

## T CELL CLONES REACTIVE WITH SPERM WHALE MYOGLOBIN

### Isolation of Clones with Specificity for Individual Determinants on Myoglobin

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Recently, in our laboratory (1) as well as others (2), it has been possible to isolate and propagate in long-term in vitro culture, T cell clones reactive with soluble, synthetic amino acid copolymer and native protein antigens. Certain T cell clones reactive to multichain poly(L-Tyr:L-Glu)-poly-DL-Ala-poly-L-Lys ([T,G]-A--L)<sup>1</sup> can provide specific help to trinitrophenyl (TNP)-primed B cells when challenged in vitro with TNP-(T,G)-A--L (3). These "helper" T cell clones exhibit antigen specificity and I region restriction in proliferative assays. Results obtained with these antigen-reactive T cell clones were analyzed in conjunction with the use of monoclonal anti-Ia antibodies and a recently described mouse strain with an I-A mutation (B6-C-H-2<sup>bm12</sup>) (4). These studies provided convincing evidence that Ia antigens on accessory cells are recognized in association with antigen by T cells and, as such, can be responsible for certain immune response (Ir) gene functions (5). Furthermore, we have shown that there is free combinatorial association of  $\alpha$  and  $\beta$  I-A polypeptides (A $\alpha$  and A $\beta$ ) as expressed on the surface of cells from animals heterozygous for I-A. These "hybrid" I-A antigens form F<sub>1</sub>-specific determinants that are functional as I region restriction elements for antigen-reactive T cells (1) and mixed leukocyte reaction (MLR)-stimulating determinants for alloreactive T cells (6). Similar hybrid F<sub>1</sub> Ia determinants are formed by combinatorial association of an  $\alpha$  polypeptide chain encoded within the I-E region (E $\alpha$ ) and a  $\beta$  chain from I-A (A $\beta$ ) (7). These hybrid molecules are also functional as MLR-stimulating determinants (8) and, as recently demonstrated (9), antigen-presenting cell-restriction sites for certain antigen-reactive T cell clones.

An important question left unanswered in previous studies using T cell clones reactive with synthetic random copolymers of amino acids is the role of Ia antigens on antigen-presenting cells (APC) in "determinant selection" for T cell recognition. It is unclear, for example, whether a number of T cell clones reactive with poly(L-Glu<sup>60</sup>, -L-Ala<sup>30</sup>, L-Tyr<sup>10</sup>) (GAT), but exhibiting different patterns of I region restriction, all

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<sup>1</sup> *Abbreviations used in this paper:* APC, antigen-presenting cell; CAS, concanavalin A-activated spleen cell supernate; CNBr, cyanogen bromide; GAT, poly(L-Glu<sup>60</sup>, L-Ala<sup>30</sup>, L-Tyr<sup>10</sup>); Ir, immune response; LDC, limiting dilution cloning in microtiter well; Mb, sperm whale muscle myoglobin; MLR, mixed leukocyte reaction; PETLES, peritoneal exudate T lymphocyte subpopulation; PPD, purified protein derivative of *Bacillus tuberculosis*; SDS-PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate; (T,G)-A-L, multichain poly(L-Tyr:L-Glu)-poly-DL-Ala-poly-L-Lys; TNP, trinitrophenyl.

see the same GAT epitope. If the same antigen epitope can be recognized in association with more than one Ia-restricting element, it would be incompatible with strict models of determinant selection.

In addition to the associative recognition of antigen and Ia molecules, T cells exhibit other differences from B cells in the manner in which they recognize antigen. It has been shown that proliferating T cells from lymph nodes of mice immunized with lysozyme are predominantly directed toward a different spectrum of determinants than B cells (10). In a system using native ovalbumin as antigen (11), T-helper cells also differ from T-suppressor cells and B cells in that the former exhibit cross-reactivity to native and denatured ovalbumin when immunized with the native molecule, whereas the latter two recognize only the native conformation. Furthermore, T-suppressor cells share idiotype with serum antibodies in the case of the antilysozyme response (12). Since hybridoma antibodies to different epitopes on lysozyme share idiotopes (13), as do antibodies to different myoglobin epitopes (14), T cells reactive to different epitopes on a complex antigen may also share idiotopes. This possibility may have physiological relevance in light of the ability to alter the immune response *in vitro* by administering minute quantities of anti-idiotypic reagents (15).

To examine in detail the mechanisms of T cell recognition of antigen, determinant selection, and idiotype sharing by T and B cells, we have begun to isolate clones of T cells from mice immunized with native sperm whale myoglobin (Mb). Its amino acid sequence (16), three-dimensional structure (17), and antigenic determinants recognized by serum antibody raised in several host species, including outbred and inbred strains of mice (18–20), are well known. The native protein is a compact, highly helical molecule of 153 amino acids in a single polypeptide chain without disulfide bonds, and carries one heme group. Mb contains two methionine residues and upon cleavage with cyanogen bromide three peptides are obtained comprising residues 1–55, 56–131, and 132–153. Serum antibody is directed predominantly, if not exclusively, to five antigenic sites, each comprising six to seven amino acids, and consisting of continuous regions of the polypeptide chain (18–20). The five sites consist of residues 16–21, 56–62, 94–99, 113–119, and 146–151. The T cell clones we have isolated display reactivity to individual determinants on cyanogen bromide (CNBr) fragments and exhibit specific I region restriction. They have helped us to significantly expand our understanding of mechanisms of T cell response to myoglobin and provide a powerful system for examining the details of T cell recognition of multideterminant antigens.

### Materials and Methods

*Mice.* Mice used in this study were either raised in the Mayo Clinic animal care facility (Rochester, Minn.) or purchased directly from The Jackson Laboratory, Bar Harbor, Maine. Mice of either sex between 7–21 wk of age were used. T cell clones were derived from (C57BL/6 × A/J)<sub>F1</sub> mice [(B6A)<sub>F1</sub>].

*Myoglobin.* The major chromatographic component (no. 10) of Mb was prepared from commercially available material (Sigma Chemical Co., St. Louis, Mo.) by ion-exchange chromatography as previously described (21).

*Immunizations.* (B6A)<sub>F1</sub> mice were immunized subcutaneously at the base of the tail with 50  $\mu$ l of a 1:1 mixture of 100  $\mu$ g Mb in saline and Freund's complete adjuvant (Bacto-Adjuvant, Difco Laboratories, Detroit, Mich.) as described previously (22). Groups of four to six mice were immunized and draining inguinal and paraaortic lymph nodes were harvested on day 7.

*Establishment of Long-Term T Cell Cultures.* Upon harvesting, lymph node cells were placed

into culture according to the method developed in our laboratory for GAT-reactive T cells (1). The cells were maintained by serial restimulation with antigen and syngeneic irradiated spleen cells. Culture media was 12 mM HEPES-buffered RPMI 1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal bovine serum (Microbiological Associates, Walkersville, Md.),  $3 \times 10^{-5}$  M 2-mercaptoethanol, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and  $2 \times 10^{-3}$  M L-glutamine. Long-term cultures of uncloned T cells were kept in upright 75-cm<sup>2</sup> culture flasks (3024, Falcon Labware, Oxnard, Calif.) in a 37°C humidified 95% air/5% CO<sub>2</sub> incubator. Into each flask was placed  $1-2 \times 10^6$  live T cells,  $60-80 \times 10^6$  irradiated (3,300 rad), syngeneic spleen cells, and 20  $\mu$ g/ml Mb in 12-15 ml media. Cultures were restimulated every 10-14 d by harvesting and resuspending live T cells in media containing Mb and spleen cells. Long-term cultures were maintained for at least 12 wk before cloning and were maintained without exogenous growth factors.

*Derivation and Maintenance of T Cell Clones.* Clones of (B6A)F<sub>1</sub> myoglobin-reactive T cells were obtained by two methods: soft agar colony formation with micropipet transfer (SAC) (1) and limiting dilution cloning in microtiter well (3040, Falcon Labware) cultures (LDC). In the SAC method,  $0.25-5 \times 10^6$  recently restimulated T cells were mixed into a 0.33% agar layer. After 2-5 d incubation, small clusters of cells were transferred by a fine-tipped Pasteur pipette into microtiter wells containing 1  $\mu$ M Mb in 0.2 ml media,  $1 \times 10^6$  irradiated syngeneic spleen cells, and 5% (vol/vol) concanavalin A-activated rat spleen cell supernate (CAS). In the LDC method, T cells were diluted in media and transferred directly into microtiter wells containing media, antigen, and spleen cells as above, with 10% CAS. In general, 0.1-1.0 T cells were placed in each well. When growth in microtiter wells was detected visually (usually after 7-14 d), the cells were transferred into 2.0-ml vol in 24-well cluster trays (3524, Costar, Data Packaging, Cambridge, Mass.) containing media,  $10 \times 10^6$  spleen cells, 1  $\mu$ M Mb, and 5% CAS. Clones were assayed for proliferation (see below) and expanded as desired. Clones were routinely maintained in upright, 25-cm<sup>2</sup> flasks (3013, Falcon Labware) containing  $5-6 \times 10^6$  T cells, 5-6 ml media, 1  $\mu$ M Mb,  $30 \times 10^6$  spleen cells, and 0-2% CAS. Cells were restimulated every 10-14 d. Addition of CAS is not necessary for proliferation as assayed below, but is required for long-term survival of certain clones.

*Assay of T Cell Proliferation.* Proliferation of long-term cultures and clones was assayed after stimulation with Mb or one of its fragments (see below) in the presence of syngeneic or other appropriate irradiated spleen cells. In a 0.2-ml culture were placed  $1 \times 10^4$  T cells,  $1 \times 10^6$  spleen cells, media, and antigen. Antigen concentration in general ranged from 0.01 to 10  $\mu$ M. After 48 h incubation, 2  $\mu$ Ci [<sup>3</sup>H]thymidine (Research Products International Corp., Mt. Prospect, Ill.) was added. After an additional 16 h incubation, cells were harvested onto filter paper disks and subjected to liquid scintillation counting. Results are the mean ( $\pm$  1 SD) of triplicate cultures and are expressed as counts per minute.

*Mb Fragments.* CNBr fragments of Mb were prepared and characterized as described previously (23, 24), except that in the present work the CNBr reaction was done in 70% formic acid (25). The resulting fragments were purified by gel filtration. Fragmentation yielded the expected peptides (1-55, 56-131, and 132-153). The purified peptides gave single spots on peptide mapping, single bands on polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE), and had the expected amino acid compositions (23, 24). Peptide 15-22, comprising an antigenic site of Mb recognized by antibody, was synthesized as described previously (26).

Fragments and peptides were dissolved in cold distilled water and sterilized by microfiltration for use in proliferative assays. Concentrations were adjusted so that 1-10  $\mu$ l of antigen solution was added to each culture to give final antigen concentrations of 0.01-100  $\mu$ M.

*Monoclonal Anti-Ia Antibodies.* Monoclonal antibodies reactive to Ia antigens were used to block T cell proliferation. The antibodies 150.13, 10.2.16, 116.32 and 116.32.8, and 118.49 (27) were provided by Dr. G. Hammerling (Institut für Immunologie und Genetik, Heidelberg, Federal Republic of Germany). Antibody Y17 (7) was provided by Dr. P. P. Jones (Stanford University, Stanford, Calif.). The specificities of these reagents are listed in Table III. To test for blocking in proliferative assays, the antibodies were added directly to the T cell cultures immediately before the addition of antigen.

## Results

*Properties of Long-Term Cultured T Cells*

**GENERAL.** A long-term culture of pooled lymph node cells from several Mb-immune B6A mice was established. This culture has been maintained continuously for nearly 1 yr by repetitive stimulation. No exogenous lymphokines have been added. The culture has maintained a stable pattern of growth, cell morphology, and antigen reactivity during this period. In general, a 5–10-fold increase in live cell count is recorded every 14 d.

The long-term T cell culture has been analyzed for cell surface markers by fluorescence-activated cell sorter and consists predominantly of cells bearing Thy-1.2 and Lyl-1 markers (data not shown).

**ANTIGEN REACTIVITY OF LONG-TERM T CELL CULTURE.** The antigen reactivity of long-term cultured T cells was tested in a proliferative assay with a panel of antigens (Table I). Within this limited spectrum, the T cells responded only to the immunogen, Mb. Reactivity to purified protein derivative (PPD) present in fresh, immune lymph node cells is lost. When Mb-reactive T cells were titrated with increasing concentrations of Mb and its three CNBr fragments, the results presented in Fig. 1 were obtained. All three CNBr fragments induced proliferation of the uncloned T cell culture. The degree of response to the peptides parallels their size: 56–131 > 1–55 > 132–153. The sum of the reactivities against the three fragments is not additive in this assay and a number of factors are probably responsible. They will be discussed below. We interpret the titration data to indicate that the long-term culture contains clones

TABLE I  
*Antigen Reactivity of Long-Term Cultured, Mb-reactive (B6A)F<sub>1</sub> T Cells*

Antigen	$\mu\text{g/ml}$	$^3\text{H}$ uptake <i>cpm</i> $\pm$ <i>SD</i>
—	—	2,441 $\pm$ 1,433
Mb	17	43,878 $\pm$ 13,835
GAT	200	2,898 $\pm$ 1,736
Lysozyme	200	2,931 $\pm$ 1,182
PPD	50	1,636 $\pm$ 777

Mb-reactive T cells were obtained and assayed as described in Materials and Methods.

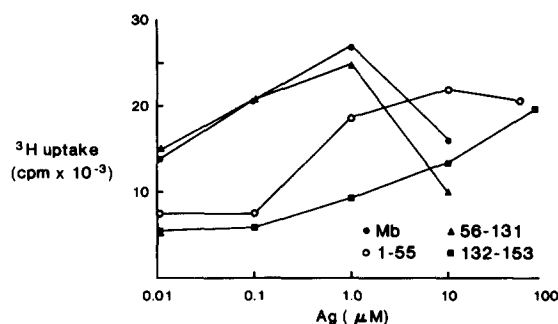


FIG. 1. Dose response of long-term cultured, Mb-reactive T cells to Mb (●) and CNBr fragments 1–55 (○), 56–131 (▲), and 132–153 (■). Antigen concentration ( $\mu\text{M}$ ) plotted vs. counts per minute of [ $^3\text{H}$ ]TdR uptake.

of Mb-reactive T cells with reactivity toward individual T cell-stimulating determinants present on CNBr fragments of Mb.

To get a better estimate of the number of clones responsive to each fragment, we performed a limiting dilution analysis of the long-term culture (10). Antigen concentration was held constant at  $1 \mu\text{M}$ . Varying numbers of responding T cells were added and  $^3\text{H}$  uptake measured. At nearly every point there is a 10-fold greater response to fragments 1-55 and 56-131 than to 132-153 (Fig. 2). We tentatively concluded that the majority of T cell clones present in long-term culture were directed to determinants located on fragments 1-55 and 56-131, with an estimated 10-fold fewer clones reactive with fragment 132-153.

**I REGION RESTRICTION.** Antigen-reactive, proliferating T cells are characterized not only by specific antigen reactivity but also by I region restriction (1). This restriction is manifested as a requirement for recognition of certain I region products on antigen-presenting spleen cells in the proliferative assay. Since responsiveness to Mb has been mapped to two different subregions within the murine I region (28-30), we assessed I region restriction of responsiveness to Mb using, as APC, spleen cells from a number of hybrid and recombinant mouse strains. Fig. 3 demonstrates the ability of irradiated spleen cells from a number of strains to present Mb to the long-term Mb-reactive (B6A) $F_1$  T cell culture. In Fig. 3A the data represent analysis after  $\sim 12$  restimulations

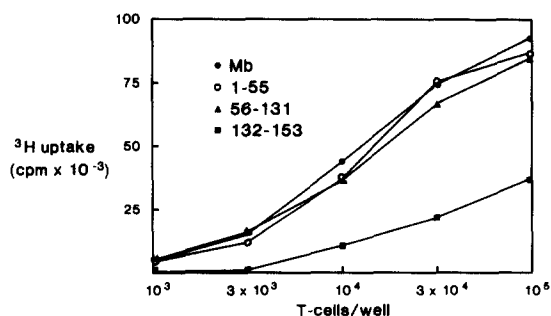


FIG. 2. Limiting dilution analysis of response of long-term Mb-reactive T cell culture to Mb and its CNBr fragments. Ag concentration was held constant at  $1 \mu\text{M}$  and [ $^3\text{H}$ ]thymidine ([ $^3\text{H}$ ]TdR) uptake of varying numbers of T cells assayed.

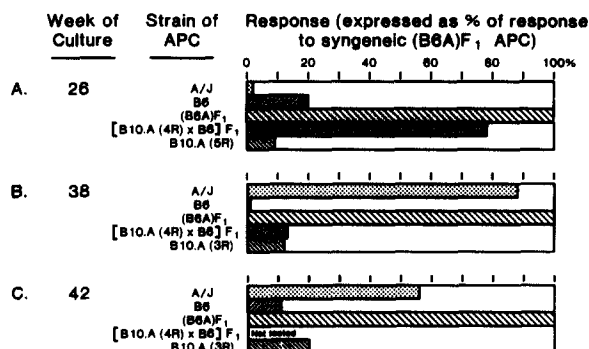


FIG. 3. APC restriction of response to Mb of (B6A) $F_1$  T cells from long-term culture. T cells assayed in the presence of  $1 \mu\text{M}$  Mb and indicated APC at week 26 (A), 38 (B), and 42 (C) of culture. [ $^3\text{H}$ ]TdR incorporation was measured and normalized by expression as percent of response to syngeneic (B6A) $F_1$  APC.

with antigen and syngeneic spleen cells. The majority of the activity is directed at I-A hybrid determinants present on (B6A)<sub>F</sub><sub>1</sub> APC. After approximately six further restimulations (Fig. 3B), there is a decided shift in reactivity, so that parental strain A/J APC determinants are predominantly recognized. A similar time-dependent shift in the specificity of I region restriction has been observed for long-term cultures of (T,G)-A-L-reactive T cells (31) and probably represents fluctuations in the growth of individual T cell clones within the culture. Fig. 3C shows the most recent results obtained by assaying the I region restriction of the long-term culture.

In summary, our observations on the reactivity of long-term cultured Mb-reactive T cells suggest that it should be possible to isolate T cell clones that (a) proliferate in response to T cell-stimulating determinants present on CNBr fragments of Mb, and (b) show a variety of patterns of I region restriction.

#### *Mb-reactive T Cell Clones*

T cell clones reactive to native, soluble Mb were isolated in two ways. Initially, soft agar colonies were generated from long-term cultures as previously described (1). After expansion and testing, they were subcloned by limiting dilution. Certain clones were isolated directly from long-term culture by limiting dilution. The properties of clones isolated by the two techniques were identical.

We used three methods to determine that a certain population of T cells was a clone. First, we applied statistical analysis to the limiting dilution experiments to confirm that there was a high probability that growth in microtiter wells was the result of proliferation of a single cell. For example, when clone 45.49 was subcloned at 0.3 cells/well growth occurred in 10 of 48 wells, giving a probability of 83% that the growth derived from a single cell. Second, we subcloned each putative T cell clone to confirm that all subclones had identical reactivity to the parent clone from which they were derived. The third method is based on the specificity of each clone for a particular antigenic determinant on Mb and a particular I region restriction pattern. We titrated each clone with increasing concentrations of CNBr fragments (see below), which were ~95–98% pure as judged by the criteria outlined above (Materials and Methods). We therefore assumed that a T cell population isolated as described above and responding to a given CNBr fragment represented a single clone if its response to the remaining two CNBr fragments (as [<sup>3</sup>H]TdR uptake) did not increase above base line with a 20–50-fold increase in the concentration of these fragments. This point will be illustrated below. In addition, Mb-reactive clones were presumed to have a specific and clearly defined I region restriction pattern. T cell populations not showing specific antigen and I region specificity were recloned.

**CLONE 26.17.** Clone 26.17 was derived by limiting dilution cloning of a soft agar colony (colony 26) that showed predominant reactivity to CNBr fragment 56–131 in addition to native Mb. When clone 26.17 was titrated with increasing concentrations of Mb and its three CNBr fragments at constant cell number, the responses shown in Fig. 4 were obtained. Clone 26.17 proliferates in response to Mb and fragment 56–131 only; responses to fragments 1–55 and 132–153 are base line over a wide range of concentrations. Moreover, the responses to Mb and fragment 56–131 are superimposable, being identical in antigen-dose response and magnitude of maximum stimulation. We interpret these results to indicate that clone 26.17 recognizes an Mb

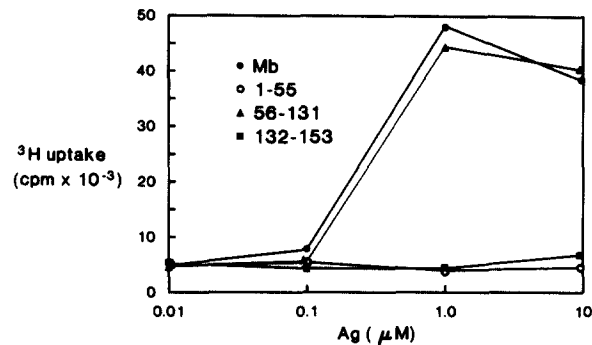


FIG. 4. Dose response of clone 26.17 to Mb and CNBr fragments. Clone 26.17 recognizes a determinant in fragment 56-131.

determinant (epitope) located within fragment 56-131 and is recognized in identical fashion whether it is part of the native molecule or the CNBr fragment.

In addition to antigen (epitope) specificity, clone 26.17 also exhibits I region restriction. Table II shows the response of clone 26.17 (and a number of other clones presented below) to Mb in the presence of spleen cells from a number of hybrid and recombinant mouse strains. The response of clone 26.17 to Mb in the presence of (B6A)<sub>F</sub><sub>1</sub> and (4R × B6)<sub>F</sub><sub>1</sub> spleen cells maps the response to the I-A subregion. In addition, the response to Mb in the presence of syngeneic (B6A)<sub>F</sub><sub>1</sub> spleen cells can be almost completely inhibited by the addition of hybridoma antibodies which react with Ia specificities 19 and 2 (a private specificity of I-A<sup>k</sup>), but not Ia.17 (Table III). These data demonstrate the associative recognition by clone 26.17 of an individual antigenic determinant on Mb fragment 56-131 and a specific I-A molecule on APC.

**CLONE 45.49.** A number of T cell clones have been isolated from (B6A)<sub>F</sub><sub>1</sub> mice immunized with Mb that proliferate in response to fragment 1-55. They fall into two groups based on their I region restriction. The first group is exemplified by clone 45.49 which was derived by limiting dilution cloning of a soft agar colony. As can be seen by the titration data presented in Fig. 5, clone 45.49 responds only to native Mb and fragment 1-55.

The I region restriction pattern of clone 45.49 is shown in Table II. The combination of reactivity to Mb only in the presence of spleen cells of (B6A)<sub>F</sub><sub>1</sub>, B10.A(3R), and (B10 × B10.BR)<sub>F</sub><sub>1</sub> mice demonstrated that gene complementation between the I-A and I-E subregions allowed antigen presentation to this clone. Thus, clone 45.49 recognizes a hybrid Ia antigen composed of the A<sub>e</sub><sup>b</sup> and L<sub>α</sub><sup>k</sup> Ia polypeptide chains. This conclusion was further supported by the inhibition of proliferation caused by the addition of the Y-17 hybridoma antibody (Table III). This antibody recognizes a combinatorial Ia determinant on certain A<sub>e</sub>E<sub>α</sub> complexes (7).

**CLONE 28.1.2.** We isolated a second group of clones that recognized Mb fragment 1-55, but differed from the pattern seen for clone 45.49 in that it exhibited a second pattern of I region restriction. Titration with increasing antigen concentration showed that clone 28.1.2 recognized fragment 1-55 (data not shown). When the I region restriction of clone 28.1.2 was analyzed by assaying proliferation induced by Mb in the presence of the APC (spleen cells) of the strains listed in Table II, the response was mapped to the I-A region. APC from all mice of haplotype I-A<sup>b</sup> could function to present antigen to clone 28.1.2. Cells from mouse strains homozygous for I-A<sup>b</sup> gave

TABLE II  
I Region Restriction of (B6A)F<sub>1</sub> Mb-reactive T Cell Clones

Clone	APC strain		MHC region							
			K	A	B	J	E	C	S	D
26.17 Experiment 1	A/J	704	k	k	k	k	k	d	d	d
	B6	490	b	b	b	b	b	b	b	b
	(B6A)F <sub>1</sub>	30,261	b/k	b/k	b/k	b/k	b/k	b/d	b/d	b/d
	[B10.A(4R) × B6]-F <sub>1</sub>	25,709	k/b	k/b	b/b	b/b	b/b	b/b	b/b	b/b
Experiment 2	(B6A)F <sub>1</sub>	48,740	b/k	b/k	b/k	b/k	b/k	b/d	b/d	b/d
	(B10.MBR × A.AL) <sub>1</sub> F <sub>1</sub>	1,187	b/k	k/k	k/k	k/k	k/k	k/k	k/k	q/k
45.49 Experiment 1	A/J	254	k	k	k	k	k	d	d	d
	B6	1,081	b	b	b	b	b	b	b	b
	(B6A)F <sub>1</sub>	25,826	b/k	b/k	b/k	b/k	b/k	b/d	b/d	b/d
	B10.A(3R)	35,893	b	b	b	b	b	d	d	d
Experiment 2	(B6A)F <sub>1</sub>	24,190	b/k	b/k	b/k	b/k	b/k	b/d	b/d	b/d
	(B10 × B10.BR)F <sub>1</sub>	21,582	b/k	b/k	b/k	b/k	b/k	b/k	b/k	b/k
Experiment 3	(B6A)F <sub>1</sub>	8,995	b/k	b/k	b/k	b/k	b/k	b/d	b/d	b/d
	[A/J × B10.A(4R)]-F <sub>1</sub>	887	k/k	k/k	b/k	b/k	b/k	b/d	b/d	b/d
28.1.2 Experiment 1	A/J	189	k	k	k	k	k	d	d	d
	B6	47,709	b	b	b	b	b	b	b	b
	(B6A)F <sub>1</sub>	39,586‡	b/k	b/k	b/k	b/k	b/k	b/d	b/d	b/d
	B10.MBR	257	b	k	k	k	k	k	k	q
Experiment 2	(B6A)F <sub>1</sub>	13,837	b/k	b/k	b/k	b/k	b/k	b/d	b/d	b/d
	B10.A(4R)	424	k	k	b	b	b	b	b	b
69 Experiment 1	A/J	19,085	k	k	k	k	k	d	d	d
	B6	377	b	b	b	b	b	b	b	b
	(B6A)F <sub>1</sub>	22,108	b/k	b/k	b/k	b/k	b/k	b/d	b/d	b/d
Experiment 1	B10.A(3R)	2,484	b	b	b	b	k	d	d	d
	B10.A(4R)	1,230	k	k	b	b	b	b	b	b
Experiment 2	A/J	11,458	k	k	k	k	k	d	d	d
	B	342	b	b	b	b	b	b	b	b
	(B6A)F <sub>1</sub>	5,943	b/k	b/k	b/k	b/k	b/k	b/d	b/d	b/d
	[B10.A(3R) × B10.A(4R)]F <sub>1</sub>	11,583	b/k	b/k	b/b	b/b	b/b	d/b	d/b	d/b
	B10.MBR	9,973	b	k	k	k	k	k	k	q

Genetic mapping of I region restriction of selected clones. Results given as cpm of [<sup>3</sup>H]TdR uptake on day 3 in the presence of antigen and listed APC.

\* 3 μM Mb. At 3 μM Mb, [<sup>3</sup>H]TdR uptake was 8,120 cpm.

‡ 10 μM Mb.

consistently higher stimulation than I-A<sup>b/k</sup> heterozygous strains, suggesting a dosage effect related to the density of Ia determinants on APC. Further results relating to the effect of Ia antigen density on APC function will be reported independently (D. J. McKean et al., manuscript submitted for publication.).

CLONE 69. As demonstrated in Fig. 3B, the long-term culture of (B6A)F<sub>1</sub> T cells demonstrated I region restriction for parental A/J strain APC after a period of in vitro growth. Therefore, the long-term culture was cloned by limiting dilution with A/J spleen cells as APC. Clone 69 was derived from this experiment. As demonstrated in Table IV, this T cell population responds predominately to fragment 56-131 (and



TABLE III  
Inhibition of T Cell Proliferation by Anti-Ia Monoclonal Antibodies

Clone		Monoclonal antibody	Specificity	cpm
26.17	Experiment 1	150.13	Ia.17	20,237 ± 4,938
		102.16.8	Ia.17	17,770 ± 2,038
		116.32.5	Ia.19	658 ± 38
		118.49	Ia.2	703 ± 196
		none	—	37,410 ± 3,574
45.49	Experiment 1	Y17	Ia.m.44	1,121 ± 545
		118.49	Ia.2	8,411 ± 1,395
		none	—	8,995 ± 2,660
	Experiment 2	Y17	Ia.m.44	5,132 ± 980
		116.32	Ia.19	24,893 ± 562
		None	—	25,826 ± 3,309

Results are expressed as cpm of [<sup>3</sup>H]TdR uptake in the presence of 3 μM Mb and syngeneic APC. Monoclonal antibodies in experiment 1 were used as ascites, 1 μl/culture well. In experiment 2, protein A-purified antibody (1 mg/ml) was used; 3 μl was added per culture well.

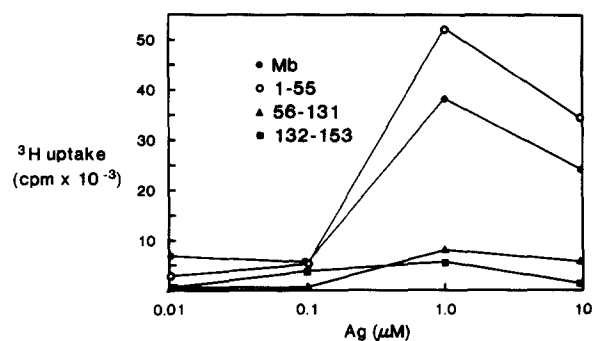


FIG. 5. Dose response of clone 45.49 to Mb and CNBr fragments. Clone 45.49 recognizes a determinant in fragment 1-55.

TABLE IV  
Response of Clone 69 to Mb and Its CNBr Fragments

Clone	Media	Mb	1-55	56-131	132-153
69	813	22,108	6,711	20,839	1,204

Results represent [<sup>3</sup>H]TdR incorporation in the presence of 3 μM antigen and syngeneic (B6A)F<sub>1</sub> APC.

probably does not truly represent a clone of a single cell; further experiments are underway with the expectation of deriving a subclone reactive only with fragment 56-131). The reactivity pattern of clone 69 in Table II demonstrates I region restriction due to recognition of the A<sub>e</sub><sup>k</sup> E<sub>α</sub><sup>k</sup> molecule. Thus, preliminary data indicate that we have identified a fourth class of Mb-reactive (B6A)F<sub>1</sub> T cell.

**FINE SPECIFICITY OF T CELL-STIMULATING DETERMINANTS.** Since CNBr fragment 1-55 contains one antigenic determinant recognized by serum antibody (residues

16-21 [18]) and the synthetic peptide 15-22 had previously been shown to induce T cell proliferation when measured in a peritoneal exudate T lymphocyte-enriched subpopulation (PETLES) assay (32) we attempted to stimulate clones 45.49 and 28.1.2 with this peptide. Over a wide range of antigen concentration (0.01  $\mu$ M - 1 mM) peptide 15-22 did not stimulate proliferation in clones 45.49 and 28.1.2. In contrast, fragment 1-55 induced the expected response.

### Discussion

The *in vitro* isolation and analysis of T cell clones have been instrumental in defining many properties of the cellular immune system. In particular, the demonstration of hybrid F<sub>1</sub> Ia antigens, formed by free combinatorial association of parental strain  $\alpha$  and  $\beta$  Ia polypeptides was particularly well suited to analysis by T cell clone (5) and monoclonal antibody (7) techniques. These studies lend strong support to the idea that Ia antigens are the gene products responsible for Ir gene function by virtue of their recognition, in association with antigen, by helper T cells. A key step in defining the mechanism of the Ir gene control of T cell responses is to determine whether the APC directs T cell reactivity by presenting only a limited number of possible antigenic determinants to T cells (determinant selection theory) or whether T cell reactivity is solely a property of the T cell receptor repertoire. We sought to explore this area by raising T cell clones reactive with a multideterminant antigen under Ir gene control. As the results above demonstrate, we have succeeded in deriving a number of clones of Mb-reactive T cells from (B6A)F<sub>1</sub> mice that recognize individual determinants on Mb and exhibit several patterns of Ia restriction.

Several points relating to the analysis of our long-term T cell culture are worth noting. (a) Upon examining the reactivity of the long-term T cell culture, we estimated that there were far fewer clones (total cell numbers) reactive with CNBr fragment 132-153 than with the remaining fragments. Indeed, to date we have been unable to derive clones reactive solely to this fragment. (b) During serial analysis of I region restriction, we noted the emergence of parental strain A restriction specificity over time (Fig. 3B). This may indicate that (B6A)F<sub>1</sub> Mb-reactive T cells restricted by strain A/J Ia determinants are inhibited *in vivo* but are free to expand in culture. (c) Despite several types of analyses (Figs. 1 and 2), reactivity of the long-term culture to the individual CNBr fragments at a given point never conveniently summed to the response to Mb alone. This may be due to the release of lymphokines that cause antigen-independent proliferation of the culture even when only a fraction of the clones are stimulated by antigen (33). Taken together, these results indicate that the long-term T cell "bulk" culture may not simply reflect the sum of the reactivities of individual clones. Cell types of particular reactivity present *in vivo* in small numbers may proliferate under favorable *in vitro* conditions. Other T cell clones, perhaps even other classes of T cells, may be present but not susceptible to isolation by cloning techniques. For these reasons, our results do not allow general conclusions relating to the T cell repertoire and the regulation of this repertoire *in vivo*.

The T cell clones for which titration data have been presented above exhibit specific responsiveness to particular CNBr fragments of Mb. In fact, this data can be used as evidence that a particular T cell population is a clone, i.e., it exhibits a specific pattern of antigen and Ia recognition. A striking finding is the identity, within statistical limits, of the titration curves for a particular clone when stimulated by Mb

and the appropriate CNBr fragment. It is noteworthy to compare these results to the findings (24) that the three CNBr fragments quantitatively bound serum antibody directed against native Mb. In the case of the antibody-binding activity of each peptide, however, a 4–10-fold M excess relative to native Mb was required for maximum binding. This was attributed to concentration-dependent conformational effects necessary to produce the native conformation required for antibody binding. Since, in the present study, the CNBr fragments were equally as efficient as native Mb in stimulating proliferation of individual T cell clones, it may be that the present class of T cells does not recognize antigen in its native conformation. If true, this finding would be consistent with other studies dealing with the recognition of native and denatured antigens by T cell subclasses (11) and would suggest that the T cell clones presented above are helper T cells. Whatever the final antigen configuration recognized by the T cells, the antigen titration data imply that the processing events required for antigen presentation do not distinguish between native Mb and its CNBr fragments.

We are currently attempting to define more precisely the antigenic determinants of Mb that are recognized by the above T cells. We are particularly interested in the relationship between determinants recognized by T cells and those recognized by B cells (antibody). For this reason, we attempted to stimulate two T cell clones, responsive to CNBr fragment 1–55, with peptide 15–22. This peptide contains the only antigenic determinant in fragment 1–55 that has been shown to be recognized by antibody (18). Peptide 15–22 produced no response in clones 28.1.2 and 45.49. Since this peptide was stimulatory to a PETLES cell population (32), it might be that clones 28.1.2 and 45.49 recognize a different epitope contained in 1–55. Alternatively, presentation and/or T cell recognition mechanisms may be significantly different in our assay system and the PETLES assay. We are currently exploring other methods to assess similarities and differences in the epitopes recognized by T and B cells. Studies aimed at further defining T cell epitopes may help us to understand the mechanism(s) of T cell recognition and activation.

The results for clone 45.49, both from genetic analysis (Table II) and anti-Ia hybridoma inhibition (Table III), provide convincing evidence that genetic control of responsiveness to certain Mb determinants is controlled by I-A/I-E subregion interaction. Specifically, clone 45.49 responds to a Mb determinant located within fragment 1–55 and this response is restricted by recognition of the Ia product  $A_e^b E_a^k$ , which is a target for the hybridoma antibody Y17 (7). Previously (28–30), response to certain Mb determinants had been mapped to the I-C subregion by virtue of the ability of B10.A(5R) macrophages to partially reconstitute the responsiveness of (B6 × DBA/2)F<sub>1</sub> (low responder × high responder) lymph node cells. Our data suggest a mechanism to explain the seemingly disparate results. Since B10.A(5R) APC express an  $A_e^b E_a^k$  complex on their cell surface, this complex might be recognized by both (b × k)F<sub>1</sub> (i.e., clone 45.49) and (b × d)F<sub>1</sub> T cells. Indeed, using alloreactive and Mb-reactive T cells that are restricted to the  $A_e^b E_a^k$  complex, we have demonstrated cross-reactivity among  $A_e^b E_a^k$ ,  $A_e^b E_a^d$ ,  $A_e^b E_a^r$ , and  $A_e^b E_a^u$  complexes when functioning as MLR-stimulating and/or I region restriction determinants (B. N. Beck, A. J. Infante, and C. G. Fathman, manuscript in preparation) (9).

Determinant selection theories propose that antigen and Ia determinants on APC form specific interaction complexes, e.g., the IAC complex of Puri and Lonai (34).

These complexes would then be the structures that are recognized by T cells. Such mechanisms explain Ir gene-controlled low responsiveness as the inability of APC of the low responder strain to form an appropriate antigen-Ia complex that can then be recognized by the corresponding T cell. Using whole immune lymph node T cells, Richman et al. (35) have shown a failure of Mb and fragment 1-55 to cause (high responder  $\times$  low responder) $F_1$  T cells to proliferate when presented by macrophages of the low responder ( $H-2^b$ ) strain. This was interpreted to mean that a defect in APC function resulted in the low responder status of  $H-2^b$  mice. Clearly, clone 28.1.2 above proliferates quite well in response to Mb presented by strain B spleen cells. We favor the interpretation that clones similar to 28.1.2 may be suppressed in vivo, but are able to expand in in vitro culture. We have demonstrated a similar situation for (T,G)-A-L reactive (A  $\times$  B) $F_1$  T cells, which can proliferate in response to antigen in the presence of low responder strain A spleen cells (31). These results indicate that genetically determined low responsiveness to Mb and (T,G)-A-L is not due to failure of antigen presentation, i.e., failure to form an appropriate Ia-antigen complex. Furthermore, the presentation of Mb to (B6A) $F_1$  clone 28.1.2 by  $H-2^b$  spleen cells might explain the seemingly anomalous finding that proliferation of (B  $\times$  D) $F_1$  T cells can be blocked by treating B10.A(5R) macrophages with anti-I-A<sup>b</sup> antibodies (36). This result was originally interpreted as indicating inhibition of a (high responder  $\times$  low responder) $F_1$  T cell response by an anti-Ia reagent directed at the low responder allele. Our findings support the conventional notion that anti-Ia antibodies inhibit T cell responsiveness by direct blocking of the APC Ia molecule recognized by the T cell.

The I region restricted, clonal T cell response to multideterminant antigens provides another useful way to probe T cell/APC interactions. A rigorous application of determinant selection theories to T cell recognition of a multideterminant antigen would lead to the conclusion that each individual determinant should be recognized in association with a separate presenting cell Ia epitope. Our results demonstrate that there are at least two Mb determinants recognized by T cells, one on fragment 1-55 (clones 28.1.2 and 45.49) and one on 56-131 (26.17 and 69). The existence of three or four distinct determinants cannot be excluded at present. Similarly, four of a possible six Ia antigen configurations are utilized by these clones as restriction elements. We should therefore be able to test for APC determinant selection by precisely defining the T cell-stimulating epitopes of Mb and mapping the corresponding Ia restriction sites. If the same Mb determinant can be recognized in association with more than one Ia restriction site, the control of T cell responsiveness should be due to T cell receptor specificity and not determinant selection by APC Ia molecules.

### Summary

We have been able to isolate clones of sperm whale muscle myoglobin (Mb)-reactive T cells from (C57BL/6  $\times$  A/J) $F_1$  [(B6A) $F_1$ ] mice. Four types of clones were isolated, distinguished by their patterns of recognition of Mb cyanogen bromide (CNBr) fragments and antigen presenting cell (APC) requirements. Individual T cell clones proliferated in response to one of three CNBr fragments of Mb. Dose-response curves of all clones were identical for native Mb and the appropriate fragment. T cell clones reactive to fragment 1-55 did not proliferate in response to peptide 15-22 (a peptide that binds to serum antibody directed against 1-55). These data support

previous findings suggesting differences between antigen recognition by T and B cells, i.e., T cells may not recognize antigen in its native conformation and/or T and B cells may recognize distinct epitopes on the same antigen.

Using T cell clones to analyze genetic control of responsiveness to Mb, we found that certain (B6A)F<sub>1</sub> T cells recognize Mb presented by low responder strain APC. Thus, genetically determined low responsiveness in this case is probably not due to failure of APC function. We also found that responsiveness to certain Mb epitopes mapped to the I-A subregion whereas others mapped, via gene complementation, to the I-A and I-E subregions. We found no examples of responsiveness mapping to the I-C subregion and suggest an alternative explanation for previous reports mapping genetic control of responsiveness to certain Mb determinants to I-C.

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