lysates were then transferred to a sterile 1.5-ml tube containing 0.3 volumes of 20× SSC (1× SSC = 0.15 M NaCl, 0.015 M trisodium citrate) and 0.2 volumes of 37% (wt/wt) formaldehyde. The mixture was then incubated at 60°C for 15 min and stored in aliquots at −70°C. The same RNA preparations from each cell line were used for all probes. For analysis, 15 μl of each sample were titrated by serial threefold dilutions in 15× SSC into a 96-well flat-bottomed microtiter plate (Falcon Labware, Oxnard, CA) in 0.1 ml. Each dilution was applied with suction to a sheet of Nytran (0.45-μm pore size; Schleicher & Schuell, Inc., Keene, NH) supported on a filter paper (No. 3 mm Chr.; Whatman Inc., Clifton, NJ) using a 96-hole Minifold apparatus (Schleicher & Schuell, Inc.). The Nytran paper was then baked (80°C, 2 h) and prehybridized for 4 h at 42°C in 50% formamide (Bethesda Research Laboratories, Gaithersburg, MD), 6× SSC, 50 μg/ml E. coli tRNA (Sigma Chemical Co.), 0.2% (wt/vol) each of Ficoll (mol wt 400,000), polyvinylpyrrolidone, and BSA. The Nytran paper was hybridized with the [32P]-labeled DNA probe in the same mixture containing 50 ng probe/ml hybridization solution at 42°C for 24 h. After hybridization, the paper was washed twice for 15 min each at room temperature in 2× SSC, then twice for 30 min each at 60°C in 2× SSC/0.5% SDS. When the background was high, a final wash for 10 min at room temperature was done in 0.2× SSC. The paper was then exposed to film using an intensifying screen and quantitated by scanning with a laser densitometer (Ultrascan XL, LKB Instruments, Inc., Gaithersburg, MD).

**Lymphokines and T Cell Supernatants.** Purified recombinant E. coli–derived mouse IL-2α was a generous gift of T. Kupper, Yale University. IFN-α and -β were obtained from Lee Biomolecular, San Diego, CA. Purified E. coli–derived recombinant mouse IL-2 and IFN-γ were provided by Schering Research, Bloomfield, NJ. Purified recombinant silkworm-derived IL-3 (37a) and purified recombinant yeast-derived GM-CSF (38) were generous gifts of J. Schreurs, DNAX. Affinity-purified mammalian-derived mouse IL-4 was a kind gift of B. Castle and M. Howard, DNAX. Purified natural mouse IL-5 (B cell growth factor II [BCGF-II], Bond, M. W., B. Shrader, T. R. Mosmann, and R. L. Coffman, submitted for publication) was a kind gift of M. Bond and R. Coffman, DNAX. T cell supernatants were prepared by incubating T cell clones with and without 5 μg/ml
Con A at 10⁶ cells/ml in RPMI 1640 plus 10% FCS. The supernatants were harvested after 24 h and tested for lymphokine activity.

**Bioassays.** IL-1 was assayed on the D10.G4.1 T cell clone (20) in the presence of 1 μg/ml Con A. The assay medium was RPMI 1640 containing 0.05 mM 2-ME and 10% FCS. Samples were diluted by twofold serial dilutions in 96-well, flat-bottomed plates (Falcon Labware) in a volume of 0.05 ml. D10.G4.1 cells were added in a volume of 0.05 ml at a final concentration of 10⁴ cells/well and plates were incubated at 37°C for 40 h. Bioassays for IL-2, IL-3, T cell growth factor 2 (IL-4), and mast cell growth factor 2 (IL-4) were modified from those described previously (13, 39). IL-2 and IL-4 were assayed on the HT2 mouse T cell line in the presence of 5 μg/ml 11B11 anti-IL-4 antibody or a 1:500 dilution of S4B6 anti-IL-2 ascites fluid, respectively. IL-3 was assayed on the MC/9 mouse mast cell line in the presence of 5 μg/ml 11B11 anti-IL-4 antibody. In all cases, a colorimetric method (40) was used to assess the extent of proliferation at the end of the incubation period. This assay detects surviving and/or proliferating cells at the end of the assay by their ability to cleave the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to produce a colored product. 1 U of activity in the MTT assay is defined as the amount of factor in 0.1 ml which results in a signal that is 50% of the maximum, using 2 × 10⁵ HT2 cells/well, 10⁴ MC/9 cells/well for 24 h, or 10⁴ D10.G4.1 cells/well for 48 h.

**ELISA Assay for IFN-γ.** Samples were assayed for IFN-γ by a two-site sandwich ELISA using the rat anti-mouse IFN-γ mAb XMG1.2 and affinity-purified rabbit anti-mouse IFN-γ antibodies described above. Flexible PVC microtitre plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated with 100 μl of 1 μg/ml XMG1.2 in PBS for 2 h at 37°C, followed by blocking with 100 μl of 20% FCS and 0.1% Tween 20 in PBS for 30 min at 37°C. Samples and recombinant mouse IFN-γ standards were diluted in RPMI plus 10% FCS, and 50 μl were added to each well. After 60 min incubation, the plates were washed three times with PBS containing 0.1% Tween 20 (PBST) and incubated with 50 μl affinity-purified rabbit anti-IFN-γ (0.2 μg/ml in PBST). After 60 min, the plates were washed four times with PBST and incubated with 75 μl of a 1:5,000 dilution of goat anti-rabbit/horseradish peroxidase conjugate (The Jackson Laboratory, Bar Harbor, ME) in PBST plus 0.1% BSA for 45 min. Trays were washed five times with PBST, and 100 μl substrate (2,2'-azino(3-ethylbenzthiazoline sulfonic acid) [ABTS] 1 mg/ml, 0.003% H₂O₂, 44 mM NaH₂PO₄·7 H₂O, 28 mM citric acid) was added. After 60 min at room temperature, absorbance was measured at a wavelength of 405 nm and a reference of 490 nm on a VMAX microplate reader (Molecular Devices, Palo Alto, CA).

**Results**

*T Cell Clones and DNA Probes.* The distinctions between Th1 and Th2 mouse helper T cell clones based on patterns of lymphokine secretion, B cell help, and ability to cause DTH have been described previously (13, 14). In this report we used a panel of 19 T cell clones, which included examples derived from different strains of mice having different antigen specificities and MHC haplotypes (Table I). The relative mRNA levels present in each of these T cell clones were assessed by hybridization with radioactively labeled DNA probes for specific lymphokines. All probes correspond to characterized lymphokine genes with the exception of TY5 and P600 which are induction-specific cDNA clones isolated from a Th2 cDNA library (Brown, K. D., and G. Zurawski, unpublished data). The functions of the induced proteins corresponding to TY5 and P600 are unknown, although it is known that TY5 mRNA is expressed as a secreted protein (Mosmann, T., and S. Zurawski, unpublished data).

*Lymphokine mRNA Synthesis of Th1 and Th2 Clones.* Fig. 1 shows representative dot blots in which Th1 and Th2 cytoplasmic mRNA preparations were hybridized with synthetic DNA probes specific for IFN-γ (A) or IL-4 (B). The IFN-γ
Table I
Properties of Mouse T Cell Clones Used for mRNA Determination

<table>
<thead>
<tr>
<th>T cell clone</th>
<th>Mouse strain</th>
<th>MHC restriction</th>
<th>Antigen specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Th1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LB2-1</td>
<td>C57BL/6</td>
<td>H-21b</td>
<td>CRBC-2,13,15,21+</td>
</tr>
<tr>
<td>LB19-1</td>
<td>C57BL/6</td>
<td>H-21b</td>
<td>CRBC-2,13,15,21+</td>
</tr>
<tr>
<td>MD13-5.1</td>
<td>BALB/c</td>
<td>H-21b</td>
<td>CRBC-2,13,15,21+</td>
</tr>
<tr>
<td>MD13-10</td>
<td>BALB/c</td>
<td>H-21d</td>
<td>CRBC-2,13,15,21+</td>
</tr>
<tr>
<td>G15-1</td>
<td>CBA/J</td>
<td>H-21b</td>
<td>CRBC-2,13,15,21+</td>
</tr>
<tr>
<td>HDK-1</td>
<td>BALB/c</td>
<td>H-21b</td>
<td>KLH</td>
</tr>
<tr>
<td>M264-20</td>
<td>A.TL</td>
<td>NA</td>
<td>H-21p</td>
</tr>
<tr>
<td>M264-37</td>
<td>A.TL</td>
<td>NA</td>
<td>H-21p</td>
</tr>
<tr>
<td>Th2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D10.G4.1</td>
<td>AKR/J</td>
<td>H-21b</td>
<td>Conalbumin</td>
</tr>
<tr>
<td>D9</td>
<td>C57BL/6</td>
<td>NA</td>
<td>Unknown</td>
</tr>
<tr>
<td>MB2-1</td>
<td>C57BL/6</td>
<td>NA</td>
<td>Spleen</td>
</tr>
<tr>
<td>HDZ-9</td>
<td>BALB/c</td>
<td>NA</td>
<td>H-21p</td>
</tr>
<tr>
<td>HDK-2</td>
<td>BALB/c</td>
<td>H-21b</td>
<td>KLH</td>
</tr>
<tr>
<td>CDC-25</td>
<td>C3D2</td>
<td>H-21a</td>
<td>F(ab)2-NRG</td>
</tr>
<tr>
<td>CDC-35</td>
<td>C3D2</td>
<td>H-21a</td>
<td>F(ab)2-NRG</td>
</tr>
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<td>M264-39</td>
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<td>M386-13</td>
<td>CB6F1</td>
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<td>CBA/J</td>
</tr>
<tr>
<td>M386-41</td>
<td>CB6F1</td>
<td>NA</td>
<td>CBA/J</td>
</tr>
</tbody>
</table>

* Chicken MHC(B) locus alleles on erythrocytes recognized by T cell clone.
† Not applicable.
‡ F(ab)2 fragment of normal rabbit IgG.
§ Specificity not determined.

A probe hybridized with the mRNA from the six Th1 clones, but not with RNA from the Th2 clones. In contrast, the DNA probe for IL-4 hybridized to the mRNA from Th2 cells but not from the Th1 clones tested. In both cases the probes only hybridized to the RNA from cells induced with Con A, and not to the RNA from uninduced cells, and this was true for all lymphokines tested in this study.

Relative amounts of lymphokine mRNA were quantitated by scanning densitometry of autoradiograms similar to the one shown in Fig. 1. Values obtained with uninduced T cell RNA were subtracted. The resultant plots of autoradiographic density versus the cell equivalents of RNA applied were linear over the ranges examined (data not shown). The amount of radioactivity hybridized to RNA derived from 10⁶ cells was calculated for each cell line. In cases where no induced RNA was detectable, the result was expressed as the detection limit of that particular probe. The value for each individual T cell clone was then expressed as a percentage of the mean mRNA content, using the average of all Th1 and Th2 clones as the 100% value. Since these numbers were directly related to the exposure time of the film and specific activity of each probe, we could only compare relative mRNA levels between cell lines for a single probe. This presentation of the results does not provide information on relative synthesis levels between lymphokines.

Fig. 2, A and B, shows the mRNA profiles of IFN-γ, IL-2, and LT for 19
UNIQUE LYMPHOKINES OF TWO TYPES OF HELPER T CELL CLONE

The mRNA for these three lymphokines was present in all induced Th1 clones and undetectable in the Th2 clones tested (except for the presence of small amounts of IFN-γ in the Th2 clone M264-39, and IL-2 in M264-39 and M386-41). In contrast, all induced Th2 clones synthesized mRNA for IL-4 and IL-5 (Fig. 2, C and D), whereas all the Th1 clones were negative for these lymphokines. The P600 probe hybridized to the mRNA from all Th2 clones, whereas all except one Th1 clone were negative. This pattern suggests that P600 encodes a protein synthesized by induced Th2 clones that is different from any of the lymphokines examined in this study.

The mRNAs for IL-3, TY5, GM-CSF, TNF and ppENK were synthesized by all the T cell clones tested. However, the levels of synthesis of GM-CSF, ppENK, TNF, and TY5 were higher in either Th1 or Th2 clones. Fig. 2 (E and F) shows that the average levels of mRNA produced for GM-CSF, TNF, and TY5 were greater for Th1 clones. In contrast, the levels of ppENK mRNA were greater for Th2 clones but were clearly detectable at lower levels in Th1 clones (Fig. 2, G and H). The only lymphokine that did not show preferential synthesis by either type of T cell clone was IL-3, which was synthesized in highly variable amounts by all clones (Fig. 2, G and H; see also Table III).
Figure 2. Induced mRNA synthesis patterns of Th1 and Th2 clones analyzed by cytoplasmic dot hybridization. Total RNA preparations from 19 Con A-stimulated T cell clones were isolated and hybridized with 11 different 32P-labeled DNA probes. Radioactivity of each DNA probe hybridized to RNA derived from 10^6 cells of each clone was determined after quantitation of autoradiogram densitometry. The 100% point was set as the average value over all clones for each individual lymphokine probe. Bars represent a percent of this average value. Values obtained with RNA from unstimulated cells were subtracted from the results shown. If no induced mRNA was detected, the result was plotted as the minimal detectable level.

Quantitative variation in mRNA levels could have been due to a combination of differences in the degree of induction of a particular batch of cells, and inherent differences in levels made by different types of clone. To gain further information on the relative contributions of these two effects, the mRNA levels for the Th2-preferential ppENK gene were plotted against mRNA levels for the Th1-preferential genes GM-CSF, TY5, and TNF, for each clone. The results in Fig. 3 show that for each pair of probes, this analysis separates the T cell clones into two clusters, and that the clones within each cluster correspond to the Th1 and Th2 sets of clones identified by the synthesis of the six type-specific lymphokines. Stated differently, there is a correlation between the levels of both lymphokines within each major group of clones, so that Th2 clones that make high enough amounts of TY5, TNF, or GM-CSF mRNA to overlap with the lowest amounts made by the Th1 clones, are also the clones that make larger amounts of ppENK mRNA. This suggests that most of the overlap in mRNA levels between Th1 and Th2 clones for these mRNAs occurred as a result of differences in the overall extent of induction, and that there are significant differences between Th1 and Th2 clones in the synthesis of each of these four lymphokines. Similar plots for IL-2 and IL-4 indicate that the low levels of IL-2 found in two Th2 clones also correlate with a high level of induction of IL-4 in these two clones.

Functionally Monospecific Lymphokine Assays. The dot-blotting procedure en-
Figure 3. For each T cell clone, relative RNA levels of the Th2-preferential gene product ppENK were plotted against the levels of the Th1-preferential gene products TNF (A), GM-CSF (B), and TY5 (C). Also shown is a plot of the Th1-specific gene product IL-2 vs. the Th2-specific product IL-4 (D). Relative RNA levels are expressed as percent average of all clones tested. If no induced mRNA was detected, the result was plotted as the minimal detectable level.

Figure 4. HT2 T cell growth factor assays. Dilutions of purified lymphokines and supernatants from Con A-induced Th1 and Th2 cells were tested for their ability to support growth of HT2 cells: without antibody present, in the presence of S4B6 anti-IL-2, in the presence of 11B11 anti-IL-4, or in the presence of both 11B11 and S4B6. Bars represent units of activity. Starting concentrations of purified lymphokines were: IL-1, 15,000 U/ml (D10/Con A proliferation assay); IL-2, 200,000 U/ml; IL-4, 50,000 U/ml (HT2 proliferation assay); IL-5, 30,000 U/ml (MC/9 proliferation assay); IL-5, 200 U/ml (BCL3 proliferation assay [41]); GM-CSF, 1,500 U/ml (NFS-60 proliferation assay [13]); IFN-γ, 0.1 µg/ml. LB2-1 (Th1) and D10 (Th2) supernatants were obtained after stimulation for 24 h with 5 µg/ml Con A at 5 × 10⁶ cells/ml. The starting dilutions of the T cell supernatants were 1:4 in each assay.

enabled us to assay a panel of different T cell clones for a large number of lymphokines with relative precision. The disadvantage of this method is that it detects mRNA and not secreted, biologically active proteins. To verify the synthesis of active lymphokines, we used mAbs to improve the specificity of some commonly used bioassays by neutralizing unwanted activities.

Fig. 4 shows that HT2 proliferated in response to either IL-2, IL-4, or supernatants from either a Th1 or Th2 Con A–induced T cell clone, but not to any of the other purified lymphokines tested. When the assay was supplemented
FIGURE 5. MC/9 growth factor assays. Dilutions of purified lymphokines and Con A-induced Th1 and Th2 supernatants were tested for their ability to support growth of MC/9 mast cells without antibody present, or in the presence of 11B11 anti-IL-4. Activities of purified lymphokines and starting dilutions of Th1 and Th2 supernatants were as described in Fig. 4.

with anti-IL-4 (11B11) the responses of HT2 to IL-2 or the Th1 supernatant were unaffected whereas the apparent titers of the purified IL-4, and IL-4-containing Th2 supernatant were reduced by ~1000-fold. Similarly, when the HT2 assay was supplemented with anti-IL-2 (S4B6), the titers of IL-2, either purified or in the Th1 supernatant, were reduced and the titers of purified IL-4 or Th2 supernatant were unaffected. Fig. 4 also shows the effect of supplementing the HT2 assay with both anti-IL-4 and anti-IL-2. The titers of IL-2, IL-4, and both Th1 and Th2 supernatants were reduced. This suggests that the responses of HT2 to the Th1 and Th2 supernatants in these assays are only due to the presence of either IL-2 or IL-4.

By comparing the units of T cell growth factor obtained in the presence of both antibodies with the units obtained in the presence of anti-IL-4 alone, the amount of IL-2 activity in the supernatant could be precisely evaluated. Similarly, IL-4 titers could be evaluated from the comparison of titrations in the presence of both antibodies, with results obtained with anti-IL-2 alone. In each case, a very large excess of the other lymphokine will prevent reliable detection, unless higher concentrations of blocking antibody are used.

We used the MC/9 mouse mast cell target line to assay for the bioactivity of IL-3. Fig. 5 illustrates that MC/9 proliferated in response to purified IL-3, IL-4, and both Th1 and Th2 supernatants but not to any of the other lymphokines tested. Data from HPLC separations of Th1 and Th2 supernatants also indicate that IL-3 and IL-4 are the only lymphokines that stimulate MC/9 proliferation (unpublished data). When IL-3 and IL-4 are both present, as in the Th2 supernatant, the maximum proliferation rate of MC/9 is greater than in the presence of either factor alone (13, 42). Supplementing the assay with anti-IL-4 antibody reduces the response to IL-4 and thus decreases the saturation level of the Th2 supernatant, but has no effect on the response of MC/9 to IL-3 or Th1 supernatant (not shown). Since the Th2 supernatant also contains IL-3, the units of activity detected are not altered in this case (Fig. 5).

It is important to note that these assays are only demonstrated to be monospecific for lymphokines present in the supernatants of induced Th1 and Th2 clones. We do not rule out the possibility that other factors, not present in these supernatants, might affect the proliferative response of the target cells.
To assay specifically for IFN-γ, an ELISA was developed using a monoclonal rat anti-IFN-γ antibody (XMG1.2) and affinity-purified polyclonal rabbit anti-IFN-γ antibodies. Only IFN-γ was detected; IFN-α, IFN-β, IL-2, and GM-CSF failed to give a significant signal at concentrations of purified protein up to 1 µg/ml (Table II). D10 (Th2) supernatant also provided no detectable signal, signifying that not only was IFN-γ absent but also that the assay did not show crossreactivity with any Th2 lymphokines. The minimum significant signal was regarded as two times the signal elicited by assay medium alone. The limit of detectability in this ELISA normally falls between 0.05 and 0.1 ng/ml IFN-γ (data not shown). Supernatants from Con A–induced Th1 cells could typically contain 1,500–6,000 ng/ml IFN-γ, whereas levels in uninduced Th1 supernatants as well as supernatants from both induced and uninduced Th2 cells fell below the sensitivity limit. Thus the IFN-γ-specific ELISA can be used to detect IFN-γ in T cell supernatants over a range of nearly 10⁵-fold.

**Correlation Between mRNA Synthesis and Protein Secretion.** To test whether there was any correlation between the levels of lymphokine specific mRNAs and the corresponding secreted proteins, we prepared Con A–induced supernatants of all the T cell clones used in RNA studies to test in our lymphokine assays. Total cell mRNA preparations (6 h) and the supernatants (24 h) were prepared from the same batch of cells in all cases. The supernatants were analyzed for IL-2, IL-3, IL-4, and IFN-γ activities in the assays described above. Results were calculated for each individual clone as units of activity for bioassays or nanograms per milliliter of IFN-γ in the ELISA, and were then converted to percent of average of all clones tested. The results obtained in this way were then compared with the mRNA values obtained by dot-blotting analysis for each corresponding lymphokine. Table III shows that there was generally good agreement between the two measures of lymphokine expression. As expected, the low-level expression of, for example, IL3, was detected only by the bioassay.

Combining the mRNA, protein, and bioactivity data, the following consensus patterns of lymphokine synthesis emerge. Compared with the average expression levels of all T helper clones tested, Th1 clones express high levels of IL-2, IFN-γ, LT, GM-CSF, TNF, TY5, and IL-3; low levels of ppENK; and no IL-4, IL-5, or
TABLE III

Correlation between Lymphokine RNA Synthesis and Protein Secretion

<table>
<thead>
<tr>
<th>T cell</th>
<th>IFN-γ</th>
<th>IL-2</th>
<th>IL-3</th>
<th>IL-4</th>
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<tbody>
<tr>
<td></td>
<td>ELISA</td>
<td>RNA</td>
<td>$\Delta$</td>
<td>Bioassay RNA</td>
</tr>
<tr>
<td>L62-1</td>
<td>279</td>
<td>267</td>
<td>236</td>
<td>341</td>
</tr>
<tr>
<td>LB19-1</td>
<td>376</td>
<td>268</td>
<td>113</td>
<td>158</td>
</tr>
<tr>
<td>MD13-5-1</td>
<td>108</td>
<td>236</td>
<td>192</td>
<td>367</td>
</tr>
<tr>
<td>MD13-10</td>
<td>209</td>
<td>148</td>
<td>56</td>
<td>13</td>
</tr>
<tr>
<td>GK15-1</td>
<td>249</td>
<td>284</td>
<td>95</td>
<td>219</td>
</tr>
<tr>
<td>HDK-1</td>
<td>191</td>
<td>228</td>
<td>183</td>
<td>74</td>
</tr>
<tr>
<td>M264-20</td>
<td>78</td>
<td>77</td>
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</tr>
<tr>
<td>M264-37</td>
<td>220</td>
<td>209</td>
<td>291</td>
<td>187</td>
</tr>
<tr>
<td>H66-61</td>
<td>155</td>
<td>79</td>
<td>513</td>
<td>319</td>
</tr>
<tr>
<td>D10</td>
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<td>&lt;4</td>
<td>&lt;0.1</td>
<td>&lt;9</td>
</tr>
<tr>
<td>D9</td>
<td>&lt;0.04</td>
<td>&lt;4</td>
<td>&lt;0.1</td>
<td>&lt;9</td>
</tr>
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<td>&lt;0.1</td>
<td>&lt;9</td>
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<tr>
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<td>&lt;9</td>
<td>234</td>
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<tr>
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<td>&lt;9</td>
<td>3</td>
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<tr>
<td>CDC-25</td>
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<td>&lt;0.1</td>
<td>&lt;9</td>
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<td>M264-39</td>
<td>54</td>
<td>68</td>
<td>21</td>
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<tr>
<td>M386-13</td>
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<td>M386-41</td>
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<td>&lt;4</td>
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<td>45</td>
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</tbody>
</table>

* IFN-γ levels in supernatant determined by ELISA assay, expressed as the percent of the average of all clones.
‡ Cytoplasmic RNA levels, determined by the dot blot procedure, expressed as a percent of the average of all clones.
\$ Supernatant bioactivity levels, determined by the bioassays shown in Figs. 4 and 5, expressed as a percent of the average of all clones.

P600. In contrast, Th2 clones express high levels of IL-4, IL-5, P600, ppENK, and IL-3; low levels of GM-CSF and TNF; and no detectable IL-2, IFN-γ, or LT.

Discussion

We have chosen in these studies to use cytoplasmic dot hybridization as the most effective way to determine the lymphokine synthesis patterns. The complexity of current bioassays means that only a few lymphokines can be unambiguously assayed without complex procedures, and so the range of lymphokines that can be stringently identified by bioassay is less than with the RNA dot-blot method. The ability of the probe to hybridize to homologous but nonidentical sequences in RNA could give an occasional false-positive reaction, although this possibility is minimized by using relatively short DNA probes (50–100 bases), and by confirming results with a second probe in certain cases. The principle drawback of the RNA procedure is that the presence of mRNA does not prove that the corresponding protein is synthesized and secreted as an active lymphokine.

We measured both RNA and protein levels of four lymphokines (IL-2, IL-3, IL-4, and IFN-γ), and the results correlate well. This supports the validity of the
RNA procedure, and suggests that at least for these four lymphokines, translational control does not significantly influence the relative levels of lymphokine produced. In addition, we have limited data that suggest that the RNA results for IL-5, met-enkephalin, TY5, and GM-CSF are also true at the level of secreted proteins (34; Mosmann, T., M. Bond, and R. Coffman, unpublished results). In general, the RNA data reported here are in agreement with the results of probing of cDNA libraries from a Th1 clone, LB2-1 (26), and a Th2 clone, D9 (27; Brown, K. D., unpublished results). The latter results are particularly important in confirming the synthesis of TNF by both types of T cell clone, since TNF has been generally regarded as a monocyte but not T cell product (43). The patterns of synthesis of TNF and IL-5 by the T cell clones in our panel have also been confirmed using second-site synthetic probes complementary to other regions of the mRNA (unpublished results).

The T cell clones assayed in this study were stimulated with Con A to induce lymphokine synthesis. In a previous study (13), we showed that for several clones, including both Th1 and Th2 examples, the same lymphokines (IL-2, IL-3, IL-4, and IFN-γ) were produced in response to either Con A or antigen plus APCs. We have carried out preliminary experiments using antigen or Con A plus PMA stimulation, and find that antigen stimulation gives lower lymphokine mRNA levels, and Con A plus PMA gives higher levels than Con A alone. In neither case was there an alteration in the patterns of lymphokine synthesis (our unpublished results). The mRNA and supernatant samples for this study were taken 6 and 24 h, respectively, after induction. These times had previously been found to be optimal for several lymphokines (our unpublished results). Levels of IL-2, IL-3, IL-4, and IFN-γ activity in supernatants correlate well with the RNA data, indicating that the major Th1/Th2 differences in synthesis of IL-2, IFN-γ, and IL-4 are not due to differences in induction kinetics. There is limited data suggesting that this is also true for IL-5 and pPENK.

In previous experiments, we noted that IL-2 mRNA or protein synthesis did not always occur in response to stimulation, in contrast to IL-3 and IFN-γ synthesis. In experiments to evaluate lymphokine mRNA synthesis, two clones (MD13-10 and LB19-1) did not synthesize IL-2, although their synthesis of all other lymphokine mRNAs showed the typical Th1 pattern. In a subsequent experiment (shown in Fig. 2), IL-2 was synthesized at significant levels by these two clones. The synthesis of the other lymphokines in our test panel has not shown this variability, which suggests that IL-2 synthesis may be regulated somewhat differently from other lymphokines.

While the Th1 and Th2 patterns of lymphokine synthesis are remarkably distinct, some of the 19 clones in our test panel (especially M264-39) did not show absolute agreement with the Th1 and Th2 consensus patterns. At present, it is not clear if this represents real heterogeneity of T cells, or if the in vitro T cell clones show some variation from normal patterns. Some of the clones in the panel have been in culture for several months, and are not completely stable with respect to Lyt-1 or IL-2 expression, although the synthesis of other cell-surface antigens and lymphokines appears to be stable over several months.

The results obtained with this panel of T cell clones suggest that the syntheses of IL-2, IFN-γ, LT, IL-4, IL-5, and P600 are mediated predominantly or entirely
by one or the other of the T cell types. In contrast, GM-CSF, TNF, TY5, and ppENK are synthesized in larger amounts by one subset, but in each case the other subset also synthesizes detectable amounts. We do not yet know the significance of these smaller differences in lymphokine synthesis between the clones, although the results of the two dimensional plots (Fig. 3) suggest that these differences are genuine.

An important question arising out of the separation of Th cell clones into two subsets is whether the same two sets of lymphokine synthesis patterns exist in vivo. Evidence from the rat suggests that two types of CD4+ Th cell exist, since OX22+ cells produce the majority of IL-2 synthesized by Th cells, and OX22- cells provide the majority of B cell help (15). These data, obtained with normal spleen cells, are consistent with the evidence derived from mouse T cell clones. Further evidence (reviewed in reference 44) also suggests that Th1 and Th2 cells may exist in normal lymphocyte populations.

In view of the major differences between Th1 and Th2 lymphokine synthesis patterns, it is perhaps not surprising that these two types of clone also differ markedly in function. When injected into the footpads of naive mice, Th1 but not Th2 clones induce an antigen-dependent swelling reaction with the characteristics of DTH (14). Only clones producing IFN-γ and LT, presumably corresponding to our Th1 clones, are capable of killing target cells (45). Although both types of clone can provide both linked and unlinked help for antibody production by B cells (21, 22), the nature of the help may differ. In particular, only Th2 clones provide strong help for IgE responses (Coffman, R., and T. Mosmann, unpublished data). This is probably related to the production of IL-4 by Th2 clones, and IFN-γ by Th1 clones, since IL-4 or monoclonal anti-IFN-γ enhances IgE responses, whereas IFN-γ or monoclonal anti-IL-4 suppresses IgE responses, both in vitro (25; Coffman, R., personal communication) and in vivo (3, 46). In contrast to the enhancement of IgE synthesis by Th2 clones, IgG2a may be positively regulated by Th1 clones, since IFN-γ is now known to enhance IgG2a production both in vitro (47) and in vivo (Finkelman, F. D., I. Katona, T. R. Mosmann, and R. L. Coffman, manuscript submitted for publication).

Summary

Lymphokine synthesis patterns of a panel of 19 T cell clones have been evaluated, using mRNA hybridization methods to examine 11 different mRNAs induced by Con A. The two types of CD4+ Th cell clone described previously (13) were clearly distinguished by this procedure, and the differences between the two types have now been extended to six induced products. With minor exceptions, only Th1 clones synthesized mRNA for IL-2, IFN-γ, and lymphotoxin, and only Th2 clones synthesized mRNA for IL-4, IL-5, and another induced gene, P600. Four more induced products were expressed preferentially but not uniquely by one or another type of clone: mRNAs for GM-CSF, TNF, and another induced, secreted product (TY5) were produced in larger amounts by Th1 clones, whereas preproenkephalin was preferentially expressed by Th2 clones. IL-3 was produced in similar amounts by both types of clone. mAbs were used to establish three bioassays that were functionally monospecific for IL-2, IL-3, and IL-4, and a new anti-IFNγ mAb, XMG1.2, was used to establish an
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ELISA for IFN-γ. These four assays were used to show that secreted protein and mRNA levels correlated well for all cell lines. The implications of these findings for normal T cells are discussed.

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