## TWO TYPES OF MURINE HELPER T CELL CLONE

## I. Definition According to Profiles of Lymphokine Activities and Secreted Proteins

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A panel of antigen-specific mouse helper T cell clones was characterized according to patterns of lymphokine activity production, and two types of T cell were distinguished. Type 1 T helper cells  $(T_{H}1)$ produced IL 2, interferon- $\gamma$ , GM-CSF, and IL 3 in response to antigen + presenting cells or to Con A, whereas type 2 helper T cells  $(T_H 2)$  produced IL 3, BSF1, and two other activities unique to the  $T_{\rm H}2$ subset, a mast cell growth factor distinct from IL 3 and a T cell growth factor distinct from IL 2. Clones representing each type of T cell were characterized, and the pattern of lymphokine activities was consistent within each set. The secreted proteins induced by Con A were analyzed by biosynthetic labeling and SDS gel electrophoresis, and significant differences were seen between the two groups of T cell line. Both types of T cell grew in response to alternating cycles of antigen stimulation, followed by growth in IL 2-containing medium. Examples of both types of T cell were also specific for or restricted by the I region of the MHC, and the surface marker phenotype of the majority of both types was Ly-1<sup>+</sup>, Lyt-2<sup>-</sup>, L3T4<sup>+</sup>. Both types of helper T cell could provide help for B cells, but the nature of the help differed.  $T_{H1}$  cells were found among examples of T cell clones specific for chicken RBC and mouse alloantigens.  $T_{H2}$  cells were found among clones specific for mouse alloantigens, fowl  $\gamma$ -globulin, and KLH. The relationship between these two types of T cells and previously described subsets of T helper cells is discussed.

Mouse helper T cells  $(T_H)^3$  appear to be fairly uniform when judged by the commonly used surface markers bearing Ly-1 and L3T4 but not Lyt-2 antigens. When assessed by functional criteria, however, at least two types of  $T_H$  cell have been described (1–6). These subtypes of T<sub>H</sub> cell have been described mainly in uncloned populations, but certain differences between them appear to be consistent. A synthesis of the work of several groups provides the following description of these T cells. One type of  $T_H$  cell ( $T_H$ 1) helps B cells in a linked, antigenspecific manner, is "monogamous" in limiting dilution cultures, and is required early in the response. These T cells may also help in an unlinked manner at high antigen concentrations (7). The other type of  $T_H$  cell ( $T_H$ 2) helps B cells in a nonlinked manner, is "polygamous" in limiting dilution cultures, and is required later in the response. In addition, T<sub>H</sub>2 cells possess determinants that map to the I region of the major histocompatibility (MHC) and bind to nylon wool, whereas T<sub>H</sub>2 cells lack both of these properties. The determinants on  $T_H2$  cells that map to the I region may actually represent structures that are controlled by the I region but are not coded by the I region (8).

We have found that in a large panel of antigen-specific and autoreactive  $T_H$  cell clones, we were able to divide the clones into two distinct groups. These groups were originally defined on the basis of patterns of lymphokine synthesis, but can also be distinguished by their characteristic mode of B cell help. The properties of these two sets of T cells agree in many respects with the two sets of T<sub>H</sub> cells described above, and we have used the nomenclature of Tada et al. (1) to describe  $T_H1$  and  $T_H2$  cells. In this study we describe the derivation of antigen-specific T cell clones of both types, and their evaluation according to the distinctive patterns of lymphokine activities produced after antigen or lectin stimulation. We have also analyzed the supernatant proteins produced after concanavalin A (Con A) stimulation and biosynthetic labeling, and have found characteristic patterns associated with each type of  $T_H$  cell clone.

#### MATERIALS AND METHODS

*Mice.* C57BL/6, CBA/J, and BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and the Institute for Medical Research (San Jose, CA). Recombinant inbred mice used for MHC restriction typing were a generous gift of Dr. H. McDevitt.

Antigens and antibodies. Chicken red blood cells (CRBC) were obtained from six partially inbred lines of chickens, homozygous for MHC alleles but differing in other blood group antigens, maintained at PMK Farms, Vacaville, CA. Keyhole limpet hemocyanin (KLH) was obtained from Pacific Biomarine, Venice, CA, and fowl  $\gamma$ -globulin (FGG) was obtained from Cappel Laboratories, Cochranville, PA. Monoclonal antibody specific for mouse interleukin 2 (IL 2) was from the S4B6 hybridoma obtained by fusing Sp2/0 hybridoma cells with spleen cells from a rat immunized with concentrated LB2-1 supernatant (manuscript in preparation).

Cell lines. The COS monkey cell line was obtained from F. Lee. The HT2 mouse T cell line (9) was obtained from S. Strober, the

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<sup>&</sup>lt;sup>3</sup> Abbreviations used in this paper: BCGF1, B cell growth factor 1; BSF1, B cell stimulating factor 1; CRBC, chicken red blood cell; FGG, fowl  $\gamma$ -globulin; GM-CSF, granulocyte-macrophage colony-stimulating factor; MCGF, mast cell growth factor; MTT, 3-(4,5-dimethylthiazol-2-yi)-2.5diphenyltetrazolium bromide: TCGF, T cell growth factor; T<sub>H</sub>1, T helper cell, type 1; T<sub>H</sub>2, T helper cell, type 2.

NFS60 cell line was obtained from J. Ihle through D. Rennick, and the MC/9 mast cell line (10) and C1.Ly-1/9 T cell line (11) were obtained from G. Nabel. The MM3 mouse mast cell line was established from BALB/c bone marrow cells by growth in medium supplemented with supernatant from Con A-induced C1.Ly-1/9 cells. All cells were grown in RPMI 1640 supplemented with 0.05 mM 2mercaptoethanol, 10% fetal bovine serum (FBS), and growth factors where necessary: recombinant mouse IL 2 (kindly provided by G. Zurawski) for HT2, Con A-induced spleen-conditioned medium (11) for C1.Ly-1/9, and supernatant from Con A-induced C1.Ly-1/9 T cells for NFS60, MM3, and MC/9.

Establishment of T cell lines. T cell lines were established as described (12)<sup>4</sup> except that T cells were primed either by adoptive transfer or by simple in vivo priming. In some cases, T cells were cloned immediately by limiting dilution without prior bulk culture in vitro. T cell lines specific for alloantigens were cloned directly from the spleens of unimmunized mice by limiting dilution on irradiated allogenic stimulator cells. T cell lines were grown on alternate cycles of antigen stimulation followed by growth in IL 2-containing medium. Antigen stimulation with antigen and 2 × 10<sup>6</sup> syngeneic spleen cells/ml in RPMI 1640 + 10% FBS + 0.05 mM 2-mercaptoethanol + recombinant mouse IL 2 (400 to 800 U/ml; see below). After 24 hr, the cultures were expanded in the same medium.

Stimulation of T cell clones. For Con A stimulation, T cells were harvested by centrifugation and were resuspended in RPMI 1640 containing 0.01 mg/ml Con A (Calbiochem, La Jolla, CA) with or without 5% FBS. Supernatants were harvested after 24 hr. T cells  $(5 \times 10^5/ml)$  were stimulated with antigen (0.02% CRBC or 0.015 mg/ml FGG or KLH) in the presence of  $5 \times 10^6$  spleen cells/ml. After 24 hr, the supernatants were harvested and were tested for lymphokine activities. Allogeneic stimulations (MHC or *Mls*) were carried out similarly except that no exogenous antigen was added.

Transfection of lymphokine cDNA clones. COS cells were transfected with cDNA clones for mouse IL 3 (13), interferon- $\gamma$ , and granulocyte-macrophage colony-stimulating factor (GM-CSF) as described (14). Supernatants containing lymphokine activity were harvested from 3 to 5 days after transfection. Plasmid DNA for these three lymphokine cDNA clones was a generous glft of K. Arai.

three lymphokine cDNA clones was a generous gift of K. Arai. Bioassays. The colorimetric MTT<sup>3</sup> proliferation assay (15) was used for all assays. The assay medium was RPMI 1640 containing 0.05 mM 2-mercaptoethanol and 10% FBS. Samples were diluted by twofold serial dilutions in 96-well, flat-bottomed trays (Falcon) in a volume of 0.05 ml. Target cells (10<sup>4</sup>/well for MC/9, MM3, and NSF60, and  $2 \times 10^3$ /well for HT2) were then added in a vol of 0.05 ml, and the trays incubated at 37°C for 22 hr. MTT solution (0.01 ml of 5 mg/ml stock in phosphate-buffered saline) was added to each well, and incubation was continued for another 2 hr at 37°C. All wells then received 0.15 ml 0.04 N HCl in isopropanol, and after thorough mixing to dissolve the dark blue crystals, the optical density was measured on a Dynatech MR580 Microelisa reader by using a test wavelength of 570 nm and a reference wavelength of 630 nm. The results were transferred directly to an Apple II computer and the units of activity were calculated. One unit is defined as the amount of factor that in 0.1 ml, resulted in a signal in the MTT assay equal to 50% of the maximum signal. In all assays, the absorbance of the blank (cells but no factor added) was subtracted from all values

Biosynthetic labeling of T cells. T cells were labeled with  $[^{35}S]$  methionine after Con A stimulation as described.<sup>4</sup> The radioactive secreted proteins were analyzed by SDS-PAGE by using the buffer system of Laemmli (16).

Protein separation methods. Secreted proteins from Con A-induced T cells were concentrated in an Amicon ultrafiltration cell (YM5 membrane) and were applied to a Vydac C4 (Separations Group. Hesperia, CA) reverse phase high pressure liquid chromatography (HPLC) column. Proteins were eluted by a gradient from 25% acetonitrile and 0.1% trifluoroacetic acid to 80% acetonitrile and 0.1% trifluoroacetic acid at a flow rate of 0.4 ml/min. Serial dilutions of the 1 min fractions were tested for bioactivity and the units of growth factor activity were calculated. SDS-PAGE was carried out by the method of Laemmli (16).

Analysis of cell surface phenotype. The surface antigens of T cell lines were evaluated by flow cytometry after reaction with the following antibodies: anti-Lyt-1, 53-7, (American Type Culture Collection; ATCC, no. TIB104), anti-Lyt-2, 53-6, (ATCC no. TIB107), and anti-L3T4, GK1.5, (ATCC no. TIB207).

*B cell assays.* T cell supernatants were tested for the ability to stimulate enhanced polyclonal secretion of IgG1 and IgE by lipopoly-

<sup>4</sup>Mosmann, T. R., D. Cher, M. A. Giedlin, and M. W. Bond. 1985. Biosynthesis of lymphokines and other secreted proteins by murine helper T cell clones after induction by concanavalin A. Submitted for publication. saccharide (LPS)-stimulated splenic B cells as described (17). After 7 days, the levels of IgG1 and IgE in the culture supernatant were measured by an isotype-specific ELISA assay.<sup>6</sup> Ia-inducing factor was measured by the method of Roehm et al. (18).

Biosynthetic labeling and immune precipitation. T cells were stimulated with Con A and were labeled with [<sup>35</sup>S]methionine as described.<sup>4</sup> Supernatant proteins were immunoprecipitated by using either normal rabbit serum or a rabbit anti-mouse interferon- $\gamma$  antiserum (Enzo Biochem, New York, NY) by described methods.<sup>4</sup>

#### RESULTS

Characteristics of bioassays. A large panel of antigenspecific T cell lines was analyzed by using several lymphokine bioassays. The cell lines could be grouped into two sets,  $T_H1$  and  $T_H2$ , on the basis of the characteristic pattern of bioactivities found in their supernatants after Con A or antigen stimulation. We used several target cell lines in bioassays to detect these lymphokine activities. Some of these assays detect more than one factor, and some activities are influenced by the presence or absence of other lymphokines. We have characterized some of these interactions on three target cell lines to use the assays as more precise tools for determining the presence or absence of certain lymphokines. In particular, we have established "dominant" assays, i.e., bioassays in which a lymphokine activity is not influenced by the presence of  $T_{H1}$  or  $T_{H2}$  supernatants. In the description of the bioassays that follows, we have used the LB2-1 T cell clone as the representative  $T_H1$  example and MB2-1 as the  $T_H2$ example.

HT2 assay. The mouse T cell line HT2, originally established by J. Watson (9), is commonly used as an assay for mouse and human IL 2. We have used this line for IL 2 assays, and have also used it to assay another lymphokine activity in supernatants of certain T cell clones. Con A does not influence the HT2 cell line at the concentrations present in the samples (results not shown). Figure 1A indicates that HT2 proliferated equally well in response to saturating amounts of either recombinant IL 2 or the Con A-induced supernatant from LB2-1, the T cell line from which the recombinant cDNA clone for IL 2 was derived (14). The addition of supernatant from the MB2-1 T cell line did not influence the stimulation due to IL 2 derived either from recombinant sources or LB2-1 supernatant (Fig. 1B). Taken together, these results suggest that HT2 cells respond to IL 2 and that this stimulation is not influenced by other factors present in induced LB2-1 or MB2-1 supernatants (examples of  $T_{H1}$  and  $T_{H2}$  cells, respectively). Figure 1A also demonstrates that the supernatant of induced MB2-1 cells contained an activity that stimulated the HT2 cell line to a lesser extent than IL 2, even at saturating amounts of supernatant. Similar results have been obtained by others (D. Rennick, personal communication). We have used a monoclonal antibody specific for mouse IL 2 to demonstrate that this second activity was distinct from IL 2. The S4B6 monoclonal antibody is a rat anti-mouse IL 2 that recognizes mouse but not human IL 2, and appears to have a high affinity for mouse IL 2 because it completely inhibits the biological activity of IL 2 at low antibody concentrations (manuscript in preparation). The bioactivity in the  $T_{H2}$ supernatant was unaffected by the monoclonal antibody S4B6, whereas IL 2 activity could be completely inhibited (Fig. 1C). Thus, the cell line HT2 can be used as a dominant assay for IL 2, i.e., the response of HT2 to IL 2 is not altered by other  $T_H1$  or  $T_H2$  supernatant activities. In



Figure 1. Response of HT2 cells to IL 2 and a second TCGF activity. A. Dilutions of Con A-induced LB2-1 and MB2-1 cell supernatants and recombinant mouse IL 2 were tested for activity in the HT2 proliferation assay. The starting dilutions in the first well were: LB2-1, 1/4; MB2-1, 1/1; recombinant IL 2, 1/4000. (-----) LB2-1, (-----) MB2-1, (--) rlL 2 B. Dilutions of MB2-1 supernatant were tested for an effect on the proliferation of HT2 cells in response to a constant amount of IL 2 (400 U/ml) supplied as a dilution of Con A-induced LB2-1 supernatant (or recombinant mouse IL 2 (--– –). A titration curve of IL 2 in LB2-1 supernatant is shown for comparison (----). Starting dilutions were: LB2-1, 1/4; MB2-1, 1/1. C. Dilutions of ascites fluid containing the monoclonal anti-IL 2 antibody S4B6 (starting at 1/40) were assayed for their effect on the response of HT2 to a constant amount of recombinant mouse IL 2 (80 U/ml, -—) or MB2-1 supernatant (1/40 final dilution, -----).

addition, HT2 cells can also be used to detect another activity in the  $T_H2$  supernatants. This second activity is obscured if IL 2 is present in the sample, because the strong IL 2 signal is dominant.

Separation of lymphokine activities. Additional evidence for the separate identities of IL 2 and T cell growth factor 2 (TCGF2), and the absence of TCGF2 in LB2-1 supernatants was obtained by column chromatography. Con A-induced supernatants from LB2-1 and MB2-1 cells were separated by chromatography on a C4 reverse phase HPLC column, and individual fractions were assayed for activity in the HT2 assay. The HT2 growth factor activities from these two cell lines eluted at very different times (Fig. 2), and each cell line produced only one activity, i.e., LB2-1 did not produce a TCGF activity eluting at the same position as the TCGF2 activity from the MB2-1 sample. Neither peak of activity could be due to Con A, which elutes at a different time. The characteristically different titration curves of IL 2 and TCGF2 from LB2-1 and MB2-1 respectively were also seen in the assay of these column fractions (results not shown).

*NFS60 assay.* The NFS60 cell line proliferated in response to recombinant IL 3 and also in response to both LB2-1 and MB2-1 supernatants (Fig. 3A). The level of proliferation in response to saturating amounts of LB2-1



#### Fraction number

Figure 2. Reverse phase HPLC of IL 2 and TCGF2. Supernatants from Con A-induced LB2-1 and MB2-1 cells were concentrated and separated on a C4 column as described in *Materials and Methods*. Dilutions of each fraction were then assayed on HT2 cells, and the units of activity per milliliter of the column fraction were calculated.



Figure 3. Response of NFS60 to lymphokines. A. Dilutions of supernatants of Con A-induced T cells or COS cells transfected with recombinant cDNA clones were assayed for their effect on NFS60 cells. Starting dilutions were: LB2-1 supernatant, 1/4 (---); MB2-1 supernatant, 1/1 (----); COS-IL 3, 1/40 (---). B. Dilutions of supernatants of Con A-induced LB2-1 and MB2-1 cells and COS cells transfected with the cDNA clone for interferon- $\gamma$  were assayed for their effect on the proliferation of NFS60 cells in response to a constant amount of recombinant IL 3 (100 U/ml). Starting dilutions were: LB2-1, 1/40 (----); MB2-1, 1/1(----); and COS interferon- $\gamma$ , 1/40 (----).

supernatant was significantly lower than the responses to the other two IL 3 sources. Mixtures of LB2-1 supernatant with recombinant IL 3 resulted in the inhibition of the strong IL 3 signal. Interferon- $\gamma$  also inhibited the proliferation of NFS60 cells in response to IL 3 (Fig. 3b). Because LB2-1 cells produce large amounts of interferon- $\gamma$ , this could explain the lower maximum proliferation level of NFS60 cells in LB2-1 supernatant. Thus, NFS60 responds strongly to IL 3, and the IL 3 response can be partially inhibited by an activity in T<sub>H</sub>1 supernatants, possibly interferon- $\gamma$ .

*MM3 assay.* The MM3 mast cell line proliferated strongly in response to MB2-1 supernatant and weakly in response to LB2-1 supernatant or recombinant IL 3 (Fig. 4A). When LB2-1 and MB2-1 supernatants were mixed, the higher signal was obtained (Fig. 4B). These results suggest that another activity in MB2-1 supernatants, in addition to IL 3, was responsible for the maximum proliferation of MM3 cells. This second activity has been separated biochemically from IL 3 and has been



Figure 4. Response of MM3 cells to IL 3 and MCGF2. Dilutions of supernatants of Con A-induced T cells or COS cells transfected with the cDNA clone for mouse IL 3 were assayed for their effect on the growth of MM3 cells. The assay medium contained no growth factor supplements [Fig. 3A] or 2% (final concentration) Con A-induced LB2-1 cell supernatant as a source of IL 3 (Fig. 3B). Starting dilutions were: MD13-5.1, 1/4 (----): COS-IL 3, 1/40 (----): MB2-1, 1/1 (----).

shown to require IL 3 as cofactor for biological activity (unpublished results). Similar results have been obtained by Rennick and Smith (19). This activity (which we shall refer to as mast cell growth factor 2; MCGF2) was not inhibited by any activity in LB2-1 supernatant (Fig. 4*B*). Growth of the MM3 cell line was used as a specific and dominant assay for the MCGF2 activity by including saturating amounts of LB2-1 supernatant containing IL 3 in the assay medium. Under these conditions, supernatants of T<sub>H</sub>1 cell lines did not cause additional stimulation of MM3 cells, whereas T<sub>H</sub>2 cell supernatants gave a strong, dose-dependent signal (Fig. 4*B*).

Classification of T cell clones according to lymphokine activities. Figure 5 shows the typical activity profiles of several T<sub>H</sub>1 and T<sub>H</sub>2 cell lines in the HT2, MM3, and NFS60 assays. T<sub>H</sub>1 cells gave results consistent with the production of IL 2, IL 3, and interferon- $\gamma$ , but not MCGF2. In contrast, T<sub>H</sub>2 cells did not appear to produce IL 2 or interferon- $\gamma$  but did produce IL 3 and the two activities described above as TCGF2 and MCGF2. It is important to note that these sets of activities were coordinate, e.g., IL 2 and interferon- $\gamma$  activities were produced by the same lines, and TCGF2 and MCGF2 activities were found together. The lymphokines that we infer were



Figure 5. Bioassay of supernatants from Con A-stimulated T cell clones. Four  $T_{H1}$  and two  $T_{H2}$  cell lines were stimulated with Con A and the resulting supernatants were assayed on HT2, NFS60, and MM3 cell lines. The MM3 assay medium was supplemented with 2% Con A-induced LB2-1 supernatant. All starting dilutions were 1/4. LB2-1 (-----), MD13-5.1 (-----), MD13-10 (----), M264-37 (-----), MB2-1 (-----), H39-34 (----).

produced by these two sets of T cell lines are summarized in Table I. IL 2 was evaluated by stimulation of HT2 cells, and MCGF2 was measured by the stimulation of MM3 cells in the presence of saturating amounts of IL 3. TCGF2 was evaluated by its weak stimulation of HT2 cells in the absence of IL 2 by using separation by HPLC in some cases to remove IL 2. IL 3 was evaluated by combining information from proliferation of NSF60 and MM3 cell lines and HPLC separations (unpublished results). Interferon- $\gamma$  was evaluated by combining data from the inhibition of NFS60 proliferation, la induction on P388D1 cells, antiviral activity, immune precipitation (see Fig. 8).<sup>4</sup> and inhibition of IgE-enhancing activity (17). B cell stimulating factor 1 (BSF1) activity was evaluated from Ia induction assays on B cells. IgE- and IgG1-enhancing activities were determined by measuring the IgE and IgG1 levels at day 7 in LPS-stimulated cultures of Tdepleted spleen cells (17).

Production of lymphokine activities in response to antigen. In the experiments described above, Con A was used to stimulate T cells to produce lymphokines without interference by spleen cells. We also assayed supernatants from T cells stimulated with antigen + irradiated spleen cells, or with MHC alloantigens. In each case, the lymphokine activities induced by antigen or by Con A were identical, i.e., the activities detected were dependent on the T cell line and not on the method of induction. Representative examples are shown in Figure 6.

Classification of T cell lines according to B cell-stimulating activities. Examples of the two sets of T cell lines were also tested for their effect on LPS-stimulated B cells. Activities in T cell supernatants have recently been described that enhance IgE production by over 100-fold (17) and IgG1 production by 10-fold (17, 20) in LPS-activated B cells. When supernatants from our panel of T cell lines were tested for these activities, the  $T_H2$  cells were found to produce IgE- and IgG1-enhancing activities, whereas T<sub>H</sub>1 cells were consistently negative in this assay (Table II). Interferon- $\gamma$  is known to be a potent inhibitor of both enhancing activities (17). Because T<sub>H</sub>1 cells produce large amounts of interferon- $\gamma$ , the absence of these enhancing activities in the T<sub>H</sub>1 supernatants does not prove that the factor or factors that mediate this activity are absent. Some T cell supernatants also contain an activity that stimulates the expression of Ia antigens on normal B cells. This activity, as well as the IgG1- and IgE-enhancing activities, may be mediated by the lymphokine BSF1 (18, 20-22). When  $T_{H1}$  and  $T_{H2}$  cell supernatants were assayed for the ability to induce Ia on B cells, T<sub>H</sub>2 supernatants were found to contain substantial amounts of this activity, whereas T<sub>H</sub>1 cell supernatants did not (Table III). Ia induction by  $T_{H2}$  supernatants is not inhibited by purified interferon- $\gamma$  (R. Coffman, unpublished observations).

Classification of T cell clones according to surface antigens. Examples of the same panel of T cell clones were tested for surface markers by using monoclonal antibodies directed against Lyt-1, Lyt-2, and L3T4 antigens (Table IV). In all cases tested, both  $T_H1$  and  $T_H2$  cell clones were L3T4<sup>+</sup> and Lyt-2<sup>-</sup>. The majority of both types of  $T_H$  cell clones were also Lyt-1<sup>+</sup>, although two  $T_H2$ and one  $T_H1$  clones were negative for this antigen. The Lyt-1 determinant may be preferentially lost during prolonged in vitro culture, because the C1.Ly-1/9 cell line was originally Lyt-1<sup>+</sup> (11). Representative examples are shown in Table IV.

Specificity or restriction for MHC determinants. Several T cell clones of both types were tested for MHC restriction or specificity by stimulation with spleen cells from recombinant inbred mice of the B10 series. The

TABLE I	
$T_H$ cell clones: specificities and lymph	okine production <sup>a</sup>

	T Cell	Specificity	Strain	MHC Restriction	IL 2	IL 3	MCGF2	TCGF2	IFN-7	BSF1	igE & lgG1 Enhancing Activities
_	T <sub>H</sub> 1										
	LB2-1	CRC-1 <sup>b</sup>	C57BL/6	Ip	+	+	-	-	+	-	-
	LB19-1	CRC-2 <sup>c</sup>	C57BL/6	Ip	+	+	-	ND <sup>e</sup>	+	ND	-
	GK15-1	CRC-1	CBA/J	Iĸ	+	+	-	ND	+	ND	-
	GK15-2	CRC-2	CBA/J	Iĸ	+	+	-	ND	ND	ND	ND
	MD13-5	CRC-3 <sup>d</sup>	BALB/c	ND	+	+	-		+	-	-
	MD13-10	CRC-3	BALB/c	Id	+	+	-	ND	+	-	-
	MDK4.1	Ik	BALB/c	NA	+	+	-	ND	+	ND	ND
	M264-15	Iq	A.TL	NA	+	+	_	ND	+	_	ND
	M264-20	Iq	A.TL	NA	+	+	_	ND	+	-	ND
	M264-37	Id	A.TL	NA	+	+	-	ND	+	-	ND
	T <sub>H</sub> 2										
	MB2-1	Spleen	C57BL/6	ND	-	+	+	+		+	+
	Cl.Lvl/9	Unknown	C57BL/6	ND	-	+	+	+	-	+	+
	MDK3.1	I,	BALB/c	NA	_	+	+	+	ND	+	ND
	MDK5.1	H2 <sup>∗</sup>	BALB/c	NA	-	+	+	+	ND	ND	ND
	HDZ-9	Ip	BALB/c	NA	-	+	+	+	ND	+	+
	H39-34	Auto	CBA/J	I*	-	+	+	+		+	+
	H39-56	FGG	CBA/J	ND	_	+	+	+	-	+	+
	H39-72	FGG	CBA/N	Iĸ	-	+	+	+	-	+	+
	H39-119	FGG	CBA/J	ND	-	+	+	+	ND	ND	ND
	H39-131	Auto	CBA/J	ND	-	+	+	+	_	+	+
	M264-39	ľ	A.TL	NA	-	+	+	+	_	ND	ND
	C71G1	H2 <sup>b</sup>	BALB/c	NA	-	+	+	+	-	ND	ND

" Lymphokine activities were evaluated by assays described in the text.

<sup>b</sup> CRBC, MHC alleles 2, 14, 15, 19, and 21, but not 13.

<sup>c</sup> CRBC, all MHC alleles.

<sup>d</sup> CRBC, MHC alleles 2, 13, 14, 15, and 19, but not 21.

" Not determined.

<sup>J</sup>Not applicable.

results in Table V demonstrate that the MHC restriction of the T cell clones specific for foreign antigens was consistent with determinations coded by the I region of the MHC, and the T cells specific for alloantigens also recognized determinants that mapped to the I region.

Secreted proteins synthesized by  $T_H1$  and  $T_H2$  cells. Several T<sub>H</sub>1 and T<sub>H</sub>2 cell lines were labeled with [<sup>35</sup>S] methionine after Con A stimulation, and the resulting induced secreted proteins were analyzed by SDS-PAGE and fluorography. We have shown previously<sup>4</sup> that under these conditions, T cell clones secrete large amounts of proteins in the 10,000 to 30,000 dalton range in response to Con A, and almost all of these proteins are induction specific. Although some differences were seen among all cell lines,  $T_{H1}$  and  $T_{H2}$  cell lines appeared to constitute two distinct and recognizable groups (Fig. 7). In particular, the discrete band at 16,000 daltons appeared to be characteristic of  $T_H2$  clones. A group of bands in the 17,000 to 18,000 dalton region was characteristic of  $T_{\rm H}$ l clones. Immunoprecipitation with an antiserum specific for interferon- $\gamma$  demonstrated that these bands were due to interferon, and that these components were absent in  $T_{\rm H}2$  samples (Fig. 8). The specificity of this antiserum has been demonstrated.4

### DISCUSSION

In our panel of antigen-specific  $T_H$  cells, we have been able to group the clones into two clearcut groups according to a variety of criteria. These include lymphokine bioactivities, helper function, and biosynthetic labeling patterns. T cell clones could be assigned to one or the other of these subclasses by using any of these three criteria, and the other properties were consistent with the assignment. The concordance of these different assays for T cell diversity, and the derivation of examples of both types of  $T_H$  cells from four different strains of mouse, suggest that these groups of  $T_H$  cells represent real subdivisions of the  $T_H$  cell class that may have important relevance in vivo.

Although the two groups of  $T_H$  cells showed very different biological activities, they shared several properties that are typical of helper cells generally. All were positive for the L3T4 surface antigen, and all lacked the Lyt-2 antigen. Examples of both types of  $T_H$  cells were restricted by or specific for the I region of the MHC, and examples of both types provide B cell help (results not shown). The lack of distinctive surface markers may have hindered past efforts to identify the two types of helper cell.

The bioactivities detected in activated T<sub>H</sub>1 supernatants were consistent with the synthesis of IL 2, IL 3, and interferon- $\gamma$ . The presence of these lymphokines in one T cell clone was also demonstrated by recombinant cloning of IL 2 (14) and Northern blot RNA analysis of IL 3 and interferon- $\gamma$  (9). All three lymphokines have also been demonstrated by biosynthetic labeling and immunoprecipitation in LB2-1 supernatants.<sup>4</sup> In contrast, T<sub>H</sub>2 clones did not synthesize detectable amounts of IL 2 or interferon- $\gamma$  assayed by bioactivity, immunoprecipitation, or sequence-specific probing of a T<sub>H</sub>2 cDNA library (K. Arai, personal communication). T<sub>H</sub>2 clones also produced several less defined lymphokine activities that were not detected in  $T_{H1}$  supernatants. These activities include IgG1- and IgE-enhancing activities and Ia-inducing activity, all of which may be mediated by BSF1 (18, 20-22), a T cell activity that was distinct from IL2, and a MCGF activity distinct from IL 3. These latter two activities have not yet been biochemically resolved from one another, and all T lines tested make both activities or neither activity. We are currently attempting to identify the molecule(s) responsible for these activities. We also have preliminary evidence that both  $T_H 1$  and  $T_H 2$  cell



Figure 6. Bioassay of supernatants from antigen-stimulated T cell clones. Three  $T_H1$  and two  $T_H2$  cell lines were stimulated with antigen in the presence of antigen-presenting cells and the resulting supernatants were assayed on HT2, NFS60, and MM3 cell lines. The MM3 assay medium was supplemented with 2% Con A-induced LB2-1 supernatant. All starting dilutions were 1/4. LB2-1 (----), MD13-10 (---), M264-37 (----), B39-34 (----), M264-39 (-----).

lines can synthesize GM-CSF, as a result of data from bioactivity assays and screening of recombinant cDNA libraries (unpublished results).<sup>4</sup>

The supernatants of the T cell clones used in these studied were rich sources of lymphokine activities. Although we used clonal assay lines to detect several of the

TABLE II IgE- and IgG1-enhancing activity in  $T_H1$  and  $T_H2$  supernatants<sup>a</sup>

			-	
T Cell Clone	Stimula			
	IgE	IgG I		
	T <sub>H</sub> 1			
	LB2.1	b	0.35	
	MD13.5	_	0.56	
	MD13.10		0.83	
	T <sub>H</sub> 2			
	C1.Ly-1/9	57°	9.4	
	HDZ-9	56	7.5	
	H39-34	13	4.5	
	H39-56	32	3.1	
	H39-131	73	9.1	

<sup>a</sup> Con A-induced T cell supernatants were tested for their ability to enhance IgE and IgG1 production by LPS-stimulated, T-depleted spleen cells. The stimulation index is the Ig level obtained with the addition of T cell supernatant, divided by the Ig level produced in control cultures stimulated only by LPS.

<sup>b</sup> lgE levels in cultures stimulated with LPS alone or LPS plus  $T_{\rm H}l$  supernatants were below the threshold of detection in the assay (1.25 mg/ml).

 $^{c}$  For the purpose of calculation, the IgE levels in cultures stimulated by LPS alone were assumed to be 1.25 mg/ml: thus, the IgE stimulation indices represent minimal estimates.

TABLE III Ia-inducing activity in  $T_{H}1$  and  $T_{H}2$  supernatants<sup>a</sup>

T Cell Clone	Units of Ia Inducing Factor/ml			
T <sub>H</sub> 1				
LB2-1	<20			
MD13-5.1	<20			
MD13-10	<20			
M264-15	<20			
M264-20	<20			
M264-37	<20			
Т <sub>н</sub> 2				
H39-72	2,000			
H39-34	>25,000			
MDK3.5	200			
C1.Ly-1/9	2,500			

<sup>a</sup> Con A-induced T cell supernatants were tested for the induction of la antigens on B cells. One unit/ml induced 50% of maximum la induction.

	TABLE	IV							
Surface marker phenotype of $T_{H}$ and $T_{H}$ cells									
T Cell Clone	Ly-1	L3T4	Lyt-2						
T <sub>H</sub> 1									
LB2-1	+	+	-						
MD13-5	+	+	-						
MB13-10	+	+	-						
GK15-1	+	+	-						
GK15-2	+	+	-						
M264-15	+	+	-						
M264-20	+	+	-						
M264-37	_	+	-						
Т <mark>н</mark> 2									
MB2-1	_	+	-						
C71-G1	+	+	-						
C1.Ly-1/9		+	-						
MDK3	+	+	-						
CTL									
M264-9	-	-	+						

biological activities, these assay lines did not always detect only one lymphokine. In some cases, more than one activity was detected by one cell line, and certain activities interacted with one another, either positively or negatively. Some examples include the inhibition by interferon- $\gamma$  of IL 3-induced proliferation of NFS60 cells and of the IgE and IgG1, enhancing activities on LPSstimulated B cells, the synergistic effect of IL 3 and MCGF2 on the growth of MM3 mast cells, and the response of HT2 cells to both IL 2 and a second factor that gave a signal in the HT2 assay. For the conclusions described above, we have relied mainly on "dominant"

	TABLE V			
MHC restriction or	specificity of	of $T_H 1$	and	T <sub>H</sub> 2 clones

	BALB/c	B10	B10.MBR	B10.BR	BIOA	B10A.(1R)	B10A.(3R)	B10A.(4R)	B10A.(5R)	DBA/2	
К	d	b	k	b	k	k	b	k	b	d	
MHC $-I^{a}$	dddd	bbbb	kkkk	kkkk	kkkk	kkkk	bbbk	kkkb	bbbk	dddd	
D	dd	bb	dd	qq	kk	bb	dd	bb	dd	dd	
T cell											
MD13-5.1	2999 <sup>b</sup>	c							ND	ND	
MD13-10	3133								ND	ND	
LB2-1	$ND^{d}$	ND	15	ND	ND	4	12549		ND	ND	
LB19-1	13	2669				12	5357		ND	ND	
GK15-2	68	56	1846	1367	1411	2265	51	1259	ND	ND	
MDK4	95	11	5413	2881	1882	2893	ND	1338	155	27	
HDZ-9	64	2105					ND	11	2387		
MDK3	100	35	8276	5553	3878	3991	ND	23	4737	60	
MDK5	26		741	773	1493	1443	ND	20		22	
H39-34	38		4229	2112	2009	2170	ND	3075	29	15	
H39-72	20		183	63	123	74	ND	134	14	-	

<sup>*a*</sup> The alleles for the  $A_{\beta}$ ,  $A_{\alpha}$ ,  $E_{\beta}$ , and  $E_{\alpha}$  genes are shown.

<sup>b</sup> Units MCGF/ml in supernatant after stimulation by antigen + antigen-presenting cells from the specified mouse strain.

<sup>c</sup> Less than 10 U/ml.

<sup>d</sup> Not determined.



*Figure 7.* Biosynthetically labeled induced, secreted proteins of T cell clones. Type 1 and type 2 T cell lines were induced with Con A and were labeled with [<sup>35</sup>S]methionine for 2 hr starting 4.5 hr after Con A stimulation. The supernatant proteins were analyzed by SDS-PAGE. Samples A to H represent T<sub>H</sub>1 cell clones, and samples I to P represent T<sub>H</sub>2 cell clones. The *arrows* indicate the position of m.w. markers (in kilodaltons). Experiment 1: A = LB2-1, B = MD13-5.1, C = MD13-10, L = H39-34, M = H39-56, N = H39-72, O = H39-119, P = MB2-1. Experiment 2: D = MD13-5.1, E = LB2-1, F = M274-15, G = M264-20, H = M264-37, I = M264-39, J = MB2-1, K = C71-G1.

assays, i.e., activities that were not inhibited or obscured by any other activities present in either  $T_H1$  or  $T_H2$  supernatants. Such assays include the IL 2 assay on HT2 and the MCGF2 assay on MM3 cells in the presence of saturating amounts of IL 3.

During the course of this study, over a period of 18 mo, the phenotypes of several  $T_{H1}$  cell clones have remained constant.  $T_{H2}$  cells in general tend to grow slowly, and we have on occasion seen overgrowth of these clones by  $T_{H1}$  cells. In some cases, these  $T_{H1}$  cells were shown by surface markers to have been derived from the feeder cells used to support growth of the  $T_{H2}$  clone. We have no evidence for interconversion of T cell clones between  $T_{H1}$  and  $T_{H2}$  phenotypes. In addition, the properties of



*Figure 8.* Synthesis of interferon- $\gamma$  by T<sub>H</sub>1 but not T<sub>H</sub>2 clones. Aliquots of the samples described in Figure 7, experiment 2 were subjected to indirect immunoprecipitation with rabbit anti-mouse interferon- $\gamma$  antiserum, and the resulting precipitates were analyzed by SDS-PAGE along with aliquots of the unprecipitated material. *A* = M264-37, *B* = LB2-1, *C* = MD13-5.1, *D* = M264-15, *E* = M264-20, *F* = M264-39, *G* = C71-G1, *H* = MB2-1.

the two types of  $T_H$  cell clones are consistent from one experiment to another. These findings suggest that the  $T_H1$  and  $T_H2$  phenotypes are stable and represent significant functional differences between two sets of  $T_H$  cells.

The T cell clones that we have designated  $T_{H}1$  may be equivalent to the Ia-negative helper cells described by Tada et al. (1) and Swierkosz et al. (2), which do not bind to nylon wool and are required early in an in vitro response. They may also correspond to the antigen-linked "monogamous" T<sub>H</sub> cells described by Waldmann (3) and Imperiale et al. (4). The clones that we have described as  $T_H2$  produce BSF1, which could account for the strong, unlinked B cell help provided by the second type of  $T_{H}$ cells described by the same investigators. In addition to their nonspecific B cell help, the second type of  $T_{\rm H}$  cell has been characterized as adherent to nylon wool (1), positive for "Ia" antigens (1, 2), and required later in the in vitro B cell response (1, 2). The full range of  $T_H$  cell phenotypes may be broader, however, and Melchers et al. (5) and Kim et al. (6) have described results consistent with more than two helper subpopulations. In particular,

the data of Kim et al. (6) when using T cell clones suggest four different phenotypes assessed by the pattern of B cell help, and therefore the two major subdivisions of  $T_{\rm H}$ cells that have described according to lymphokine production may be additionally subdivided when more data are available. We have obtained data on several  $T_{H1}$ clones that suggest that these may differ in B cell helper function (manuscript in preparation), and the patterns of biosynthetically labeled secreted proteins suggest additional differences between the T cell clones. Although similarities could be seen between all T<sub>H</sub>1 clones and between all  $T_{\rm H}2$  clones, there were individual differences between the clones, especially in the  $T_H2$  group. The presence or absence of some of the proteins in particular T cell clones suggest that additional bioassays may reveal functional heterogeneity within the  $T_{H1}$  and  $T_{H2}$  groups. This is also suggested by the two-dimensional electrophoresis results of Kettman and Lefkovits (23), who argue for considerable T cell heterogeneity.

There appears to be some preference for the type of  $T_{\rm H}$ cell clone isolated in different experiments. We have isolated CRBC-specific T cell clones from three different mouse strains, and in over 200 antigen-specific clones we have not found any T<sub>H</sub>2 examples. In contrast, FGGspecific clones in one experiment were all  $T_H 2$ . Both  $T_H 1$ and  $T_{H2}$  clones were found among allospecific  $T_{H}$  cells specific for the I region of the MHC. This apparent association of certain antigen specificities with one or another type of T cell may represent genuine differences in the induction of T cells in a manner analogous to the induction of different antibody isotypes by different antigens. In fact, these two phenomena may be related, because the  $T_H1$  and  $T_H2$  cells may have differential effects on the Ig isotype produced in vitro.  $T_H 2$  cells produce activities that strongly enhance IgE and IgG1 production relative to other isotopes in a polyclonal activation system. whereas  $T_{\rm H}$  cells produce interferon- $\gamma$ , which strongly inhibits the enhancing activities in T<sub>H</sub>2 supernatants.<sup>5</sup> If the two types of  $T_H$  cells produce similar activities during an in vivo immune response, the following hypothesis can be advanced. An antigen, such as FGG adsorbed to alum, that induces a substantial IgE response may do so by preferentially inducing T<sub>H</sub>2 cells, whereas CRBC, which induce large IgM but very small IgE responses, may do so by preferentially activating  $T_{\rm H}1$  cells that help IgM but suppress IgE production. We are also investigating the association of the type of  $T_H$  cell in relation to the antigen and the adjuvant used for immunization.

The derivation of two distinct types of  $T_H$  cell clones raises the question of the total diversity of T cell phenotypes. The procedures used to generate our T cell clones may be highly selective for particular types, and it is quite possible that other T cell types exist in vivo. This is underscored by the uniform phenotype of our anti-CRBC T cell lines, which were all  $T_H1$ . Only when we examined T cell clones specific for FGG or alloantigens did we discover antigen-specific T cells of the second phenotype. The study of additional antigens or alterations in culture conditions may reveal additional types of  $T_H$  cell.

The two types of helper cells described in this report differ in several respects, and yet they have not been extensively described before at the clonal level. Several features of these cells may have contributed to reducing the number of  $T_H2$  clones isolated. Because  $T_H2$  cells are dependent on IL 2 for growth, and yet do not produce IL 2, these cells must be grown on a constant supply of exogenous IL 2. In addition, we have found that the  $T_H2$  clones do not grow as rapidly as  $T_H1$  clones, and they may have extra requirements for growth or a limited lifespan. We have been much more successful in growing large numbers of  $T_H1$  clones than  $T_H2$  clones.

Several questions about the two types of  $T_H$  cells now need to be answered. Because both types bear the same cell surface markers as the delayed-type hypersensitivity (DTH) effector cells, is one or both types identical to the DTH cell? What is the relative contribution of each type of T cell to normal in vivo responses? Do the differences between  $T_H 1$  and  $T_H 2$  cells represent separate lineages of  $T_H$  cells, or do the two types represent different stages of the same lineage? Is the apparent selectivity of the antigen or adjuvant for  $T_H 1$  or 2 a real phenomenon, and what determines this selectivity? How is IL 3 regulated coordinately with IL 2 and interferon- $\gamma$  in  $T_H 1$  cells and yet noncoordinately in  $T_H 2$  cells? The T cell clones described in this report should be useful in answering these and many other questions.

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*Note Added in Proof:* We have recently obtained evidence from serological data<sup>5</sup> and recombinant DNA cloning of BSF-1<sup>6</sup> that the activities described as MCGF2 and TCGF2 are mediated by BSF-1.

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