MURINE CD4+ T CELL SUBSETS DEFINED

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Numerous studies have related differences in phenotype with functional hetero-geneity in lymphocytes. Recently attention has focused particularly on the CD4+ T cell subset, since the surprising finding that murine CD4+ T cell lines could be divided into at least two types based on their differential ability to synthesize and secrete distinctive sets of lymphokines (1, 2). Furthermore, this distinction is associated with different functions, such as delayed-type hypersensitivity or help for antibody secretion (3-7). However, the relationship between such in vitro T cell lines and the normal CD4+ T cell population found in vivo remains unclear.

In this study, we have been able to discriminate murine CD4+ T cell subsets by differential expression of antigenic determinants using monoclonal anti-T cell au-toantibodies (SM3G11 and SM6C10). These antibodies were derived from hybrid-omas made with a distinctive mouse B cell subset, Ly-1 B (8-10). Both antibodies react with determinants expressed on thymocytes and peripheral T cells, but not found on B cells, myeloid cells, or erythrocytes. Application of these two antibodies, together with anti-CD4 antibody in multi-color FACS analysis and sorting experiments, resolves four different CD4+ T subsets.

Studies have been carried out to examine these CD4+ T subsets, primarily for the types of lymphokines secreted. Furthermore, we address the question of whether different CD4+ T cell subsets require particular types of APC and how this might relate to the distinct sets of lymphokines elaborated by such subsets. We present here: (*a*) the response to Con A in the presence of either B cell or non-B cell acces-sory cells; (*b*) lymphokine (IL-2 or IL-4) secretion induced by such activation; and (*c*) localization of the memory T cells responsible for secondary antibody formation initiated by antigen-pulsed memory B cells. We then discuss the significance of differen-tial expression of antigenic determinants that coincides with these distinct functions and how the differentiation and maturation of normal CD4+ T cells might produce such heterogeneity.

Materials and Methods

Mice.   BALB/cAnNIcr mice were bred and maintained in our animal facility. 2-4-mo-old female mice were used in most experiments, except where noted. 3-mo-old female SM/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used for establishing anti-T cell hybridomas.

Anti-T Cell Hybridomas and Antibody Purification.   Cells expressing Ly-1 and IgM on the sur-

face (Ly-1 B) (8) were sorted from a pool of spleen cells from five SM/J mice using the FACS. Sorted Ly-1 B were cultured for 2 d in the presence of LPS from *Escherichia coli* (No. L2880; Sigma Chemical Co., St. Louis, MO) and then used for cell fusion with a HAT-sensitive MPC11 myeloma cell line (11). SM3G11 and SM6C10 were selected and cloned as were 13 other IgM antithymocyte antibody-secreting hybridomas simultaneously established (12). Ascites were obtained by injection of hybridomas into C.B-17*scid* mice and purified by precipitation with 50% saturated ammonium sulfate, followed by gel filtration (Ultrogel AcA34; IBF Biotechnics, Villeneuve-la-Garenne, France). Both antibodies are IgMκ, with some association of γ_{2b} H chain contributed by MPC11.

Fluorescence Staining Reagents. The hybridomas producing anti-CD4 (GK1.5), anti-CD8 (53-6), anti-CD5 (53-7), anti-B220 (RA3-6B2), anti-IgM (331.12), and anti-CD3 (145-2C11) (13) were cultured in serum-free HB101 medium (Hana Biologics, Berkeley, CA), and secreted antibody was purified as described elsewhere (8, 14, 15). Purified anti-CD3 antibody (500A-A2) (16) was provided by Dr. J. Allison (University of California, Berkeley, CA). The mouse hybridomas producing anti-IgD (10-4.22) and anti-Ia^d (MKD6) were injected into C.B-17*scid* mice and antibodies were purified from ascites (8). Phycobiliproteins (Phycoerythrin [PE],[1] phycocyanin [PC], allophycocyanin [AP]) were purified from seaweed or cyanobacteria as described previously (17). "Avidin D" was purchased from Vector Laboratories, Inc. (Burlingame, CA). Conjugation of antibodies and avidin with either biotin, fluorescein, phycobiliproteins, or Texas Red have all been described previously (8, 17).

Immunofluorescence Staining, Analysis, and Sorting. Staining for multicolor analysis was performed as described previously (8, 18). Data analysis and cell sorting were carried out using a FACStar plus (Becton Dickinson Immunocytometry Systems, Mountain View, CA) equipped with two lasers, the second laser being a tunable dye laser. The FACS was equipped with appropriate filters and compensators for collecting four-color immunofluorescence together with forward and right angle light scatter. Data were collected in list mode on a Micro VAX II computer (Digital Equipment Co., Maynard, MA) using the DESK software developed by Mr. W. Moore in Dr. L. A. Herzenberg's laboratory, Stanford University, Stanford, CA. Techniques of multicolor FACS analysis (18) and data manipulation (19) have been described elsewhere. Cells were typically sorted at rates of 7,000 cells/s into microcentrifuge tubes. Reanalysis of the sorted cells show >99% purity for T cell subsets.

Bioassay for Lymphokine Secretion. The growth factor–dependent cell line HT-2 (20) was obtained from D. Hansburg (Fox Chase Cancer Center) and maintained in RPMI 1640 medium supplemented with 10% FCS (Gibco Laboratories, Grand Island, NY), 50 μM 2-ME, and 2% culture supernatant from Con A–stimulated rat spleen cells. Bioassay for lymphokine (IL-2 and IL-4) secretion was determined by the MTT assay as described by Mosmann (21), with slight modification. 10^4 HT-2 cells were used per well and the development of color was read using an ImmunoReader NJ2000 (InterMed, Tokyo, Japan) equipped with a 570-nm sample filter and a 650-nm reference filter. Serial dilutions of supernatant of Con A–stimulated mouse spleen cell culture provided a standard where 50% of maximum growth was determined to be 1 U in 0.1 ml. Anti-IL-4 (11B11) (22) containing ascites was a generous gift of Dr. J. Ohara (NIH, Bethesda, MD). The anti-IL-2 (S4B6) cell line established by Mosmann (1), was obtained from H. C. Morse (National Institutes of Health, Bethesda, MD), adapted to growth in HB101 medium, and antibody was purified as described previously.

Con A Stimulation of T Cells. CD4+ T cell subsets were sorted from spleen after three-color immunofluorescence staining. In particular, sample tubes containing stained cells were kept on ice while sorting. As B lymphocyte accessory cells, RA3-6B2+ (B220, reference 23) CD4– cells of typical small lymphocyte size (determined by forward light scatter) were sorted as a population enriched for nonactivated cells. This is based on the fact that activation of B cells induces a decrease and eventual loss of expression of the 6B2 determinant from the B220 molecule (Hayakawa, K., and R. R. Hardy, unpublished observations). As non-B accessory cells, either cells in the peritoneal cavity showing large forward and right angle light scatter measurements (large granular fraction) or Thy-1– B220– cells from spleen were used.

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[1] *Abbreviations used in this paper:* AP, allophycocyanin; KLH, Keyhole limpet hemocyanin; PC, phycocyanin; PE, phycoerythrin.

5 × 10⁴ of these sorted accessory cells were cultured (without treatment) in microtiter wells (No. 3598; Costar, Cambridge, MA) together with 10⁵ cells of selected T cell subsets and Con A (No. C5275; Sigma Chemical Co.) at 5 µg/ml in 200 µl of RPMI 1640 + 10% FCS + 2-ME (culture medium) for 2 d.

Secondary Antibody Response. BALB/c mice were immunized with 100 µg of Keyhole limpet hemocyanin (KLH; Pacific Bio-Marine Laboratories, Inc., San Rafael, CA) on alum with 10⁹ of heat-inactivated *Bordetella pertussis* vaccine. More than 3 mo after immunization, spleen cells were used for memory B cell and T cell sorting. Spleen cells were incubated with KLH (1 µg/ml) simultaneously with the process of immunofluorescence staining (at 4°C for 30 min for each step); then the B220⁺ (IgM, IgD)⁻ cell fraction (15) was sorted and used as a population enriched for antigen (KLH) pulsed-memory B cells. In parallel, spleen cells from the same primed mice were stained using three-color immunofluorescence for sorting CD4⁺ T cell subsets. 10⁴ KLH-pulsed B220⁺ (IgM, IgD)⁻ cells and 10⁵ cells of selected T cell subsets were cocultured in 200 µl culture medium for 7–14 d.

Specific IgG anti-KLH antibody secretion at 7 and 14 d of culture was measured by ELISA assay as described elsewhere (15, 24). For detection of IgG1 or IgG2a antibody, biotinated (Bi-) rabbit anti–mouse IgG1 purified from serum purchased from Bethyl Laboratories (Montgomery, TX) or Bi-monoclonal anti-Igh-1ᵃ (20-8.3, reference 25) were used, followed by alkaline phosphatase–conjugated avidin. Anti-KLH secondary antiserum in BALB/c mice from 14 d after boost was used as standard. The amount of anti-KLH antibody in the culture supernatant is expressed as units, 1 U is activity at a 1:10⁵ dilution of standard antiserum.

Results

CD4⁺ T Cell Subsets in Spleen Revealed by SM3G11 and SM6C10 Anti-T Cell Autoantibodies. Two hybridomas, SM3G11 and SM6C10, established from SM/J Ly-1 B cells were selected for their secretion of antithymocyte autoantibody. They recognize determinants expressed both on thymocytes and peripheral T cells of all mouse strains tested, including BALB/c mice (Hayakawa, K., and R. R. Hardy, manuscript in preparation).

However, they each have a characteristic expression on thymocytes as demonstrated by fluorescence staining analysis. SM3G11 stains 20% of thymocytes, whereas SM6C10 stains most thymocytes (90%), which suggests that they react with different antigenic determinants (3G11, 6C10) (Fig. 1 *a, left*). Using three- and four-color FACS analysis, subpopulations were revealed based on the correlated expression of 3G11 and 6C10. In the thymus, most cells expressing undetectable (or low) levels of CD3 (immature thymocytes) show high levels of 6C10 expression with only a small percentage expressing low levels of 3G11 (Fig. 1 *a, middle*). On the other hand, cells expressing high levels of CD3 (mature thymocytes) express 3G11 and show a decreased and more heterogeneous level of 6C10 (data not shown). In particular, the mature CD4⁺CD8⁻ cells in thymus can be divided into four different populations based on expression of these determinants (Fig. 1 *a, right*).

We have compared CD4⁺ cells in spleen (and lymph node) with the most mature fraction of CD4⁺CD8⁻ cells in thymus and find a similar distribution, although the subsets are more distinct in spleen (Fig. 1 *b, left*). Furthermore, the correlated expression of these determinants on CD8⁺ spleen cells is different from the expression on CD4⁺ cells, with most CD8⁺ cells expressing neither determinant (Fig. 1 *b, middle*). Finally, spleen cells lacking CD3 expression (non-T cells) do not express these determinants (Fig. 1 *b, right*). In addition, 6C10 and 3G11 are not expressed to any appreciable extent on erythrocytes or bone marrow cells (data not shown). Therefore, we conclude that among lymphoid, erythroid, and myeloid cell
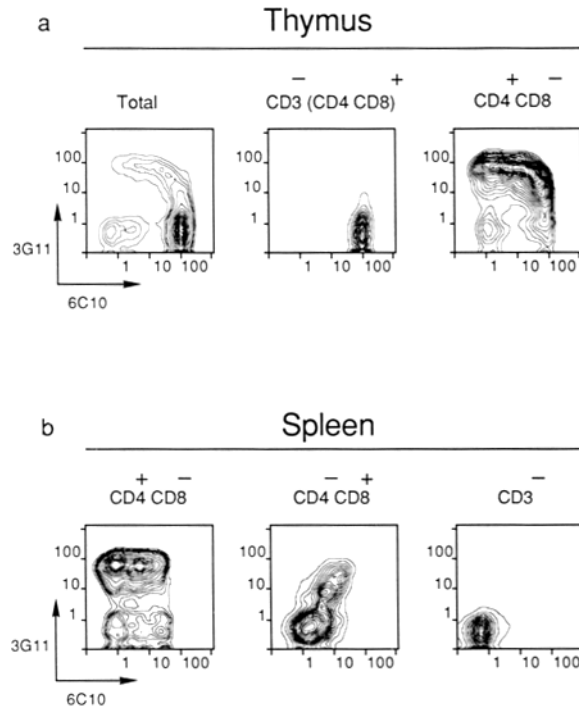
a **Thymus**

b **Spleen**

FIGURE 1. Distribution of 3G11- and 6C11-expressing cells in the thymus and spleen. 3-mo-old BALB/c mice were used for immunofluorescence staining analysis. (*a*) Thymocytes were stained with two different four-color combinations. For the first two panels, FL-SM6C10, Bi-3G11 plus TR-avidin, PE-anti-CD3(500A), and a mixture of AP anti-CD4 and AP-anti-CD8 were used. For the right panel, FL-SM6C10, Bi-3G11 plus TR-avidin, AP-anti-CD4, and PE-anti-CD8 were used. CD4⁺,CD8⁻ cells constitute 9% of thymus. (*b*) Spleen cells were stained with the four-color combinations described for the upper right panel thymocyte analysis; CD4⁺ (CD8⁻) or CD8⁺ (CD4⁻) cells are shown. For analysis of CD3⁻ cells in spleen, cells were stained with a three-color combination: FL-SM6C10, Bi-SM3G11 plus PC-avidin, and PE-anti-CD3. 6C10⁻3G11⁺, 6C10⁺3G11⁺, 6C10⁺3G11⁻, 6C10⁻3G11⁻ cell fractions in CD4⁺ cells are 31, 46, 13, and 10%, respectively. 40% of CD8⁺ cells do not express either determinant.

lineages, the expression of 3G11 and 6C10 is T lineage restricted and is preferentially found on CD4⁺ cells.

Responsiveness to Con A in the Presence of Two Types of Accessory Cells. To investigate any preference for a particular type of accessory cell among the CD4⁺ subsets, three fractions (I, II, III) were sorted, as shown in Fig. 2 *a*. Since activation of CD4⁺ T cells by the mitogen Con A is completely dependent on accessory cells (26), contamination of Ia⁺ non-T cells obscures the accessory cell dependence in this system, and we found such cells only in fr. IV (Fig. 2 *b*). After we specifically sorted to deplete this fraction of Ia⁺ cells, no activation by Con A occurred unless accessory cells were added to these fractions (data not shown). All fractions express readily detectable levels of CD3 on their surface (Fig. 2 *c*).

The four CD4⁺ T cell fractions were examined for their responsiveness to Con A. Either B cells from spleen (B220 [6B2]⁺ B cells) or non-B cells (principally peritoneal nonlymphoid cells enriched for large granular cells; or B220⁻ Thy-1⁻ cells from spleen) were used separately as accessory cells. Activation of T cells was measured by secretion of growth factors using a bioassay with the growth factor-dependent cell line HT-2. In fact, such supernatant growth factor activity (for HT-2 cells) could always be detected if there were metabolically active cells (as measured by the MTT assay) growing in the T cell culture as the result of their activation during the 2-d Con A stimulation (data not shown).

The results, as summarized in Table I, showed a differential response by 6C10⁺ or 3G11⁺ cells depending on the source of accessory cells. When non-B cells were
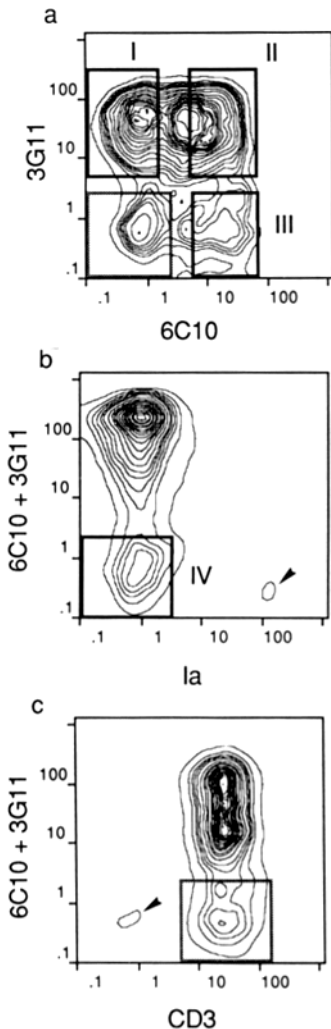
FIGURE 2. The four CD4$^+$ T cell subsets. Spleen cells were stained with FL-SM6C10, Bi-SM3G11 plus PE-avidin, and PC-anti-CD4. Regions for the sorting gates for Fr. I, Fr. II, and Fr. III are boxed (*a*); all cells are also gated as CD4$^+$. Percentages of cells sorted as Fr. I, Fr. II, Fr. III were 23, 26, and 9% of total CD4$^+$ cells; these percentages reflect 70, 50, and 70% of the total cells in each fraction. The amounts of staining reagents used did not significantly decrease staining compared with any reagent used alone. Fr. IV (8% of CD4$^+$ cells) was obtained by staining with FL-anti-Ia, a mixture of SM6C10 and SM3G11, followed by PE-anti-IgM, and PC-anti-CD4 and then sorting CD4$^+$ but FL$^-$, PE$^-$ cells (*b*). These Fr. IV cells express CD3 (*c, dashed boxed*). Arrows point out infrequent cells (1–2% of CD4$^+$ cells) that are CD4$^+$,Ia$^+$,CD3$^-$ found in the 6C10$^-$ 3G11$^-$ cell population. Comparison between *b* and *c* suggests some inhibition of the 3G11 and 6C10 mixture staining by anti-CD3 antibody.

used as accessory cells, 3G11$^+$ cells (Fr. I, Fr. II) responded better than 3G11$^-$ cells (Fr. III, Fr. IV). Furthermore, a notable difference was seen between Fr. I and Fr. III. Although Fr. III responded less well to Con A in the presence of non-B cells compared with another two fractions, they also could respond in the presence of B cells (although the B cell-dependent Con A response was generally weaker). In contrast, Fr. I, which was activated well by Con A together with non-B cells, failed to respond (or responded only weakly) if B cells were used as accessory cells at the limiting number used in this experiment (see ratio Fr. I/III in Table I). Fr. II, which expresses both 3G11 and 6C10, responded in both assays and is not functionally discriminable. Curiously, Fr. IV cells, which lack expression of both of these determinants, showed the least response under either condition despite the fact that they expressed CD3. Inclusion of Ia$^+$ cells found in the CD4$^+$ cell fraction in Fr. IV did not improve its responsiveness to Con A (data not shown).

TABLE I

Differential Accessory Cell Effect Comparing Fr. I and Fr. III in Con A Activation

CD4+ T subset	Accessory cells	Growth factor secretion					
		U/ml					
Fr. I	Non-B cells	414	113	160	70	76	162
Fr. II		672	142	160	152	157	273
Fr. III		57	40	53	32	64	ND
Fr. IV		ND	ND	<1	<1	ND	9
Ratio units I/III		7	3	3	2	1	
Fr. I	B cells	<1	6	2	<1	17	7
Fr. II		2	17	ND	19	50	37
Fr. III		20	20	187	11	53	ND
Fr. IV		ND	ND	<1	<1	ND	6
Ratio units I/III		<0.1	0.3	<0.1	<0.1	0.3	

Spleen cells from 2–4-mo-old BALB/c were stained and CD4+ T cell fractions were obtained as described in Fig. 2. Non-B cells were sorted from peritoneal washout cells or cells lacking FL-anti-B220 and PC-anti-Thy-1 staining from spleen (see Materials and Methods). B cells were obtained from spleen as small sized FL-anti-B220+ and PC-anti-CD4⁻ cells. 10^5 T cell subset and 5×10^4 accessory cells were cultured for 2 d. Results showing levels of growth factor activity in the supernatant between fractions are expressed vertically and data from individual experiments are shown horizontally.

Distinctive Lymphokine Secretion. Proliferation of the HT-2 cell line can be induced by either IL-2 or IL-4 (1). We sought to determine the presence of each growth factor in culture supernatant by using mAbs reactive with these two lymphokines. A mixture of B cells and peritoneal granular cells was used as an accessory cell population in this experiment to avoid any possible preferential induction of lymphokines due to accessory cell difference. The result (Fig. 3) showed that different lymphokines are secreted by Fr. I and Fr. III. Fr. I secretes IL-2 but not IL-4, whereas Fr. III secretes IL-4 but not IL-2. As was also the case with accessory cell dependence, Fr. II did not show restricted lymphokine secretion. The majority of activity from Fr. II was due to IL-2 and this was found when either B cells or non-B cells were used
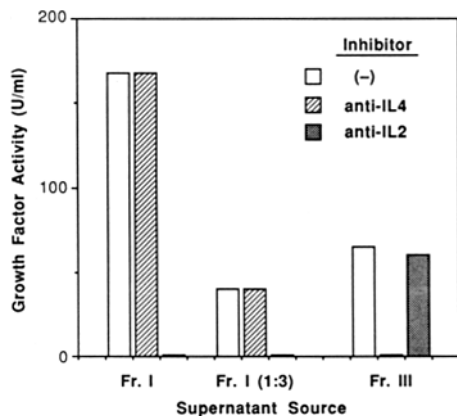

FIGURE 3. IL-2 secretion from Fr. I and IL-4 secretion from Fr. III. Fr. I or Fr. III T subsets (10^5 cells) were cultured with Con A and a mixture of B cells and peritoneal non-B cells (10^4 cells each). Growth factor activity was measured with the HT-2 cell line in the presence or absence of antibody to IL-2 (10 µg/ml) or IL-4 (1:300 of ascites). Data from a 1:3 dilution of Fr. I supernatant is also shown to confirm the distinctive inhibition seen with Fr. III.

in separate experiments (Fig. 4). Whereas IL-2 was readily found, IL-4 was not detectable in the supernatant of unseparated CD4$^+$ cultured cells (data not shown).

Helper T Cells Responsible for Secondary Antibody Response are 6C10$^+$. As we have previously reported, the secondary antibody response is initiated by the presentation of antigen by memory B cells that express surface Ig with high affinity for antigen (15). Such memory B cells in long-term KLH-primed mice are enriched in the B220$^+$ cell fraction that lacks surface IgM and IgD (Ig isotype-switched cells; reference 15). Thus, use of this cell fraction, B220$^+$ (IgM, IgD)$^-$, provides an experimental system where the interaction between isotype-switched memory B cells and T cells can be investigated.

Spleen cells from KLH-immunized mice were incubated with antigen (KLH) at 4°C (pulsed), then B220$^+$ (IgM, IgD)$^-$ cells were sorted and cocultured with CD4$^+$ T cell subsets from primed mice. As shown in Table II, 6C10$^+$ fractions (II, III) showed helper activity resulting in IgG1 antibody secretion whereas the 6C10$^-$ fraction (I) did not.

Use of aged long-term primed mice in the secondary antibody formation system revealed the most distinctive result. Splenic CD4$^+$ cells in old mice (>8 mo) also comprise four T cell subsets similar to those shown in Fig. 2, although there is relative increase in the level of Fr. III (from 10 to 35% among CD4$^+$ cells) and a decrease of Fr. I (from 35 to 15%), leaving Fr. II and Fr. IV relatively constant. Similar frequencies were observed in comparisons of long-term primed and unprimed aged mice (data not shown). In these old mice, Fr. III responded most strongly to nonspecific activation by Con A in the presence of B cells (Table III).

Furthermore, as Table IV shows, helper activity for secondary antibody secretion was restricted to Fr. III (6C10$^+$ 3G11$^-$) and only this group showed activation and proliferation by microscopic survey. The possibility of a functional effect by SM3G11 and SM6C10 antibodies at the amounts used in the staining was unlikely, since, as Table IV (Exp. 2) shows, simple positive and negative fractionation based on staining with either antibody alone agrees with the data when they were used simultaneously. Data in Table IV (Exp. 2) demonstrate that help for secondary IgG secretion is not present in the fraction lacking 6C10 which shows that Fr. IV lacks this activity in addition to Fr. I. Finally, this helper activity was not restricted to IgG1 secretion.
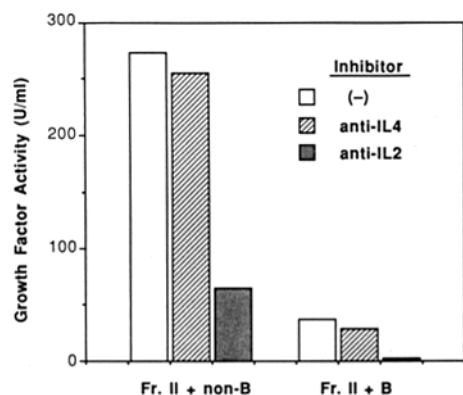

FIGURE 4. Inhibition of Fr. II supernatant by anti-IL-2 or anti-IL-4 suggests that both lymphokines are secreted. Fr. II cells were cultured with Con A together with either peritoneal non-B cells or B cells, and 2-d supernatant was tested for the presence of IL-2 or IL-4. Compared with the data using Fr. I supernatant (where no inhibition with anti-IL-4 and complete inhibition with anti-IL-2 was constantly seen in several experiments), marginal inhibition (~5-20%) by anti-IL-4 was found. Inhibition by anti-IL-2 varied from 70 to 100%.

TABLE II

A Secondary IgG Antibody Response Did Not Occur from Fr. I Cells

| KLH-pulsed B cells | CD4⁺ T subsets | IgG1 anti-KLH | |
		Exp. 1	Exp. 2
		U	
B220⁺(IgM, IgD)⁻	+ Unseparated	95	ND
	+ Fr. I (6C10⁻3G11⁺)	<2	<3
	+ Fr. II (6C10⁺3G11⁺)	110	1,500
	+ Fr. III (6C10⁺3G11⁻)	135	1,060

Spleen cells from KLH primed mice were incubated with KLH, FL-anti-B220, and a mixture of Bi-anti-IgM and Bi-anti-IgD at 4°C in medium containing 0.1% azide, washed, and then incubated with PC-avidin to reveal the biotin reagents. The sorting region for B220⁺ (IgM, IgD)⁻ cells, corresponding to 1–2% of spleen cells, has been shown in a previous paper (15). CD4⁺ T cell subsets were sorted from primed mice. Cells stained with PC-anti-CD4⁺, but not with FL-anti-Ia were used as total unseparated CD4⁺ T cells. 10⁴ KLH-pulsed B cells and 10⁵ CD4⁺ T cells were cultured to elicit antibody secretion. Antibody secretion was not detectable if the B cells were not preincubated with antigen, or in any culture containing only B or only T cell fractions. Numbers represent the mean of duplicate cultures; intersample deviation was always within 10%.

Activation of Fr. III resulted in both IgG1 and IgG2a antibody secretion from isotype-switched memory B cells.

Discussion

Four functionally distinct subpopulations of murine CD4⁺ T cells are separated by differential expression of determinants recognized by a pair of mAbs. Two of these subsets (Fr. I, Fr. III), which express these determinants in a mutually exclusive manner, exhibit distinctive functions. Both fractions can be induced to proliferate in the presence of accessory cells by the addition of the T cell mitogen Con A. However, after such activation, one subset, Fr. I, secretes IL-2 but not IL-4 into the supernatant. In contrast, nonspecific activation by Con A of the other subset, Fr. III, results in IL-4 but not IL-2 secretion. This latter subset, in turn, includes memory

TABLE III

Fr. III Sorted from Old Mice Responds Well to Con A Stimulation in the Presence of B Cells

CD4⁺ T subset	Accessory cells	Growth factor secretion	
		U/ml	
Fr. I	B cells	<1	<1
Fr. II		20	10
Fr. III		70	50

T cell subsets were sorted from the spleen of 9–12-mo-old BALB/c mice. B cells were sorted from 3-mo-old mice. Results are shown for two representative experiments.

TABLE IV

In Aged Long-Term Primed Mice, Fr. III Alone Contributes to the Secondary IgG Response

| Exp. | KLH pulsed B cells | CD4$^+$ T subset | Anti-KLH | |
			IgG1	IgG2a
			U	
1	B220$^+$ (IgM, IgD)$^-$	+ Unseparated	540	320
		+ Fr. I (6C10$^-$3G11$^+$)	<1	<1
		+ Fr. II (6C10$^+$3G11$^+$)	<1	<1
		+ Fr. III (6C10$^+$3G11$^-$)	510	430
2	B220$^+$ (IgM, IgD)$^-$	+ Unseparated	1,160	410
		+ 6C10$^-$	<1	<1
		+ 6C10$^+$	1,020	880
		+ 3G11$^-$	1,020	630
		+ 3G11$^+$	<4	<4

B cells and T cell subsets were sorted from spleens of 10-mo-old BALB/c mice that had been primed with KLH 8 mo earlier. In Exp. 2, spleen cells were simultaneously stained with PC–anit-CD4, SM6C10 (or SM3G11) plus PE–anti-IgM, and FL–anti-Ia. Ia$^-$ CD4$^+$ T cells were then separated into 6C10 (or 3G11)$^+$ fractions showing PE-anti-IgM staining and 6C10 (or 3G11)$^-$ cells that lacked PE–anti-IgM staining.

T cells capable of inducing large amounts of IgG secretion from memory B cells, whereas the former subset lacks such activity.

Our finding explains why murine CD4$^+$ T cell lines can be classified into two subsets (1) based on their differential secretion of certain lymphokines. Data suggest that this division actually reflects CD4$^+$ T subsets present in the normal CD4$^+$ population in mice. That is, Th1 clones are similar to the Fr. I CD4$^+$ subset and Th2 clones are similar to the Fr. III CD4$^+$ subset. However, our results further demonstrate that many CD4$^+$ cells (40%) in vivo apparently do not belong exclusively to either distinctive subset and raise the question as to how these populations may be related in differentiation and maturation of T cells. As yet, the developmental relationships between the CD4$^+$ subsets that we have defined is unclear.

A major question remaining is whether Fr. II consists of a single homogeneous population of cells or alternatively is a mixed population composed of cells committed to either Fr. I or Fr. III. Because of its intermediate function and lymphokine secretion, we might speculate that Fr. II could represent either the uncommitted precursor before maturing into two distinctive functional populations (I or III) or else an intermediate stage between Fr. I and Fr. III. Activation of Fr. II resulted predominantly in IL-2 secretion compared with very slight IL-4 secretion (~9:1). Since using B accessory cells did not increase IL-4 secretion and still induced IL-2 secretion, some cells in this fraction do not exhibit the exclusive functions found with Fr. I or Fr. III. Although inhibitory effects on IL-4 secretion by other lymphokines might complicate the picture, we suggest that Fr. II likely contains cells functionally distinct from either I or III.

Furthermore, it remains to be determined whether the four distinctive CD4$^+$ T cell subsets that we have observed are related to the "maturation" of T cells that may

naturally occur in mice. We found that whereas memory T cells responsible for antibody secretion were present in both Fr. II and III 2-5 mo after priming, they were limited exclusively to Fr. III in aged long-term primed mice. If this change is related to maturation of T cells, it suggests that such maturation is accompanied by phenotypic changes. Phenotypic changes of determinants expressed on CD4+ T cells during maturation have previously been suggested primarily by work with human CD4+ subsets (27, 28). Two determinants, CD45R (highest molecular weight form of common leukocyte antigen) and CDw29, have been used to delineate such subsets. These determinants are predominantly expressed in a mutually exclusive fashion on peripheral CD4+ cells. Recent work suggests that one of these subsets (expressing CD45R alone) is the precursor of the other (expressing CDw29 alone) and that this transition results from activation (27). Admittedly, these observations found in humans introduce further complications for CD4+ T cell subsets in terms of distinctive lymphokine secretion in relation to distinctive cell surface phenotype.

The fractionation of CD4+ T cells and the observation of their differential accessory dependence provides a new perspective for the study of T cell activation. Activation of CD4+ T cells absolutely requires Ia+ APC or Ia+ accessory cells in certain nonspecific stimulation systems such as Con A. It is puzzling that regardless of their Ia levels, resting B cells show poor accessory function in several experimental systems when compared with other Ia+ non-B cells (29). Although there has been accumulating evidence demonstrating that resting B cells can act as APC (30), some T cell lines fail to respond with B accessory cells (30). Most explanations for this behavior have focused on the concept of an impairment in B cells, such as biochemical differences in the Ia molecules (31), ability to process antigen (32), or cytokine secretion as compared with non-B accessory cells (33). However, our data using different CD4 subsets suggest a further possibility; that is, T cell effector differences may distinguish between B cells and non-B cells as accessory cells.

Although specialization of CD4+ T cells for particular types of APC has been suggested by earlier work with T cell lines (6, 30), it was not clear whether such distinction exists among normal CD4+ T subsets. We found here that the ability of B cells or non-B cells to act as accessory cells is not uniform when subsets of CD4+ T cells were examined separately. That is, when non-B cells are used as accessory cells, Fr. I shows considerably better activation by Con A than Fr. III. However, when B cells are used as accessory cells, Fr. III responds as well or better, but Fr. I responds only poorly (or fails to respond). Therefore, two T cell subsets exhibit differential accessory dependence.

We consider this finding that B cell–dependent Con A stimulation activates Fr. III (and Fr. II), carries significant implications in consideration of the secondary antibody response. This response is rapid and is initiated by the administration of small amounts of antigen when compared with the primary response. In earlier work we have suggested that specific antigen presentation by long term memory B cells is the essential event in the initiation of CD4+ T cell activation which, in turn, induces differentiation and secretion of the memory B cells. The frequency of such specific memory B cells, particularly with high affinity, must be low (on the order of 10^{-3} to 10^{-4} as estimated by the frequency of antigen binding cells, reference 15). Thereby, a preference by this antigen-specific memory T cell population for B cell antigen presentation would guarantee that the T cells would indeed be activated

appropriately. Consequently, particular lymphokines secreted from T cells after activation will likely be important in the expansion and differentiation of antigen-specific memory B cells as was found on resting virgin B cells (34, 35).

The observation of different accessory cell interactions associated with differential expression of the two determinants described here is intriguing. Although extensive studies have been carried out on the TCR and its associated molecules (CD3) (36), the mediator of the elusive accessory cell signal for T cell activation is not well understood. Considering the importance of molecules that mediate adhesion of T cells and APC, it is not unreasonable to expect that differential expression of 3G11 and 6C10 might be associated with the restriction of the initiation of T cell activation. Among several determinants reported that show heterogeneous expression on $CD4^+$ cells (in human, rat, and mouse; references 37–41), expression of 3G11 and 6C10 seems to be uniquely T lineage restricted. The relationship of these determinants to others described previously awaits further biochemical characterization of 6C10 and 3G11. Such studies are currently underway.

Summary

We have used two monoclonal anti-murine T cell autoantibodies (SM3G11 and SM6C10) and multi-color immunofluorescence staining to resolve splenic $CD4^+$ cells into four populations. Two of these populations (Fr. I and Fr. III, 35% and 10% of $CD4^+$ cells) show mutually exclusive expression of these determinants and exhibit distinct functions. Fr. III secretes IL-4, but not IL-2 when activated by Con A, and includes memory T cells responsible for secondary antibody formation. In contrast, Fr. I secretes IL-2 but not IL-4 in response to Con A, and does not contribute to the secondary antibody response. Furthermore, these two fractions exhibit differential accessory cell dependence. Whereas Fr. III responds with B cells (and also non-B cells) as accessory cells in Con A–induced activation, Fr. I requires non-B cells. However, we found that many $CD4^+$ cells (Fr. II, 40% of $CD4^+$ cells) express both determinants and are not distinguishable with regard to lymphokine secretion, accessory cell effect, and memory T cell activity. Curiously, the fraction expressing neither determinant (Fr. IV, 10% of $CD4^+$ cells) is unresponsive to experimental conditions used here. We discuss the possible relationships between these T cell subsets and the implications of differential expression of these determinants.

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