

# FUNCTIONAL PROPERTIES OF T CELL CLONES WITH A DOUBLE SPECIFICITY FOR ALLOANTIGENS AND FOREIGN ANTIGENS

BY STANLEY J. WATERS, PAOLO R. LUZZATTI, AND  
CONSTANTIN A. BONA

*From the Department of Microbiology, Mount Sinai School of Medicine,  
New York, New York 10029*

One of the intriguing properties of T cell population is that the frequency of the precursors that respond to major histocompatibility complex (MHC)<sup>1</sup>-encoded alloantigens are several orders of magnitude higher than the frequency of precursors responding to foreign antigens (1, 2). To explain this observation, it has been postulated that at least some T cells responding to the MHC alloantigens are also capable of responding to various foreign antigens.

Studies on the specificity of T cell clones provided direct support for this hypothesis. Indeed, several T cell clones recognizing foreign antigens in association with self-MHC-encoded antigens and displaying alloreactivity have been reported (3-6). The observation that half of the clones having identical specificity for an insulin epitope recognized in association with syngeneic I-A<sup>b</sup> gene product also respond to alloantigens of H-2<sup>u</sup> haplotype, indicated that a certain degree of mimicry exists between alloantigens and foreign antigens associated with syngeneic H-2 gene products (7). Although these studies demonstrate that a single T cell clone specific for antigen in association with a syngeneic H-2 gene product can also recognize alloantigenic determinants, the question as to whether there are two T cell recognition units, one specific for the alloantigens and the other for foreign antigens, or whether a single receptor is responsible for this recognition event, has not definitely been resolved.

Information regarding the genetic restriction of B cells with T cell clones possessing double specificity is limited. However, this issue is of theoretical interest, particularly with regard to the relationship between the receptor recognizing the foreign and alloantigens and the products of T clones critical for the activation of B cells.

This communication presents the results of a functional analysis of four clones originating from a CB6F<sub>1</sub> mouse immunized with keyhole limpet hemocyanin (KLH). Two clones expressing a helper phenotype develop proliferative response not only to KLH in association with syngeneic Ia but also to H-2<sup>k</sup> and H-2<sup>d</sup>

This work was supported in part by grant PCN 1105788 from the National Science Foundation and grant IM-275A from the American Cancer Society.

<sup>1</sup> *Abbreviations used in this paper:* APC, antigen-presenting cells; BUdR, 5-bromo-deoxy uridine; FACS, fluorescence-activated cell sorter; HGPRT, hypoxanthine guanine phosphoribosyltransferase; KLH, keyhole limpet hemocyanin; MHC, major histocompatibility complex; MLR, mixed lymphocyte response; OVA, ovalbumin; PFC, plaque-forming cell; TNP, trinitrophenyl.

allogeneic cells, respectively. Both antigen- and alloantigen-induced proliferative responses were inhibited by an anti-clonotypic monoclonal antibody. These two clones provided help via a hapten carrier bridge for only syngeneic B cells.

### Materials and Methods

**Mice.** BALB/c, C57/6, CB6/F<sub>1</sub> (BALB/c × C57BL/6), C3H/He, A/J, RIII, and PL/J, 6–8 wk old were purchased from The Jackson Laboratory, Bar Harbor, ME. B10.Q and C3H.Q were obtained from the colony of Dr. Dan Meruelo of the New York University School of Medicine.

**Antigens.** KLH (Calbiochem-Behring Corp., San Diego, CA) and ovalbumin (Sigma Chemical Co., St. Louis, MO) were conjugated separately with 2,4,6-trinitrobenzene sulfonic acid (Eastman Kodak Co., Rochester, NY) in 0.083 M sodium bicarbonate buffer and then dialyzed against 0.1 M potassium bicarbonate buffer. The degree of conjugation was determined by relative optical densities to be 18 trinitrophenyl (TNP) groups per 100,000 mol wt KLH(TNP-KLH) and 12 TNP groups per molecule of ovalbumin (TNP-OVA). Horse cytochrome *c* (Sigma) and an isolate of type A influenza virus, PR8, were used as controls in determining the antigen specificity of T cell clones.

**Monoclonal Antibodies.** Hybridoma cell lines with defined specificities for I-A<sup>b</sup> (25-9-3s) and I-A<sup>d</sup> (MK-D6) were purchased from the American Type Culture Collection, Rockville, MD. Ascites of anti-Lyt-1.2 and anti-Lyt-2.2 were purchased from New England Nuclear, Boston, MA. Ascites of hybridoma 30-H12 (anti-Thy-1.2) and 10.2-16 (anti-I-A<sup>b</sup>) originally defined by Drs. Ledbetter and Herzenberg were generously provided by Dr. Benvenuto Pernis, Columbia University, NY. All anti-I-A antibodies of IgG class used in T cell proliferative blocking experiments were purified on a protein A-Sepharose 4B column.

**Preparation of T Cell Clones.** Lymph node T lymphocytes were obtained from CB6/F<sub>1</sub> mice primed with 100 μg KLH in Freund's complete adjuvant (Difco Laboratories, Detroit, MI). The mice were injected subcutaneously in the base of the tail and in the hind foot pads. 7 d later, T cells from the periaortic popliteal and inguinal lymph nodes were purified on a nylon wool column (8).

The KLH-reactive T cells were further enriched by culturing the nonadherent population from the nylon wool column with 50 μg KLH/ml for 4 d in T cell medium (RPMI 1640), containing 10% fetal calf serum (Reheis Chemical Co., AZ), 12 mm Hepes, 100 μg/ml penicillin, 100 mg/ml streptomycin, 2 × 10<sup>-3</sup> M glutamine, and 3 × 10<sup>-5</sup> M 2-mercaptoethanol. After 4 d of incubation at 37°C and 7% CO<sub>2</sub>, lymphoblasts were isolated on a discontinuous Percoll density gradient (9). The enriched blasts were established as long-term T cell lines by serial restimulation *in vitro* as described by Kimoto and Fathman (10). The T cell line was then cloned by stringent limiting dilution (0.5 cells/well) condition as previously described (10).

**Proliferation Assays.** All T cell clones and subclones were tested for proliferation to a panel of soluble protein antigens and allogeneic lymphocytes. 7 d after subculture without antigen, 10<sup>4</sup> cloned T cells and 5 × 10<sup>4</sup> irradiated syngeneic or allogeneic spleen cells were cultured in 0.2-ml microtiter wells. After 3 d, the cultures were pulsed with [<sup>3</sup>H]thymidine (1 μCi/well) and harvested 18 h later. No exogenous IL-2 was added to the cultures. All responses were measured in triplicate cultures.

**BUdR Suicide Experiment.** These experiments were performed according to a previously described technique (11). Briefly, 5 × 10<sup>4</sup> cells were incubated for 36 h at 37°C in medium containing KLH or allogeneic cells without or with 10<sup>-5</sup> M BUdR. The cells were then exposed to fluorescent light for 2 h (40 W, F40 D light fluorescent bulbs; Sylvania Lighting Products Div., Hillsboro, NH). The cultures were placed at a distance of 20 cm from the plane of the bulbs. After three washings, the cells were incubated an additional 36 h with KLH or allogeneic cells.

**In Vitro Antibody Synthesis Assay.** TNP-OVA-primed spleen cell suspensions were depleted of T cells by treatment with anti-Thy-1.2 and anti-Lyt-1.2 plus complement (12). A total of 3 × 10<sup>6</sup> T-depleted, primed B cells were cultured with 1 × 10<sup>4</sup>–1 × 10<sup>6</sup> lymph

nodes from KLH-primed mice or cloned T helper cells in 2 ml of T cell media. Three 16-mm diam wells (3524; Costar, Cambridge, MA) were used for each T cell concentration tested. Cultures were incubated for 5 d at 37°C in a 7% CO<sub>2</sub>-humidified incubator. Cells were cultured in either the presence or absence of 0.001–10 µg/ml TNP-KLH. An additional culture containing 10 µg/ml or 0.001 µg/ml TNP-OVA was used as specificity control.

*Plaque-forming Cell Assay.* Sheep erythrocytes (SRBC) were coupled with TNP (TNP-SRBC) by the method of Rittenberg and Pratt (13). The above cultures were harvested, washed, and assayed for plaque-forming cells (PFC) on TNP-SRBC. Both direct (IgM) and indirect (IgG) plaques were measured according to the method described by Cunningham et al. (14).

*Preparation of Monoclonal Anti-clonotype Hybridomas.* CB6/F<sub>1</sub> mice were immunized with the parental KLH-specific T cell line from which all the T cell clones were derived. The initial intraperitoneal immunization with 2 × 10<sup>6</sup> syngeneic T cell line was followed by three weekly intravenous boosts with 10<sup>6</sup> T cell line. 5 d after the final boost, 10<sup>8</sup> lymph node cell or spleen cell suspensions from the immunized mice were fused with SP2/0 myeloma cells according to a previously described method (10). The antibodies secreted by these hybridomas were screened for their ability to bind a single T clone among our panel of KLH-specific T cell clones. Binding was detected using indirect fluorescence staining technique and radioimmunoassay. T cell clones were incubated for 45 min with culture supernatant, washed, and stained with fluoresceinated goat anti-mouse Ig. A fluorescence-activated cell sorter (FACS III; B-D FACS Systems, Sunnyvale, CA) was used to determine the percentage of positively stained cells. In radioimmunoassay, 3 × 10<sup>5</sup> T cells were incubated for 45 min at 4°C in PBS supplemented with 1% BSA, 0.05% azide, and 10 µg purified antibodies. After three washings, the cells were incubated for 2 h at 4°C with <sup>125</sup>I-rat anti-murine kappa chain antibodies (50,000 cpm) and then washed extensively. The radioactivity was counted in a γ-spectrometer.

## Results

*Establishment of KLH-specific T Cell Clones.* Several T cell clones were derived by limiting dilution culture at 0.25 cells per well of a T cell line obtained from KLH-primed CB6/F<sub>1</sub> mice. Four clones were chosen to be studied in detail because they exhibited an antigen-specific proliferative response to KLH, and not to other unrelated antigens (cytochrome *c* and PR8 influenza virus) in the presence of irradiated CB6/F<sub>1</sub> splenic cells (Table I). These clones also did not exhibit a proliferative response to syngeneic CB6/F<sub>1</sub> cells in the absence of antigen. Subcloning by limiting dilution culture was done at least once with each of the four clones. Subclone C1.4 derived from clone C1 and subclone A12.11 derived from clone A12 were utilized in the present study. In some cases, as with clone A12, the magnitude of proliferation to KLH was enhanced after subcloning. The cell surface phenotype of the clones were analyzed by indirect immunofluorescence. The clones and subclones in Table I expressed Thy-1.2 and Lyt-1.2 differentiation antigens and lacked Lyt-2.2 antigens. Fluorescence profiles gave no indication of subpopulations of cells within the clonal population (data not shown).

*Characterization of the Genetic Restriction of T Cell Clones.* The KLH-induced proliferative response of T cell clones was studied in the presence of CB6F<sub>1</sub>, BALB/c, and C57BL/6 irradiated spleen cells. The data in Table II illustrates two patterns of genetic restriction: clones D18, F6, and C1.3 proliferate only in the presence of CB6F<sub>1</sub> and C57BL/6, whereas clone 12.11 proliferates only in the presence of CB6F<sub>1</sub> and BALB/c. These results indicated that while D18, F6,

TABLE I  
*KLH-specific Proliferative Responses*

Responder cells	Nil	CB6 irradiated cells plus <sup>‡</sup>		
		KLH (40 µg)	PR8 (10 µg)	Cytochrome (50 µg)
I (Line)	214 ± 112*	13,781 ± 1,789	733 ± 62	193 ± 40
A12 (clone)	137 ± 19	1,381 ± 113	145 ± 11	110 ± 18
A12.11 (subclone)	497 ± 51	16,279 ± 287	520 ± 58	570 ± 173
Cl (clone)	210 ± 27	5,481 ± 17	182 ± 12	189 ± 13
Cl.3 (subclone)	495 ± 18	5,137 ± 800	465 ± 33	516 ± 43
F6 (clone)	451 ± 4	16,602 ± 478	376 ± 70	386 ± 62
D18 (clone)	488 ± 12	15,359 ± 478	367 ± 61	471 ± 36
Cytochrome primed	5,315 ± 813	ND <sup>§</sup>	ND	32,386 ± 687
KLH primed	1,446 ± 54	9,925 ± 1,242	3,105 ± 69	2,143 ± 42

\* 10<sup>4</sup> responder cells/culture for the parental line and all clones or subclones. Cytochrome of KLH-primed lymph node cells were cultured at 10<sup>6</sup> cells/culture. Results are tritiated thymidine incorporation of triplicate cultures presented in cpm ± SD.

<sup>‡</sup> 10<sup>6</sup> (cells/culture) syngeneic splenic cells were added for all clones. PR8 is a solubilized glycoprotein from an isolate of influenza virus. Optimal antigen concentrations for KLH, cytochrome *c*, and PR8 were determined by in vitro proliferation.

<sup>§</sup> ND, not done.

TABLE II  
*Genetic Restriction of T Cell Clones*

Responder cells	Irradiated cells*						
	CB6/F <sub>1</sub>			BALB/c		C57BL/6	
	nil	KLH	KLH	nil	KLH	nil	KLH
A12.11	142 ± 30 <sup>‡</sup>	121 ± 10	23,685 ± 3,116	86 ± 8	22,740 ± 2,170	238 ± 26	733 ± 283
Cl.3	85 ± 6	142 ± 25	20,445 ± 6,006	113 ± 62	237 ± 71	235 ± 69	17,348 ± 2,314
D18	241 ± 76	191 ± 7	31,963 ± 2,528	130 ± 26	304 ± 50	213 ± 19	14,549 ± 1,755
F6	73 ± 11	159 ± 32	20,445 ± 6,006	113 ± 62	249 ± 32	143 ± 40	32,023 ± 3,296

\* Responder T clones (10<sup>4</sup> cells/culture) stimulated with syngeneic CB6/F<sub>1</sub> or parental BALB/c, C57BL/6 (10<sup>6</sup> cells/culture), and 50 µg/ml KLH.

<sup>‡</sup> Tritiated thymidine incorporation of triplicate cultures presented as cpm ± SD.

and Cl.3 are restricted to H-2<sup>b</sup> determinants, the clone A12.11 was restricted to H-2<sup>d</sup> determinants for KLH-specific proliferation.

*Alloreactivity of T Cell Clones.* The ability of four clones to proliferate upon in vitro culture with stimulating cells of various H-2 haplotypes was further investigated. Clone D18 and subclone Cl.4 did not exhibit any proliferative response to the panel of stimulating cells used. (H-2<sup>d,b,k,q,a,s,r,u</sup>) (data not shown).

In contrast, clone F6 and subclone A12.11 showed a strong proliferative response to H-2<sup>q</sup> and H-2<sup>k</sup>, respectively. The data depicted in Table III strongly suggest that the proliferation was related to Ia determinants and not to MLS determinants of stimulating cells. Indeed, the A12.11 clone proliferated upon culture with spleen cells from C<sub>3</sub>H (H-2<sup>k</sup>, MLS<sup>c</sup>) and CAB/N (H-2<sup>k</sup> MLS<sup>-</sup>), but not with SJL (H-2<sup>s</sup> MLS<sup>c</sup>), mice, whereas clone F6 proliferated upon culture with spleen cells from C<sub>3</sub>H.a (H-2<sup>q</sup> MLS<sup>c</sup>) and B10.q (H-2<sup>q</sup> MLS<sup>b</sup>) mice (Table III). These clones did not proliferate in the presence of spleen cells of other haplotypes (H-2<sup>b,d,r,u,s</sup>) (data not shown).

TABLE III  
<sup>3</sup>H]Thymidine Incorporation of T Cell Clones Stimulated by Syngeneic and Allogeneic Cells

Stimulating cells	Haplotype		Responder T cell clones		
	H-2	MLS	F6	A12	A12.11
nil			105 ± 17*	131 ± 19	121 ± 20
BALB/c	d	b	400 ± 16	246 ± 10	539 ± 172
C57BL/6	b	b	414 ± 40	350 ± 20	431 ± 23
CB6F <sub>1</sub>	d/b	b	451 ± 14	755 ± 17	497 ± 51
C3H/He	k	c	471 ± 43	ND <sup>†</sup>	66,617 ± 10,760
CBA/N	k	—	536 ± 64	6,371 ± 280	12,871 ± 3,070
CE/J	k	c	ND	10,063 ± 85	ND
SLJ	s	c	373 ± 48	ND	425 ± 55
B10.q	q	b	65,663 ± 5,996	ND	502 ± 29
C3H.q	q	c	42,289 ± 9,410	ND	455 ± 24

\* Mean cpm ± SD of triplicate culture.

<sup>†</sup> ND, not done.

**BUDR Suicide Experiments.** The clone F6 and subclone A12.11 have been obtained under stringent limiting dilution conditions and, as shown in Table's I and III, the A12.11 subclone exhibited the same antigenic specificity and allogeneic reactivity pattern as the A12 parental clone. However, because these clones exhibited alloreactive proliferation property, we carried out BUDR suicide experiments to assess whether or not the same cells that respond to KLH in association with parental Ia determinants exhibited the ability to proliferate to Ia allodeterminants.

In these experiments, the cells were cultured in the presence of minute amounts of IL-2, KLH, or alloantigens, and on day 2, BUDR was added for 18 h and then the cultures were exposed to light for 2 h. The cells cultured for 4 d were reciprocally stimulated with KLH, fresh irradiated syngeneic splenic cells, or allotype stimulator cells. The data presented in Table IV show that the clones cultured with small amounts of IL-2 followed by stimulation with KLH or alloantigen, exhibited significant proliferation with the second round of stimulation. In contrast, clone F6 and subclone A12.11, when stimulated with either KLH or alloantigen on the first round, incubated with BUDR, and exposed to light, did not mount a proliferative response when stimulated with the reciprocal antigen. The results support the clonal nature of F6 and A12.11 T cell clones displaying a double specificity.

**Helper Function of T Cell Clones.** The helper activity of clones expressing Lyt-1<sup>+</sup>, 2<sup>-</sup> surface markers was tested in an in vitro antibody synthesis assay by the incubation of KLH-specific T clones or lymph node cells with highly purified B cells from TNP-OVA-primed CB6/F<sub>1</sub> mice in the presence of TNP-KLH. Similarly, cultures were incubated with TNP-OVA as a specificity control. The results depicted in Fig. 1 show the effect of antigen dose on the generation of an anti-TNP response. All the clones tested provided a KLH carrier-specific helper activity at various antigen doses for IgM and IgG response. While with KLH-primed lymph node T cells a higher IgG PFC response was observed with low antigen dose, no significant variations in the magnitude of IgM and IgG PFC

TABLE IV  
*Reciprocal Stimulation with KLH and Allogenic Cells of T Cell Clones with Double Specificity after BUdR-light Treatments*

Responding clones	Response							
	day 0	day 2	day 3	day 3	day 4	day 7	day 8	
A12.11	nil	—	—	3H-T	439 ± 22*			
	KLH	—	—	3H-T	3,168 ± 361			
	C3H	—	—	3H-T	3,974 ± 74			
	nil	BUdR <sup>‡</sup>	light	3H-T	320 ± 15			
	KLH	BUdR	light	3H-T	468 ± 15			
	C3H	BUdR	light	3H-T	511 ± 34			
	IL-2 <sup>‡</sup>	BUdR	light	nil		3H-T	505 ± 53	
	IL-2	BUdR	light	KLH		3H-T	3,717 ± 712	
	IL-2	BUdR	light	C3H		3H-T	16,003 ± 2,330	
	KLH IL-2	BUdR	light	nil		3H-T	392 ± 15	
	KLH IL-2	BUdR	light	KLH		3H-T	579 ± 153	
	KLH IL-2	BUdR	light	C3H		3H-T	473 ± 45	
	C3H IL-2	BUdR	light	nil		3H-T	343 ± 32	
	C3H IL-2	BUdR	light	KLH		3H-T	585 ± 74	
	C3H IL-2	BUdR	light	C3H		3H-T	450 ± 45	
	F6	nil	—	—	3H-T	844 ± 170		
		KLH	—	—	3H-T	12,835 ± 836		
		B10.Q	—	—	3H-T	16,541 ± 1,238		
		nil	BUdR	light	3H-T	794 ± 341		
KLH		BUdR	light	3H-T	1,308 ± 248			
B10.Q		BUdR	light	3H-T	2,463 ± 457			
IL-2		BUdR	light	nil		3H-T	639 ± 381	
IL-2		BUdR	light	KLH		3H-T	4,462 ± 1,016	
IL-2		BUdR	light	B10.Q		3H-T	5,015 ± 499	
KLH IL-2		BUdR	light	nil		3H-T	260 ± 100	
KLH IL-2		BUdR	light	KLH		3H-T	257 ± 224	
KLH IL-2		BUdR	light	B10.Q		3H-T	350 ± 154	
B10.Q IL-2		BUdR	light	nil		3H-T	879 ± 168	
B10.Q IL-2		BUdR	light	KLH		3H-T	1,025 ± 217	
B10.Q IL-2		BUdR	light	B10.Q		3H-T	984 ± 143	

\* Mean cpm ± SD of triplicate cultures.

<sup>‡</sup> Cultures contained 10<sup>4</sup> responding clone A12.11 or F<sub>6</sub> and 5 × 10<sup>5</sup> splenic filler cells on day 0. After stimulation with KLH or allogeneic stimulator cells on day 2 BUdR was added for 18 h followed by exposure to light for 2 h. Fresh splenic fillers plus KLH or allogeneic filler cells were added to reciprocally stimulated cultures. Tritiated thymidine incorporation was measured on day 4 and day 8.

<sup>‡</sup> Cultures contained 0.1% IL-2 instead of 2% in standard culture.

response were noted upon incubation of T cell clones with various doses of antigen (0.001–10 µg).

The experiments presented in Fig. 2 compare the helper capacity of the T clones with KLH-primed lymph node cells in the generation of an anti-TNP response. In this experiment, 4 × 10<sup>6</sup> B cells were incubated with various concentrations of T cell clones per culture. In this particular experiment, we observed that clone F6 and subclone C1.3 provided stronger helper activity than did a heterogeneous lymph node population of KLH-primed cells.

*Genetic Restriction of Helper Property of A12.11 T Cell Subclone Exhibiting Double Specificity.* Further studies on helper property of T cell clone exhibiting double specificity were focused on only A12.11 T cell subclone, because of the lack of monoclonal antibodies specific for H-2<sup>d</sup> and the poor availability of monoclonal antibodies specific for I-A<sup>d</sup> and I-A<sup>k</sup>. The data depicted in Table V show that A12.11 cooperated with only CB6F<sub>1</sub> B cells to provide carrier-specific help. In

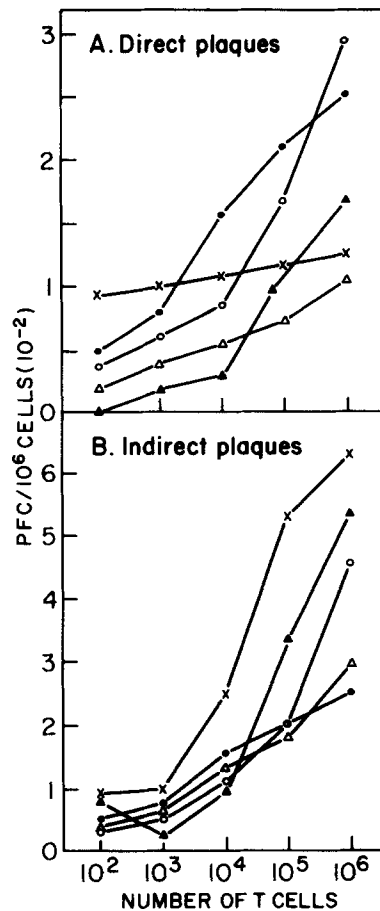


FIGURE 1. Dose-effect relationship between the number of T cells and anti-TNP PFC response. Various numbers of (O) lymph node cells from KLH primed CB6/F<sub>1</sub> mice, (●) clone D18, (Δ) subclone C1.3, (X) clone F<sub>6</sub>, and (▲) subclone A12.11 cultured with  $21 \times 10^6$  TNP-OVA-primed B cells in the presence of (0.001  $\mu\text{g}/\text{ml}$ ) TNP-KLH. The response was measured on day 4.

contrast, no significant helper activity was observed with C<sub>3</sub>H/He TNP-primed B cells, in three independent experiments upon the culture of  $2 \times 10^6$  B cells with  $3 \times 10^5$  A12.11 cells. Furthermore, no help for C<sub>3</sub>H/He and CB6F<sub>1</sub> B cells was observed upon the culture of A12.11 T cells in presence of TNP-OVA and KLH. Similarly, the clone F<sub>6</sub> also did not provide help to allogeneic B cells (H-2<sup>d</sup>) data not shown). Because of these surprising results, which suggested that A12.11 KLB-specific and H-2<sup>k</sup>-reactive clones require covalent hapten-carrier linkage and lack helper activity for alloreactive B cells, in further experiments, we investigated the effect of various concentrations of T cells on the anti-TNP response.

The results illustrated in Fig. 3 show that the incubation of  $2 \times 10^6$  C<sub>3</sub>H/HeJ TNP-primed B cells with various concentrations of ( $10^4$ – $10^6$ ) A12.11 T cells does not lead to a significant increase of anti-TNP PFC above background. In

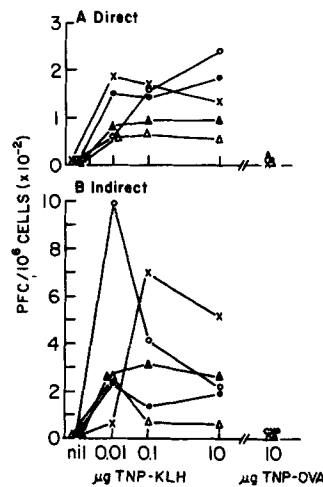


FIGURE 2. Dose-effect relationship between antigen concentration and anti-TNP PFC response.  $3 \times 10^5$  cells from (○) lymph node cells from KLH-primed CB6F<sub>1</sub> mice, (●) clone D18, (△) subclone C1.3, (X) clone F<sub>6</sub>, and (▲) subclone A12.11 were cultured with  $2 \times 10^6$  TNP-OVA-primed B cells. The response was measured on day 4.

contrast, a dose-dependent increase of the anti-TNP PFC response was observed when B cells were incubated with various concentrations of T cells ( $10^4$ – $3 \times 10^5$ ), i.e., C<sub>3</sub>H/HeJ TNP-primed B cells with C<sub>3</sub>H/HeJ KLH-primed T cells or CB6F<sub>1</sub> primed B cells with A12.11 cells. Furthermore, we investigated whether A12.11 cells were capable of providing help in a nonspecific manner to syngeneic B cells (CB6F<sub>1</sub>) or to the allogeneic B cells (C<sub>3</sub>H/HeJ and CBA/N) expressing H-2<sup>k</sup> haplotype. This was particularly important, since Asano et al. (15) reported data indicating that the same T cell can help in a MHC-restricted manner, the Lyb-5<sup>-</sup> B cells, and in a MHC nonrestricted fashion, the Lyb-5<sup>+</sup> B cells. The Marrack and Kappler assay system (16) was employed to investigate whether clone A12.11 stimulated by KLH in the presence of syngeneic APC, can help normal B cells to mount a SRBC PFC response upon the culture in presence of SRBC.

The data depicted in Table VI show that spleen cells from SRBC-primed CB6F<sub>1</sub>, C<sub>3</sub>H/HeJ and CBA/N develop an excellent anti-SRBC PFC response upon in vitro culture with SRBC. No, or a very weak, anti-SRBC-PFC response was observed in the cultures where A12.11 clone was incubated in the presence of KLH and SRBC with B cells from CB6F<sub>1</sub>, C<sub>3</sub>H/HeJ, or CBA/N. Furthermore, the addition to the culture of CB6F<sub>1</sub> spleen cells did not augment the anti-SRBC PFC response.

*Effect of Anti-Ia and Anti-clonotype Antibodies on the Proliferative Response of A12.11 T Cell Subclone.* Several syngeneic monoclonal anti-Id antibodies were generated by a fusion of SP2/0-Ag14 myeloma cells with spleen cells and lymph node cells obtained from CB6F<sub>1</sub> mice immunized with the KLH-specific T cell line. The hybridomas secreting monoclonal antibodies specific for T cell clones were selected by IF and then were cloned. The clones secreting monoclonal antibodies were expanded in ascites and their products after typing of heavy and



TABLE V  
Genetic Restriction and Requirement for Linked Carrier Hapten Recognition of Helper Effect A12.11 Clone

TNP-OVA primed cells	KLH-specific T cells	Anti-TNP PFC/culture									
		Exp. 1		Exp. 2				Exp. 3			
		TNP-KLH 10 µg		TNP-KLH 10 µg		TNP-KLH 0.001		TNP-OVA 0.001		KLH 40 µg + TNP-OVA 0.001 µg	
		Direct	Indirect	Direct	Indirect	Direct	Indirect	Direct	Indirect	Direct	Indirect
CB6F <sub>1</sub> *	—	18 ± 11	0	6 ± 3	3 ± 1	2 ± 1	0	6 ± 2	16 ± 4	4 ± 2	4 ± 6
C3H/He	—	13 ± 3 <sup>†</sup>	2 ± 1	9 ± 6	21 ± 12	0	4 ± 2	4 ± 2	12 ± 4	2 ± 1	0
CB6F <sub>1</sub>	CB6F <sub>1</sub> LN <sup>‡</sup>	354 ± 12	756 ± 33	435 ± 21	944 ± 42	88 ± 24	948 ± 11	11 ± 7	30 ± 20	33 ± 8	122 ± 36
C3H/He	C3H/HeLN	386 ± 42	633 ± 26	321 ± 12	846 ± 6	27 ± 7	382 ± 82	6 ± 2	12 ± 4	45 ± 10	128 ± 23
C3H/He	CB6F <sub>1</sub> LN	33 ± 9	55 ± 20	30 ± 3	48 ± 3	5 ± 2	24 ± 11	2 ± 1	8 ± 4	2 ± 2	8 ± 4
CB6F <sub>1</sub>	12.11 <sup>§</sup>	174 ± 31	213 ± 21	54 ± 7	218 ± 12	38 ± 5	344 ± 40	2 ± 2	0	4 ± 2	6 ± 2
C3H/He	A12.11	126 ± 83	0	9 ± 3	36 ± 3	4 ± 2	10 ± 2	1 ± 1	4 ± 2	3 ± 1	8 ± 4

\*  $4 \times 10^6$  TNP-OVA-primed B cells per culture.

†  $10^6$  lymph node cells per culture.

‡  $3 \times 10^5$  T cell clone per culture.

§ PFC mean response  $\pm$  SEM, day 5.

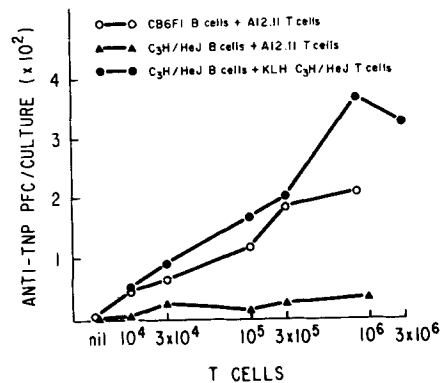


FIGURE 3. Dose-effect relationship between number of T cells and anti-TNP-PFC response developed by TNP-OVA-primed syngeneic and allogeneic B cells. The cultures were incubated with 0.10  $\mu$ g TNP-KLH and the direct PFC response was measured on day 4.

light chain isotypes, were purified on a protein A-Sepharose 4B column. The specificity of the monoclonal obtained was studied in a radioimmunoassay in which their binding to T cell clones was measured using  $^{125}$ I-rat anti-murine kappa monoclonal antibody.

The data illustrated in Table VII show the binding of two monoclonal anti-clonotype antibodies to three KLH-specific T cell clones and two KLH-specific T cell hybridomas already described (17, 18). While the monoclonal anti-clonotype antibody S2a6.18, binds only to the T cell subclone A12.11 and T hybridoma FN1-18, the antibody L9.3 displays a low binding to T cell hybridomas and no binding to T cell clones. It should be noted that the lack of binding of our monoclonal antibodies to thymocytes, two T cell clones specific for OVA and PR8 influenza virus and BW5147, an AKR lymphoma line. These findings exclude the possibility that antibody S3a.6-18 reacted with other T cell antigens such as T300, LAF-1, L<sub>3</sub>T<sub>4</sub>, T<sub>4</sub>, or gp54 protein (data not shown). Therefore, these data suggested that the syngeneic S3a6.18 monoclonal antibody obtained from a CB6F<sub>1</sub> mouse immunized with a KLH-specific line, recognizes antigenic determinants shared by only A12.11 and FN1-18 T cell clones.

This antibody, as well as anti-I-A monoclonal antibodies, were used to investigate their effect on KLH- and allogeneic-induced proliferative responses. The results shown in Table VIII illustrate that the monoclonal anti-clonotype S2a.6-18 inhibits the proliferative response of A12.11 induced by KLH, as well as C<sub>3</sub>H/He allogeneic stimulator cells. In contrast, only anti-I-A<sup>d</sup> antibodies blocked the proliferative response to KLH. These data are in agreement with Table II, which shows that the proliferation to KLH only occurs in the context of H-2<sup>d</sup> antigen-presenting cells. The allogeneic response to H-2<sup>k</sup> stimulating cells, however, was only blocked by anti-I-A<sup>k</sup> monoclonal antibodies. These results demonstrate that the binding of anti-I-A antibody to KLH associated with I-A<sup>d</sup> determinants on the surface of antigen-presenting cells or to I-A<sup>k</sup> determinants on stimulating cells mask these determinants, leading to the inhibition of the proliferation of A12.11 clone. In contrast, the binding of an anti-clonotypic

TABLE VI  
*In Vitro* Anti-SRBC PFC Response After Culturing B Cells with A12.11 T Cell Clone in Presence of SRBC and KLH

Origin of B cells ( $2 \times 10^6$ )*	T cells ( $3 \times 10^5$ )	Irradiated spleen cells	Antigen (SRBC $10^7$ KLH 40 $\mu$ g)	Anti-SRBC PFC/ culture
CB6F <sub>1</sub>	—	—	—	0
	—	—	SRBC KLH	26 $\pm$ 4
C3H/HeJ	—	—	—	2 $\pm$ 2
	—	—	SRBC KLH	19 $\pm$ 4
CBA/N	—	—	—	0
	—	—	SRBC KLH	8 $\pm$ 3
CB6F <sub>1</sub>	A12.11	—	—	2 $\pm$ 2
	A12.11	—	SRBC KLH	36 $\pm$ 5
C3H/HeJ	A12.11	—	—	12 $\pm$ 3
	A12.11	—	SRBC KLH	4 $\pm$ 2
CBA/N	A12.11	—	—	0
	A12.11	—	SRBC KLH	10 $\pm$ 3
CB6F <sub>1</sub>	A12.11	CB6F <sub>1</sub>	—	6 $\pm$ 3
	A12.11	CB6F <sub>1</sub>	SRBC KLH	31 $\pm$ 5
C3H/HeJ	A12.11	CB6F <sub>1</sub>	—	0
	A12.11	CB6F <sub>1</sub>	SRBC KLH	20 $\pm$ 3
CBA/N	A12.11	CB6F <sub>1</sub>	—	2 $\pm$ 2
	A12.11	CB6F <sub>1</sub>	SRBC KLH	18 $\pm$ 4
Control for in vitro SRBC PFC response: spleen cells from SRBC-primed mice				
CB6F <sub>1</sub>	—	—	—	204 $\pm$ 111
	—	—	SRBC KLH	1,432 $\pm$ 13
C3H/HeJ	—	—	—	192 $\pm$ 10
	—	—	SRBC KLH	1,072 $\pm$ 14
CBA/N	—	—	—	91 $\pm$ 6
	—	—	SRBC KLH	839 $\pm$ 20
Control for helper function of A12.11 cells				
B cells from TNP-OVA primed ( $2 \times 10^6$ )	T cells ( $3 \times 10^5$ )	Antigen (0.01 $\mu$ g/ml)	Anti-TNP PFC/ culture	
CB6F <sub>1</sub>	nil	TNP-KLH	8 $\pm$ 4	
CB6F <sub>1</sub>	A12.11	TNP-KLH	184 $\pm$ 42	

\* B cells were prepared as described in Material and Methods.

antibody to the receptor of A12.11 blocked its ability to interact with altered self or alloantigens.

### Discussion

The central finding of this communication has been to characterize the properties of four KLH-specific clones and particularly of two of them, A12.11 and F<sub>6</sub>, exhibiting proliferation to allogeneic cells from mouse strains bearing H-2<sup>k</sup> and H-2<sup>q</sup> haplotype, respectively. The antigen-induced proliferative response of T cell clones was genetically restricted to parenteral MHC-encoded antigens. Clone A12.11 proliferates in response to KLH in the presence of either CB6F<sub>1</sub> or BALB/c(H-2<sup>d</sup>) APC, whereas clones C1.2, D18, and F<sub>6</sub> proliferate in response

TABLE VII  
*Binding of Monoclonal Anti-clonotype Antibodies to KLH-specific T Cell Clones and Hybridomas*

Monoclonal antibodies	Specificities	T clones			T hybridomas		Bw5147
		A12.11	D18	C1.4	FN1-18	FN13-21	
Nil	—	876 ± 178*	1,056 ± 392	947 ± 334	274 ± 49	257 ± 49	331 ± 62
3D H.12	Thy-1.2	7,690 ± 418	5,964 ± 219	8,208 ± 3,706	19,980 ± 2,965	17,347 ± 229	ND
NEI.017	Lyt-1.2	11,054 ± 380	11,034 ± 248	14,948 ± 1,018	7,810 ± 1,165	7,843 ± 661	472 ± 95
NEI 006	Lyt-2.2	1,328 ± 708	1,456 ± 183	1,719 ± 66	ND <sup>‡</sup>	ND	ND
10-2.16	I-A <sup>k</sup>	1,644 ± 418	1,140 ± 69	1,382 ± 342	ND	ND	ND
S3a 6.18	KLH-T cell line	8,376 ± 1,776	1,434 ± 440	1,510 ± 410	5,134 ± 1,358	440 ± 69	264 ± 49
L9.3	KLH-T cell line	1,300 ± 108	1,042 ± 40	1,98	3,760 ± 1,331	2,878 ± 164	240 ± 62

3 × 10<sup>5</sup> cells were incubated for 45 min at 4°C in PBS 1% BSA, 0.01% azide containing 10 µg monoclonal antibodies. After incubation, the cells were washed 3× and incubated for 2 h at 4°C with <sup>125</sup>I-rat anti-mouse K mono-clonal antibodies (70,000 cpm) and radioactivity was measured in γ-spectrometer after extensive washing.

\* Mean cpm ± SD of triplicate.

<sup>‡</sup> ND, not done.

TABLE VIII  
*Effect of Anti-Ia and Anti-Id Antibodies on the Proliferative Response of A12.11 T Cell Clone*

Antibody added to the culture	Specificity	A12.11	CB6/F <sub>1</sub> + KLH*	C3H/HeJ
nil	—	—	130 ± 34 <sup>‡</sup>	90 ± 8
nil	—	+	19,588 ± 2,699	24,636 ± 386
25-9-3s	I-A <sup>b</sup>	+	18,691 ± 2,221	23,079 ± 3,234
MK-D6	I-A <sup>d</sup>	+	2,673 ± 383	20,711 ± 1,055
10.2=16	I-A <sup>k</sup>	+	20,301 ± 3,588	2,928 ± 421
S3a.6-18	KLH T clone	+	3,343 ± 1,367	3,450 ± 824
L9-3	KLH T clone	+	20,207 ± 351	22,611 ± 487
UPC-10	β2-1 fructosan	+	17,261 ± 1,517	22,399 ± 780
76-42	Anti(anti-A48Id)	+	19,275 ± 2,110	18,912 ± 319

\* Cultures contained Iscove's serum-free medium supplemented with 1% purified monoclonal antibodies.

<sup>‡</sup> Tritiated thymidine incorporation of triplicate cultures presented in cpm ± SD.

to KLH in the presence of either CB6F<sub>1</sub> or C57BL/6(H-2<sup>b</sup>) cells. These results are in agreement with previously reported data that T cells from F<sub>1</sub> animals recognize the foreign antigens in association with Ia determinants expressed by one parent.

The clonal nature of T cell clones exhibiting double specificity was demonstrated by subcloning under stringent limiting dilution (0.5 cells/well) and BUdR experiments. The subclone exhibited the same reactivity pattern as parental clones. Furthermore, in BUdR-suicide experiments, both KLH and allogeneic-induced reciprocal proliferative responses were ablated upon BUdR and light exposure.

Two major hypotheses can be entertained to explain the ability of a single T cell to recognize foreign antigens in association with syngeneic MHC-encoded determinants and to proliferate in response to allogeneic Ia antigens: (a) a certain degree of mimicry between the alloantigens and the neoantigens created by the association of foreign antigens with the syngeneic MHC determinants; and (b) that T cells carry two receptors with different recognition specificities.

The first hypothesis is supported by a recent finding demonstrating the inhibi-

tion of an alloreactive (H-2<sup>d</sup>) T cell hybridoma by a synthetic polypeptide antigen (19). Our results are also congruent to the first hypothesis: the incubation of A12.11 subclone with anti-I-A<sup>d</sup> inhibited the KLH proliferative response in the presence of BALB/c APC and the anti-I-A<sup>k</sup> antibody inhibited the proliferative response induced by C<sub>3</sub>H/HeJ stimulating cells. The anti-clonotype antibody inhibited both responses. Thus, when anti-I-A antibodies were added to the cultures, they masked I-A<sup>d</sup> determinants crucial for KLH-induced proliferative response or I-A<sup>k</sup> determinants required for alloproliferative response. In contrast, the anti-clonotypic antibody subsequent to the binding to T cell receptor prevented the recognition and triggering of the proliferation by KLH plus I-A<sup>d</sup> or I-A<sup>k</sup> allogeneic cells. Therefore, our data strongly favor a single receptor hypothesis and a mimicry between antigen plus self and alloantigens. However, the inability of anti-I-A<sup>k</sup> antibody to inhibit KLH-induced proliferation and of anti-I-A<sup>d</sup> antibody to inhibit allogeneic proliferation suggests only a certain degree of mimicry, since the alloantigens are only "infidèle" copies or "internal image" of neoantigens created by association of foreign antigens with self MHC-encoded determinants. It should be mentioned that our experiments do not formally rule out the two-receptors hypothesis, since it is possible to imagine that V genes encoding for recognition of KLH plus I-A<sup>d</sup> and I-A<sup>k</sup> share a common clonotypic determinant or that our anti-clonotypic antibody was specific for the product of DNA segment encoding for constant region of T cell receptor (20). This is highly unlikely, since the data presented in Table VII show that the monoclonal anti-clonotype binds to only A12.11 and FN1-18 KLH-specific clones.

The second set of findings reported in this communication deals with the helper function of the KLH-specific clones. All four clones were able to provide help for CB6F<sub>1</sub> TNP-OVA-primed B cells to mount an IgM and IgG PFC response in the presence of 0.001–10 µg/ml TNP-KLH conjugate. The helper function was dependent of carrier-hapten linkage, since no significant anti-TNP-PFC response was observed upon the culture of CB6F<sub>1</sub> TNP-OVA-primed B cells and A12.11 T clone in the presence of TNP-OVA alone or TNP-OVA and KLH mixture.

Thus, it appears that the properties of our clones, depending on hapten-carrier linkage and able to provide help for IgM and IgG with both low and high antigen doses, are very different from the properties of clones described by Asano et al. (15). However, our data using KLH-heterogeneous T cells clearly show that the clones described by Asano et al. (15) exist in the repertoire, since we observed, indeed, a significant high IgG response with lymph node T cells in the presence of a low dose of antigens. The ability of our clones to provide help with a dose of antigen as low as 0.001 µg/ml suggests that the clones described in this paper have a high affinity for the carrier determinant(s). The study of helper T cells recognizing carrier determinants in association with self-encoded MHC determinants and exhibiting alloreactivity provided an excellent tool for investigating the rules that govern the genetic restriction of T-B cell cooperation.

There are numerous reports suggesting the existence of two antigen-specific helper T cell subsets. The first subset recognizes carrier determinants in association with self MHC-encoded determinants that cooperate with B cells via an

antigen-carrier bridge (21) in an MHC-restricted manner (22). The existence of this subset of helper T cells was confirmed by cloning experiments (see data reviewed in reference 23). A second subset, first clearly demonstrated by the experiments of Marrack and Kappler (16), can induce the differentiation of unprimed B cells in response to a particulate antigen or even a polyclonal activation of B cells (24) through the releasing of lymphokines. Asano et al. (15) described an antigen-specific T cell clone exhibiting the properties of both subsets which were MHC restricted in their interaction with Lyb5<sup>-</sup> B cells and helped Lyb5<sup>+</sup> B cells in MHC-unrestricted fashion. DeFranco et al. (25) have also shown that antigen-specific T cell clones can induce the activation and differentiation of resting B cells in a MHC-restricted manner in the presence of antigen for which T cells are specific as well as a polyclonal activation of both Lyb5<sup>+</sup> and Lyb5<sup>-</sup> subsets in the presence of antigen and APC of proper MHC haplotype. These reports suggest that the same antigen-specific T cells can cooperate with B cells in a MHC-restricted and unrestricted manner. However, it clearly appears from our results that at various T:B cell ratios ( $10^4$ – $10^6$  T:  $2 \times 10^6$  B), the A12.11 KLH-specific helper T clone was able to activate B cells only via a carrier-antigen bridge and in a MHC-restricted fashion. No polyclonal activation of IgM secretion, or specific activation of SRBC precursors was observed with syngeneic B cells or allogeneic Lyb5<sup>-</sup> and Lyb5<sup>+</sup> B cells. These data are particularly important, since other investigators showed that allospecific T cells can provide help to B cells (26–28).

The ability of T cell clones specific for a foreign antigen and exhibiting a secondary alloreactive to provide help through only MHC-restricted T-B cell interaction could be explained: (a) by differences in the affinity of the receptor of T cell clones for the carrier determinants versus alloantigen or alternatively; (b) by the inability of T cells recognizing foreign antigens in association with self MHC-encoded determinants to recognize antigen in the context of alloantigens, since determinant selection (29) requires different association of the same antigen with various Ia determinants due to the allelic polymorphism of genes encoding for these determinants. Whether or not this is a general property of antigen-specific helper cells exhibiting alloreactivity, remains to be elucidated.

### Summary

Four keyhole limpet hemocyanin (KLH)-specific clones prepared from the lymph node of CB6F<sub>1</sub> mice immunized with KLH had a proliferative response restricted to parental major histocompatibility complex (MHC)-encoded antigens. These clones provided help for CB6F<sub>1</sub> trinitrophenyl-ovalbumin (TNP-OVA)-primed B cells to mount IgM and IgG plaque-forming cell (PFC) responses in the presence of KLH-TNP conjugate. In addition, two of these clones (A12.11 and F6) proliferated in response to allogeneic cells from mice strains bearing H-2<sup>k</sup> or H-2<sup>q</sup> haplotypes, respectively. However, they did not provide help for C<sub>3</sub>H/He or B10.Q primed B cells. The clonal nature of A12.11 and F6 was demonstrated by subcloning and in BUdR-suicide experiments. The proliferative response to KLH was ablated by anti-Ia<sup>d</sup> antibodies, whereas the proliferation induced by C<sub>3</sub>H/HeJ stimulating cells was ablated by anti-Ia<sup>k</sup> antibodies. Furthermore, both responses were inhibited by a monoclonal anti-clonotype (idi-

otype) antibody. Taken together, these results strongly support the hypothesis that the same receptor recognizes alloantigens and KLH associated with self-antigens.

*Received for publication 4 June 1984.*

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