

CLONAL ANALYSIS OF F₁ HYBRID HELPER T CELLS
I-A Subregion-encoded Hybrid Determinants Restrict the
Activity of Keyhole Limpet Hemocyanin-specific Helper T Cells*

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The I region of the murine major histocompatibility complex (MHC)¹ encodes Ia antigens and determines the functional reactivity of T lymphocytes (1). F₁ hybrids of strains that carry different I region alleles express unique hybrid Ia antigen specificities (2, 3). Hybrid determinants also mediate other I region-controlled functions. We have recently reported (4) that the activity of many keyhole limpet hemocyanin (KLH)-specific F₁ hybrid helper T cells is restricted by unique MHC-encoded hybrid determinants. This identifies a hybrid restriction element for the helper T cell response to a complex antigen and extends to this I region-determined function earlier observations of unique F₁ hybrid mixed lymphocyte response (MLR)-stimulating determinants (5) and dual complementing immune response (Ir) genes (6).

In many of these instances, unique properties of the F₁ hybrids are accounted for by complementation between genes of the I-A and I-E subregions (2, 7). Complementation within the I-A subregion has, however, also been observed for dual Ir gene-regulated responses (6) and for the proliferative response of F₁ hybrid T cell clones (8, 9). In the present report we show that both parental and F₁ hybrid restriction elements for KLH-specific helper T cells are encoded within the I-A subregion and reflect little if any contribution from I-E.

Materials and Methods

Mice. Mice used in these studies were bred in our own animal facilities at Columbia University, New York. Breeding pairs of C57BL/10 Sn (B10) and B10.A(5R)/SgSn were purchased from The Jackson Laboratory, Bar Harbor, Maine. Breeding pairs of B10.A(4R) were kindly provided by Dr. Donald Schreffler, Department of Genetics, Washington University, St. Louis, Mo.

Antigens. KLH was purchased from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif. Ovalbumin (OA) was purchased from Miles Laboratories, Inc., Elkhart, Ind. Dinitrophenylated (DNP) conjugates were prepared as previously described (4). Molar substitution ratios were DNP₄KLH (per 10⁵ daltons KLH) and DNP₄OA.

B Cell Source. Mice were immunized intraperitoneally with 100 μg DNP₄OA precipitated in alum and 2 × 10⁸ *Bordetella pertussis* and were boosted 10–20 d before sacrifice with 50 μg of the same antigen in phosphate-buffered saline. Spleen cell suspensions were treated with monoclonal anti-Thy-1.2, and monoclonal anti-Lyt-2.2 and rabbit complement as previously described (4).

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¹ *Abbreviations used in this paper:* BDNP, anti-Thy-1.2, anti-Ly-2.2, and complement-treated spleen cells; CAAb, concurrent antibody; DNP, dinitrophenyl(ated); Ir, immune response; KLH, keyhole limpet hemocyanin; MHC, major histocompatibility complex; MLR, mixed lymphocyte response; OA, ovalbumin.

T Cell Sources. Mice were immunized subcutaneously at the base of the tail with 100 μg of KLH in complete Freund's adjuvant. 4 d later the inguinal and paraaortic lymph nodes were removed and a single cell suspension prepared. Limiting dilution cultures of primed lymph node cells were initiated in 0.1 ml round-bottomed wells and helper activity was determined for each well by transfer after 3–4 wk to 10- μl secondary in vitro antibody-forming cell cultures as previously described (4).

Concurrent Antibody (CAb) Hemagglutination Assay. Each in vitro antibody-forming cell culture is washed free of accumulated antibody on day 5 and then incubated for an additional 2 h at 37°C in the presence of trinitrophenylated horse erythrocytes. The sensitivity of this hemagglutination assay and correlation with plaque-forming cells has been previously described (4).

Results

Isolation of KLH-specific (B10.A(4R) \times B10)F₁ Hybrid Helper T Cells Restricted to Unique I-A-encoded Hybrid Determinants. We have recently (4) described the isolation and expansion of clonal precursors to KLH-specific helper T cells in limiting dilution cultures of KLH-primed lymph node cells. The MHC restriction of each clone was determined by independently testing the helper activity of its progeny on a panel of haplotypes. It was demonstrated that KLH-primed lymph node cells from F₁ hybrids between two MHC congenic strains include independent clonal precursors for helper T cells restricted to either parental MHC haplotype as well as a large number of precursors for clones able to cooperate with B cells of F₁ origin only. To assess the relative contribution of I-A and I-E subregions to the expression of parental and unique F₁ hybrid MHC determinants that restrict the activity of KLH-specific helper T cells, we have examined the cooperation specificity of clones derived from F₁ hybrids with recombinant MHC haplotypes.

(B10.A(4R) \times B10)F₁ KLH-primed lymph node cells were cultured at between 4×10^3 and 16×10^3 viable cells/100 μl culture and supplemented to a final concentration of 50×10^3 cells/well with mitomycin C-treated normal F₁ spleen cells. After 3–4 wk the cellular contents of 40–100 cultures at each initial concentration were individually transferred in equal aliquots to six 10 μl culture wells. Helper activity was assayed by the addition of 3×10^4 DNP-OA-primed, anti-Thy-1.2, anti-Lyt-2.2, and complement-treated spleen cells (B_{DNP}) to each 10- μl well. Each set of six test cultures originating from a single 100- μl lymph node cell culture consisted of duplicate wells that received B_{DNP} of B10, B10.A(4R) or (B10.A(4R) \times B10)F₁ origin. DNP-specific antibody secreted in each individual 10- μl test well was measured on day 5 by hemagglutination assay as previously described (4).

A titration curve for precursors to helper T cells in one such lymph node cell population is plotted in Fig. 1. As decreasing numbers of KLH-primed lymph node cells are added at the time cultures are initiated, a larger fraction of cultures (F₀) fails to transfer help to any test well. The linear relationship between log F₀ and the number of KLH-primed lymph node cells added per culture indicates that, in the presence of mitomycin-C-treated splenic filler cells, only one cell in the KLH-primed lymph node population limits the ability of a culture to transfer help. The frequency of the limiting cell may be determined from the Poisson relationship $F_0 = e^{-\mu}$; where F₀ is the fraction of lymph node cultures that fail to transfer any helper activity and μ is the mean number of limiting cells added per culture (10). In this particular experiment the limiting cell was present in the initial lymph node cell suspension at a frequency of $\sim 1/6 \times 10^3$ cells. When a lymph node cell culture transferred helper

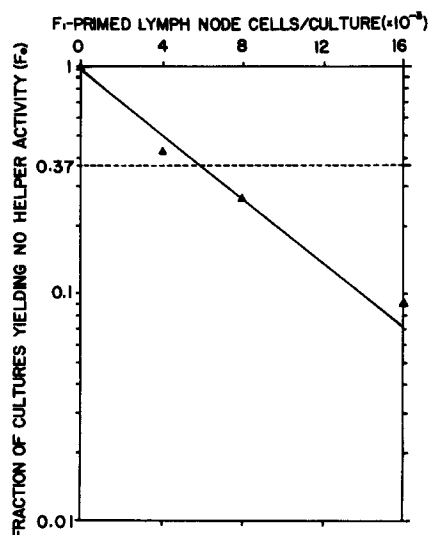


FIG. 1. Semilog plot of the fraction of lymph node cell cultures that fail to transfer any helper activity (F_0) vs. the number of $(B10.A(4R) \times B10)F_1$ hybrid KLH-primed lymph node cells added to each 100- μ l culture. If a DNP-specific response was induced in any test culture of either B10.A(4R), B10 or F_1 hybrid origin, the donor F_1 lymph node cell culture was scored positive for helper activity. The points conform to a straight line determined by least square analysis with correlation coefficient >0.95 .

activity to test wells of a particular type, help was most often transferred to both test wells of that type. A minimal estimate for the mean number of helper T cells arising from a single precursor in any positive lymph node cell culture can be calculated from a Poisson analysis of the responses in the test wells (4). For the experiments reported in Tables I and II this value is ≥ 15 functional progeny per precursor.

In Table I data for this experiment are shown for three initial lymph node cell concentrations at which a significant proportion of cultures failed to transfer help to any of the six test wells. Helper activity of each of the 240 F_1 hybrid lymph node cell cultures is tabulated. Under these conditions a large proportion of the cultures transfer helper activity to test wells of only a single parental type (in addition to F_1), indicating that helper T cells restricted to cooperate with one or the other parental haplotype segregate in different cultures. In addition, an equally high proportion of lymph node cell cultures gave rise to helper activity restricted to spleen cells of F_1 origin, but failed to provide any help to spleen cells of either parental type. Because in this experiment the two parental haplotypes differ only in the K and I-A subregions, these results clearly indicate that unique F_1 hybrid MHC determinants that serve as restriction elements for KLH-specific helper T cells can arise by complementation between the k and b haplotypes at this locus. As in previous experiments (4) a limited number of cultures with helper activity for parental but not F_1 spleen cells were detected (Table I, column 5). An interpretation of this observation is suggested below.

Clonal Analysis of $(B10.A(5R) \times B10)F_1$ Hybrid Helper T Cells. To assess the contribution of genes that map to the right of the I-A subregion to the expression of determinants that restrict the activity of KLH-specific helper T cells, we have analyzed the cooperation specificity of limiting dilution clones of lymph node cells

TABLE I
Isolation of (B10.A(4R) × B10)F₁ KLH-specific Helper T Cell Clones

(B10.A(4R) × B10)F ₁ lymph node cells/culture	Number of cultures assayed	Helper activity (number of cultures)						
		B10.A(4R) and F ₁	B10 and F ₁	F ₁ only	B10.A(4R) only	B10 only	B10.A(4R) B10 and F ₁	None
× 10 ³								
16.0	100	6	25	13	0	2	45	9
8.0	100	13	18	16	0	2	25	26
4.0	40	5	8	5	0	0	5	17
Total		24	51	34	0	4		

(B10.A(4R) × B10)F₁ hybrid KLH-primed lymph node cell cultures were initiated at between 4×10^3 and 16×10^3 cells in 100- μ l round bottomed wells and supplemented to 50×10^3 cells with mitomycin C-treated normal F₁ filler cells. The helper activity of each culture was assayed after 3–4 wk by transfer to duplicate 10- μ l microculture wells with DNP₄OA-primed B_{DNP} of either B10.A(4R), B10, or (B10.A(4R) × B10)F₁ origin. If a DNP-specific response was detected in at least one test well in a duplicate set, the donor lymph node cell culture was scored positive for helper activity restricted to that haplotype. DNP-specific responses were detected in a CA₁ hemagglutination assay of antibody secreted on day 5 of secondary culture as previously described (4). In plates that received the same number of B_{DNP} but to which F₁ T cells were added in excess, a DNP-specific response was detected in every culture, indicating that B_{DNP} are present in excess.

TABLE II
Restriction Elements for KLH-specific (B10.A(5R) × B10)F₁ Helper T Cells Are Encoded Predominantly in the I-A Subregion

Experiment	(B10.A(5R) × B10)F ₁ primed lymph node cells/culture	Number of cultures assayed	Helper activity (number of cultures)			
			B10	B10.A(5R)	B10 and B10.A(5R)	None
	× 10 ³					
1	8	50	1	0	32	17
2	10	60	0	2	42	16
3	3	40	0	0	14	26
3	8	40	0	1	17	22
Total			1	3	105	

(B10.A(5R) × B10)F₁ hybrid KLH-primed lymph node cell cultures were initiated at the indicated number of cells. Helper activity was assayed by transfer to triplicate 10- μ l microculture wells with DNP₄OA-primed B_{DNP} of either B10.A(5R) or B10 origin. In control plates that received the same number of B_{DNP} but to which F₁ T cells were added in excess, a DNP-specific response was detected in every culture well, indicating that B_{DNP} are present in excess.

from KLH-primed (B10.A(5R) × B10)F₁ hybrids. The helper activity of each lymph node cell culture was assayed by transfer to triplicate test wells with B_{DNP} of either B10 or B10.A (5R) origin. The helper activity of 190 limiting dilution cultures from three different F₁ hybrid lymph node populations is shown in Table II. In each case data are presented for those lymph node cell concentrations at which a large proportion of cultures failed to transfer helper activity to any of the six test wells. In contrast to the previous experiment, the helper activity arising from limiting dilution cultures of (B10.A(5R) × B10)F₁ hybrid lymph node cells did not distinguish between

the B10 and the B10.A(5R) parental haplotypes. This result suggests that restriction elements encoded within the I-E subregion of the k and b haplotypes, in particular a previously described restriction element encoded by complementing genes in I-A^b and I-E^k (7), are not utilized to any appreciable extent by KLH-specific helper T cells in these strains.

Discussion

These studies demonstrate that unique F₁ hybrid MHC determinants that restrict the activity of KLH-specific helper T cells arise through complementation within the I-A subregion. Immunoprecipitation studies have resolved two noncovalently associated chains, A_α and A_β, both encoded within I-A (1). As suggested by Kimoto and Fathman (8, 9), unique F₁ hybrid determinants could arise through combinational association of parental A_α and A_β chains.

Hybrid determinants are also known to be expressed on a second Ia antigen comprised of an α chain encoded within I-E and a second β chain encoded within I-A (2). Cell surface expression of this Ia molecule requires that both chains be expressed in the same cell (2). Immune responses to a number of antigens controlled by complementing Ir genes that map to the I-A and I-E subregions have been clearly associated with expression of this Ia molecule (7, 11). The recombinant strain, B10.A(5R), expresses this molecule and is a responder to some of these antigens. Our failure to demonstrate a significant level of (B10.A(5R) × B10)F₁ hybrid clones restricted to the B10.A(5R) parent (Table II) suggests that restriction elements associated with this Ia molecule are not utilized by KLH-specific helper T cells to the same extent as are others encoded entirely within the I-A subregion. This restriction element might, nevertheless, be significant for helper T cells specific for other antigens. Sprent (12) has, in fact, recently demonstrated I-A/E-encoded restriction elements for sheep erythrocyte-specific responses. The use of different restriction elements by T cells reactive to different antigens can fully account for the apparent antigen specificity of Ir genes.

As noted in the results presented in Table I, four F₁ hybrid lymph node cultures had helper activity restricted to one parental haplotype but failed to cooperate with F₁ hybrid spleen cells. Unique homozygous MHC determinants have been previously reported by others (9). It is possible to account for the occurrence of homozygous restriction elements if multiple functional determinants are associated with each α, β complex. Hybrid molecules (α^b β^k, α^k β^b) might then share some but not all determinants expressed in homozygous molecules (α^b β^b, α^k β^k). Determinants that fail to be expressed in hybrid complexes would then represent unique homozygous restriction elements. To account for the absence of homozygous complexes on F₁ hybrid cells, we would propose that for some haplotype combinations hybrid complexes are formed preferentially and occur at a significantly higher density than homozygous complexes on the surface of F₁ hybrid spleen cells. The expression of multiple Ir gene determinants in a single individual may have more general significance in the selection of multiple independent variable-region genes for expression in T lymphocytes (13).

Summary

Clonal expansion of isolated precursors to helper T cells was induced in limiting dilution cultures of keyhole limpet hemocyanin (KLH)-primed F₁ hybrid lymph node

cells. Progeny of each isolated precursor was tested for helper activity by transfer to independent cultures with hapten-primed B cells of either parental or F₁ hybrid origin. The major histocompatibility complex (MHC) restriction specificity of each F₁ hybrid helper T cell clone was determined. To assess the contribution of I-A and I-E subregion-encoded genes to the expression of these restriction elements, helper T cell cultures derived from F₁ hybrids between strains with recombinant H-2 haplotypes were analyzed. Parental and unique F₁ hybrid MHC determinants that are encoded entirely within the I-A subregion were found to restrict the activity of KLH-specific helper T cells.

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