CLONAL ANALYSIS OF F_1 HYBRID HELPER T CELLS RESTRICTED TO PARENTAL OR F_1 HYBRID MAJOR HISTOCOMPATIBILITY DETERMINANTS*

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The activity of antigen-specific helper T cells is restricted by genes that map to the *I* region of the major histocompatibility complex (MHC)[1] (1). Helper T cells of an F_1 hybrid have been shown by positive selection with antigen-presenting cells of parental origin to consist of at least two independent populations, each specific for antigen, and one parental MHC (2–4). It appears, therefore, that the ability to recognize specific MHC-encoded determinants is clonally distributed in helper T cells.

A number of experiments have demonstrated the existence of unique *I* region-encoded F_1 hybrid determinants. Fathman et al. (5–7) have detected mixed lymphocyte response- (MLR) stimulating determinants expressed in an F_1 hybrid but absent in either parent. Jones et al. (8) found that cell surface expression of an Ia molecule that maps to the *I-E* subregion is determined by two *I* region genes that complement in an F_1 hybrid. More recently, hybrid Ia antigen specificities have been identified serologically (9). The relevance of such hybrid determinants to helper T cell function was suggested by the observation that responses to certain protein and polypeptide antigens are regulated by dual MHC-linked immune response (*Ir*) genes that complement in F_1 hybrids of two independent low responders (10, 11). If helper T cells specific for these antigens are restricted to unique F_1 hybrid determinants, then expression of such determinants could account for complementation of these *Ir* genes. A relationship between *Ir* gene products and *I* region-encoded restriction elements has been suggested by a number of experiments. In particular, it has been demonstrated that the high-responder phenotype in (high-responder × low-responder)F_1 hybrid T cells is manifested only in those F_1 T cells restricted to the high-responder MHC haplotype (12, 13).

We have, for these reasons, investigated in an F_1 hybrid population whether some helper T cells specific for a randomly chosen antigen are restricted to F_1 hybrid

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[1] *Abbreviations used in this paper:* B_{DNP}, anti-Thy-1.2, anti-Ly-2.2, and complement-treated spleen cells; C′, complement; CAb, concurrent antibody; complete medium, RPMI-1640 medium supplemented with 100 mM (final) Hepes, 1 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 5×10^{-5} M 2-mercaptoethanol, and 10% FCS; DNP, dinitrophenyl(ated); F_0, fraction of lymph node cultures that fail to transfer helper activity; CFA, complete Freund's adjuvant; FCS, fetal calf serum; FGG, fowl gamma globulin; GLϕ, poly($Glu^{53}Lys^{36}Phe^{11}$); HBSS, Hanks' balanced salt solution; HRBC, horse red blood cells; KLH, keyhole limpet hemocyanin; MHC, major histocompatability complex; MLR, mixed lymphocyte response; OA, ovalbumin; PFC, plaque-forming cell(s); RBC, erythrocytes; TNP, trinitrophenyl; T_{KLH}, KLH-specific helper T cells.

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determinants. In this report we describe the isolation and expansion in vitro of clonal precursors to keyhole limpet hemocyanin (KLH)-specific F_1 helper T cells restricted to either parental or unique F_1 hybrid MHC determinants.

Materials and Methods

Mice. The *H-2* congenic strains, BALB.c (*H-2^d^*), BALB.B (*H-2^b^*), BALB.K (*H-2^k^*), and F_1 hybrids were bred in our own animal facilities at Columbia University, New York, from breeding pairs provided by Dr. Frank Lilly of the Albert Einstein College of Medicine, Bronx, N. Y.

Antigens. KLH was purchased as an ammonium sulfate slurry from Calbiochem-Behring Corp., American Hoechst Corp., La Jolla, Calif. Fowl gamma globulin (FGG) was prepared by ammonium sulfate fractionation of chicken serum from Gibco, Grand Island Biological Co., Grand Island, N. Y. Ovalbumin (OA) was purchased from Miles Laboratories, Inc., Elkhart, Ind. Dinitrophenylated (DNP) conjugates were prepared by reaction with dinitrobenzene sulfonate in 0.15 M potassium carbonate overnight at 4°C. Substitution ratios were DNP_8KLH (per 10^5 daltons KLH), DNP_7FGG, and $DNP_{13}OA$.

B Cell Source. Mice were immunized with 100 μg of DNP_7FGG or $DNP_{13}OA$ emulsified in complete Freund's adjuvant (CFA) intraperitoneally, and boosted with 50 μg of the same antigen in phosphate buffered saline 10–20 d before they were killed. Spleen cell suspensions were prepared in Hanks' balanced salt solution (HBSS) at 10^8 cells/ml and treated with monoclonal anti-Thy-1.2, and monoclonal anti-Ly-2.2 (gifts of U. Hämmerling, Memorial-Sloan Kettering Cancer Institute, N. Y.) for 45 min at 4°C followed by resuspension in selected rabbit complement (1:9) for 45 min at 37°C. The treatment was repeated once. Concanavalin A reactivity of treated spleen cells relative to controls was reduced to background levels.

T Cell Sources

BULK T CELLS. Mice were immunized subcutaneously at the base of the tail with 100 μg of KLH emulsified in CFA in a total volume of 50 μl (14). 4 d later the inguinal and paraaortic lymph nodes were removed and teased through a stainless steel mesh into HBSS. Lymph node cell cultures were initiated in Falcon flasks (3013; Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) at 3×10^6 cells/ml in 5 ml of RPMI-1640 medium supplemented with 10 mM (final) Hepes, 1 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 5×10^{-5} M 2-mercaptoethanol, and 10% FCS (complete medium). KLH was added to a final concentration of 40 μg/ml. Cultures were kept at 37°C in a humidified incubator with 7.5% CO_2 in air and fed with 2 ml of complete medium, without KLH, on days 4, 9, and 14. Cells were harvested after 15–30 d, washed, resuspended to 2×10^6/ml in HBSS, and treated with 25 μg/ml mitomycin C for 30 min at 37°C. Cells were then washed extensively and used as a source of KLH-specific helper T cells (T_{KLH}).

"CLONED" F_1 T CELLS. KLH-primed lymph node cells were obtained as above and 100 μl cultures of between 2×10^3 and 30×10^3 cells were initiated in 96-well round-bottomed culture plates (Linbro Chemical Company, Hamden, Conn.). The total cell number was adjusted to 35×10^3/well by addition of normal F_1 spleen cells treated with anti-Thy-1.2 and complement (C') and with 25 μg/ml mitomycin C. Cells were transferred for assay of helper activity on day 21 to 10-μl culture wells of Falcon 3034 MicroTest plates and washed as described below for concurrent antibody (CAb) assay, except that complete culture medium was used in the last wash.

In Vitro Antibody-forming Cell Cultures. Secondary antibody responses were induced in a 10-μl microculture system (15). 3×10^4 primed B cells and macrophage, and various numbers of T_{KLH} were added to each well of Falcon 3034 MicroTest (Terasaki) plates in complete medium as for T cell cultures except with 20% FCS. Antigen was added to a final concentration of 0.1 μg/ml. Cultures were incubated without feeding in a humidified incubator at 37°C with 7.5% CO_2 in air. Specific antibody secretion was assayed in each well on day 5 by one of two methods described below.

Plaque-forming Cell (PFC) Assay. The number of hapten-specific PFC was determined using Cunningham and Szenburg modification (16) of the Jerne and Nordin hemolytic plaque assay

(17). Trinitrophenyl (TNP) was coupled to HRBC by the method of Rittenberg and Pratt (18). All TNP-specific PFC were developed in the presence of a polyvalent rabbit anti-mouse Ig antiserum. After our immunization protocol, secondary in vitro responses to DNP gave >95% indirect PFC on TNP-conjugated horse red blood cells (HRBC). Addition of 10^{-6} M ϵ-DNP-lysine inhibited >95% of all PFC.

CAb Hemagglutination Assay. The CAb assay replaces the above PFC assay in the last two experiments. At the end of the 5-d culture period, the microculture plates are flooded with 12 ml of sterile HBSS. The walls of these plates (Falcon 3034) allow a reservoir of this size to collect above the culture wells. The plates are then rocked for 10 min on a mechanical rocking platform (Bellco Glass, Inc., Vineland, N. J.) in a laminar flow hood after which the HBSS reservoir is aspirated. This is repeated twice with HBSS and once with HBSS supplemented with 10 mM Hepes, 0.04% $NaHCO_3$, and 1% heat-inactivated FCS. The four washes give a $>2^{17}$ dilution of the 60 original 10-μl culture supernates in each plate. Spleen cells, which during 5 d of stationary culture settle to the bottom of the culture wells, remain undisturbed. 2 μl of a 2.5% suspension of haptenated HRBC are added to each well with a Hamilton repeating dispenser (Hamilton Industries, Two Rivers, Wis.). The culture-plate lids are replaced and the plates are incubated at 37°C in a humidified incubator with 7.5% CO_2 in air for 2 h to accumulate secretion by any antibody-forming cells. Cells are sedimented by centrifugation at 100 *g* for 3 min and are then assayed for agglutination of haptenated HRBC by inclining the plates at an angle of ~80°. The slanted geometry of the Terasaki wells allows unagglutinated erythrocytes (RBC) to run. Negative controls, which receive either no T cells or no antigen on day zero, or nonhaptenated HRBC in the assay, run completely in 10-15 min. At that time, all the remaining wells are scored for hemagglutination with the aid of an illuminated colony counter (American Optical Corp., Scientific Instruments Div., Buffalo, N. Y.). Wells were scored as (++) for complete agglutination, in which case all the the RBC form a tight button at the bottom of the well; (+) wells also have a button covering the bottom of the well; however, some of the RBC accumulate at the vertex of the side and bottom of the well; in (−) wells, all the RBC run clearly to the outer rim of the well. Wells can be scored up to 30 min after inclining the plate with no change in agglutination pattern. Culture plates, which were washed on day 5 and receive haptenated HRBC but are assayed immediately without further incubation at 37°C, are uniformly negative.

Correlation between the conventional PFC assay and CAb assay is excellent. This was determined by scoring the same cultures first in the CAb assay and then for PFC. After a 2-h incubation in the CAb assay for DNP-specific responses on day 5, >15 PFC score as (++), 10-15 PFC as (+), and <10 as (−). This is convenient because the background PFC level without added T cells is <10 PFC/well, and the mean number of PFC/positive test culture in the experiments reported here was >100.

Results

Selection of MHC-restricted Antigen-specific Helper T Cells In Vitro. Lymph node cells from recently primed mice are induced to extensive proliferation upon restimulation by specific antigen in vitro (14). Augustin et al. (19, 20) observed that cells selected in such antigen-stimulated cultures are depleted of alloreactive cells and are highly enriched in helper T cells required for the induction of specific antibody responses in vitro. This helper function is antigen specific and restricted to the *I* region of the donor MHC (20). We have determined, by limiting-dilution analysis, that, under our own slightly modified culture conditions, >1 in 300 viable cells recovered after 18 d of culture are specific helper T cells (21). Results shown in Table I demonstrate, for two combinations of H-2-congenic strains, that helper T cells selected in KLH-primed lymph node cultures are restricted to cooperate with syngeneic spleen cells in secondary in vitro responses. Apparent MHC restriction in this system cannot be ascribed to negative allogeneic effects (22) because no suppression is observed in

TABLE I

Helper T Cells Selected in Primed Lymph Node Cultures Are MHC Restricted

B_{DNP}	T_{KLH}	PFC/well*
Experiment 1		
BALB.B	BALB.B	374
BALB.B	BALB.K	27
BALB.B	—	2
BALB.B	BALB.B + BALB.K	337
BALB.K	BALB.K	370
BALB.K	—	2
Experiment 2		
BALB.B	BALB.B	150
BALB.B	BALB/c	14
BALB.B	—	0
BALB.B	BALB.B + BALB/c	158
BALB/c	BALB/c	100
BALB/c	—	0
Experiment 3		
(BALB.B × BALB/c)F_1	(BALB.B × BALB/c)F_1	252
(BALB.B × BALB/c)F_1	BALB.B	280
(BALB.B × BALB/c)F_1	BALB/c	323
(BALB.B × BALB/c)F_1	—	2
(BALB.B × BALB/c)F_1	BALB.B + BALB/c	232

* Mean number of PFC/well determined from six replicate cultures assayed individually on day 5.

Secondary in vitro DNP-specific responses were determined for various syngeneic, allogeneic, and semiallogeneic combinations of T_{KLH} and B_{DNP}. Helper T cells were selected in 18-d bulk cultures of KLH-primed lymph node cells and treated with 25 μg/ml mitomycin C as described in Materials and Methods. In experiments 1 and 2, each well received 1,500 T_{KLH} of each type indicated. In experiment 3, a total of 6,000 T_{KLH} were added/well. Each well received, in addition, 3×10^4 B_{DNP} derived from spleens of donors primed and boosted with DNP_7FGG (experiments 1 and 3) or $DNP_{13}OA$ (experiment 2). The number of PFC induced in cultures that received either no antigen or optimal concentrations (0.1 μg/ml) of DNP_7FGG were uniformly <10% of those receiving DNP_8KLH (0.1 μg/ml).

mixtures of T cells syngeneic and allogeneic to the B cell donor (experiments 1 and 2). In addition, parental T cells cooperate as well as F_1 hybrid T cells with F_1 hybrid B cells and macrophage (experiment 3).

Isolation of F_1 Hybrid Helper T Cells Restricted to Parental MHC Haplotypes. The considerable enrichment of T_{KLH} in appropriately stimulated lymph node cultures (21) suggested that this might, in part, reflect clonal expansion of specific precursors. We undertook, therefore, to isolate clonal precursors in limiting-dilution cultures of primed F_1 hybrid lymph node cells. Others have shown through selective T cell binding or activation by parental macrophage that F_1 hybrid helper T cells comprise two independent populations, each specific for antigen and one parental MHC haplotype (2-4). If, as is likely, a similar restriction applies at the level of clonal precursors, then clones of F_1 T cells should be restricted to cooperate with hapten-primed spleen cells of only one parental type.

(BALB/c × BALB.B)F_1 KLH-primed lymph node cell cultures were initiated at between 2×10^3 and 30×10^3 viable cells per 100 μl culture. Normal anti-Thy-1.2

and C'- and mitomycin C-treated F_1 spleen cells were added as fillers to a final concentration of 35×10^3 cells/well. After 21 d, the cellular contents of 30-50 cultures at each initial input concentration were individually resuspended and transferred in equal aliquots to six 10-μl culture wells. Cells were washed in the new culture plates and helper activity was assayed by addition of 3×10^4 DNP-FGG-primed, anti-Thy-1.2-, anti-Ly-2.2-, and C'-treated spleen cells (B_{DNP}), and DNP-KLH (0.1 μg/ml) to each well. In each set of six test cultures, three received B_{DNP} of BALB/c origin and three of BALB.B origin. DNP-specific responses were assayed as PFC 5 d later.

The lymph node cell titration curve for one experiment is plotted in Fig. 1. At 30 $\times 10^3$ primed lymph node cells per culture, T cell precursors are in excess and virtually every culture transfers helper activity on day 21 to test wells of both parental types. As decreasing numbers of lymph node cells are added per culture, a required cell type becomes limiting and an increasing number of cultures fail to transfer helper activity to test wells of either parental type. The frequency of the limiting cell in the F_1 hybrid population can be determined from the Poisson relationship $F_0 = e^{-\mu}$; where F_0 is the fraction of lymph node cultures that fail to transfer helper activity and μ is the mean number of limiting cells added per culture (23). In this particular primed lymph node population, the limiting cell occurred at an initial frequency of ~1 in 10^4 cells. The linear relationship between log F_0 and the number of lymph node cells added/culture indicates that, in the presence of mitomycin C-treated splenic filler cells, only one cell in the F_1 population limits the ability of a lymph node culture to transfer helper activity (23). When titrations were carried out in the absence of filler cells, second-

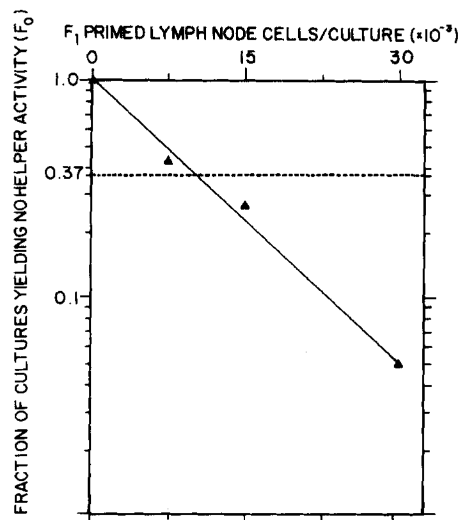

FIG. 1. Semilog plot of the fraction of lymph node cultures that fail to transfer any helper activity (F_0) vs. the number of (BALB/c × BALB.B) F_1 KLH-primed lymph node cells added to each 100-μl culture. Each culture was supplemented with syngeneic filler cells to 35×10^3 cells/well. This is the same experiment as experiment 1 of Table II but with additional data points. Helper activity was assayed as described in the legend to Table II. If a DNP-specific response was induced in any test culture of either BALB/c or BALB.B origin, the donor F_1 lymph node culture was scored positive for helper activity. The points conform to a straight line determined by least squares analysis with correlation coefficient >0 95.

order curves were obtained (not shown). This is consistent with an additional requirement of an accessory cell for T cell activation (24).

The results of two independent experiments are presented in Table II. For each experiment, data are shown for those initial lymph node cell concentrations at which a significant proportion of cultures failed to transfer helper activity to any of the six corresponding test wells. Under these conditions, the majority of F_1 lymph node cultures transfer helper activity to recipient wells of only one parental type. As discussed below, this is not caused by a limitation in the number of helper T cells present in a single culture on day 21 because when helper activity is transferred to test wells of one parental type it is most often transferred to all three test wells of that type. These results indicate that induction of F_1 helper T cells restricted to cooperate with BALB/c or BALB.B is dependent on cells that segregate in the limiting-dilution. The frequency of lymph node cultures, which should include limiting cells for both H-2 specificities, is the product of the individual frequencies and could, in fact, account for all of the cultures that were observed to transfer helper activity to recipient wells of both parental types. This analysis does not, however, exclude the possibility that a minority of precursors may be specific for a cross-reactive determinant shared by both parental haplotypes.

It is most important for these experiments that a lymph node culture that receives a limiting cell gives rise to an adequate number of helper T cells so that their specificity may be characterized by assay of helper function in independent test cultures with B_{DNP} of diverse origin. A minimal estimate for the mean number of helper cells present in a positive culture on day 21 can be derived from a Poisson analysis of the distribution of helper activity from lymph node cultures initiated at a particular limiting-dilution. For this purpose, data are pooled from all sets of three test wells for which a DNP-specific response is detected in at least one well. The fraction of test cultures in which a DNP-specific response is not detected (F_0) then

TABLE II

Isolation of (BALB/c × BALB.B)F_1 Helper T Cells Restricted to Parental MHC Haplotypes

Experiment	(BALB/c × BALB.B)F_1 primed lymph node cells/culture	Number of cultures assayed	Helper activity (number of cultures)			
			BALB/c only	BALB.B only	BALB/c and BALB.B	None
	× 10^3					
1	7.5	30	***5***	***10***	2	13
	15.0	30	***5***	***8***	9	8
2	7.5	50	***7***	***16***	10	17

(BALB/c × BALB.B)F_1 KLH-primed lymph node cultures were initiated at the indicated number of cells in 100-μl round-bottomed wells and supplemented with anti-Thy-1.2 and C′- and mitomycin C-treated F_1 filler cells as described in Materials and Methods. The helper activity in 30 or 50 identical lymph node cultures was assayed on day 21 by transfer to triplicate 10-μl microculture wells with DNP_7FGG-primed B cells and macrophage of either BALB/c or BALB.B origin. If a DNP-specific response was induced in at least one test well in a set of three, the donor lymph node culture was scored positive for helper activity restricted to that haplotype. In control plates that received the same number of B_{DNP}, but to which T cells were added in excess, a DNP-specific response was detected in every culture, indicating that B_{DNP} are also in excess. DNP-specific responses were detected as PFC on day 5 of secondary culture. The mean PFC/positive well for each B cell donor in both experiments was ≥100.

represents the fraction that did not receive a helper T cell. The mean number of helper T cells transferred to each test culture is determined from the Poisson relationship, $F_0 = e^{-\mu}$, and the mean number of helper T cells derived from the original 100-μl-vol lymph node cultures can be calculated. For the experiments in Table II, this value was at least 9–10 T_{KLH}/culture. In subsequent experiments (Table III), higher expansion factors of 19–22 were obtained.

Isolation of F_1 Hybrid Helper T Cells Restricted to F_1 Hybrid MHC Determinants. We have extended this analysis to determine whether clonal precursors of F_1 hybrid helper T cells may be restricted to unique F_1 hybrid MHC determinants. To facilitate assays of helper activity in the large number of individual cultures generated in such experiments, we have adopted an *in situ* hemagglutination assay for specific antibody secretion on day 5 of secondary culture. Details of this assay and its correlation with the PFC assay are presented in Materials and Methods.

Cultures of lymph node cells from KLH-primed (BALB.K × BALB.B)F_1 mice were initiated at various concentrations in the presence of normal F_1 splenic filler cells as described above. The cellular contents of each culture were resuspended on day 21 and transferred to six 10-μl culture wells for assay of helper activity. Each set of six test cultures consisted of duplicate wells that received B_{DNP} of BALB.K, BALB.B, or (BALB.K × BALB.B)F_1 origin. The helper activity, which arises from precursors isolated at the limiting-dilutions in two different experiments, is shown in Table III. As in the experiments described above, F_1 helper T cells restricted to cooperate with spleen cells of one or the other parental type segregate in different cultures. The inclusion of F_1 hybrid indicator B_{DNP} in these experiments revealed, in addition, a large number of cultures that give rise to helper activity restricted uniquely to F_1 spleen cells. T_{KLH} from these cultures fail to cooperate with spleen cells of either parental type. Most lymph node cultures with helper activity for parental type spleen cells also demonstrate helper activity for the F_1 hybrid. The possible significance of the low frequency of cultures with helper activity for parental but not F_1 spleen cells will be discussed below.

It needs to be emphasized that, in all the experiments reported here, B_{DNP} are in excess when 3×10^4 primed spleen cells are added/well because, when T_{KLH} were also provided in excess, a DNP-specific response was induced in every test well. As indicated above, a minimal estimate for the number of helper T cells derived from cultures that received a single limiting lymph node cell in the experiments of Table

TABLE III

Isolation of (BALB.K × BALB.B)F_1 Helper T Cells Restricted to F_1 Hybrid MHC Determinants

Experiment	(BALB.K × BALB.B)F_1 primed lymph node cells/culture	Number of cultures assayed	Helper activity (number of cultures)						
			BALB.K and F_1	BALB.B and F_1	F_1 only	BALB.K only	BALB.B only	BALB.K, BALB.B, and F_1	None
	$\times 10^3$								
1	2.0	50	***6***	***9***	***7***	0	1	2	25
	6.0	40	***6***	***11***	***4***	0	1	12	6
2	2.0	50	***4***	***8***	***8***	2	0	5	23

(BALB.K × BALB.B)F_1 KLH-primed lymph node cultures were initiated at the indicated number of cells supplemented with normal F_1 filler cells. Helper activity was assayed on day 21 by transfer to duplicate 10-μl microculture wells with DNP_7FGG-primed B cells and macrophages of either BABL.K, BALB.B, or (BALB.K × BALB.B)F_1 origin (F_1). Induction of DNP-specific responses was detected in a hemagglutination assay of antibody secreted on day 5 of secondary culture as described in Materials and Methods. In control plates that received the same number of B_{DNP}, but to which T cells were added in excess, a DNP-specific response was detected in every culture.

III is 19–22. In practice this meant that, out of a total of 93 pairs of duplicate wells scored as positive in the two experiments, 87 pairs gave a DNP-specific response in both wells and 6 gave a DNP-specific response in only one well.

We have consistently observed in these and similar experiments that, in (H-2^d × H-2^b) and (H-2^k × H-2^b) F_1 hybrids, a substantially higher frequency of precursors are induced for KLH-specific helper T cells restricted to H-2^b than to either H-2^d or H-2^k. This, in effect, defines H-2^b as a relatively high responder to KLH. The system described here can reliably detect quantitative differences in the response to this complex antigen because frequency determinations are made from a single source of F_1 cells.

Discussion

We have demonstrated in an F_1 hybrid population the presence of helper T cells restricted to unique F_1 hybrid MHC determinants. It is particularly striking that helper T cells selected only on the basis of specificity for a complex antigen include T cells restricted to F_1 hybrid determinants at a frequency approximately equal to that of T cells restricted to either parental MHC. The F_1 hybrid specificities detected may be directed at determinants that arise through gene complementation within or between different *H-2* subregions. Gene complementation between *I-A* and *I-E* subregions has been previously demonstrated for other *I* region-determined functions (8–11). Experiments are in progress to test whether helper T cells may be restricted to F_1 hybrid determinants expressed in strain combinations that differ only in the *K* and *I-A* subregions. The choice of KLH for this analysis, a complex antigen whose diverse determinants may be recognized by helper T cells with different restriction specificities, increases the likelihood that if hybrid determinants exist then they will be detected.

Clones with discrete restriction specificities were shown to segregate in limiting-dilution cultures of primed F_1 lymph node cells. It was determined from a distribution analysis of the helper activity transferred from individual limiting lymph node cultures that positive cultures give rise to an initial burst of 10–20 helper T cells with apparently identical specificity. The limiting cells in the lymph node populations may themselves be clonal precursors or regulatory cells. If they are regulatory cells, then they must be specific for a marker that distinguishes precursors of helper T cells restricted to different MHC determinants. A single culture, even at limiting concentrations of lymph node cells, might then include progeny of more than one precursor with identical H-2 specificity. This would, or course, in no way affect the analysis of these specificities. In any case, that clonal precursors do indeed proliferate was suggested by the very high frequency of helper T cells recovered in lymph node cultures after 2 wk (21) and has been demonstrated more directly in experiments of Julius and Augustin (20) and Schrier et al. (25) in which it was shown that helper activity is recovered in a blast cell fraction.

We noted in the results presented in Table III that, in each experiment, two cultures with helper activity restricted to one parental haplotype failed to transfer help to F_1 hybrid test cultures. This contrasts with helper activity in 44 other cultures in the two experiments that was also restricted to one parental haplotype but could be transferred to test cultures of the F_1 hybrid. Fathman and Hengartner (6, 26) have described T cell clones reactive to a unique homozygous (H-2^b) MLR-stimulating determinant

that is not expressed in an F_1 hybrid (H-2^b × H-2^a). It is possible that a minority of helper T cell clones in our experiments have a similar specificity. The definitive identification of minor helper T cell restriction specificities will require further purification and expansion of representative clones.

A major implication of these and related experiments of others is that there is a relationship between the phenomena of *Ir* gene regulation and *I* region restriction of helper T cell function. This was first suggested by the observation that the high-responder phenotype in (high responder × low responder)F_1 hybrids is associated only with helper T cells restricted to the high-responder MHC haplotype (12, 13). This relationship was further supported by the demonstration that both of these *I* region-determined functions are expressed in the inductive environment during T cell maturation and that they need not be expressed directly in T cell precursors (27). In the absence of fine structure genetic mapping, these parallel phenomena are the best available evidence that *Ir* gene products and *I* region-encoded restriction elements are identical. The experiments reported here extend these parallels in that they demonstrate that, just as two complementing *Ir* genes may regulate responses to specific antigens such as poly($Glu^{53}Lys^{36}Phe^{11}$) (GLϕ) (11), complementing genes in an F_1 hybrid may form the basis for H-2 restriction in helper T cells. It becomes necessary then to account for how genes for restriction elements that function in both T cell maturation and in physiological interactions are associated with antigen-specific effects. A minimal requirement is that there be some relationship between the receptor for antigen and the receptor for a restriction element expressed on a single T cell. This relationship might be that they are, in fact, the same receptor or, as suggested by von Boehmer et al. (28), that they are different receptors derived from a single germ-line gene. In principle, however, a more complex genetic relationship, which links the expression of independent germ-line genes, is also possible. Whatever the mechanism, a receptor relationship is both necessary and sufficient to account for how T cells selected for restriction to one MHC determinant may recognize a specific antigen such as GLϕ (29), whereas T cells restricted to another determinant do not.

In our own experiments, we have examined restriction of KLH-specific helper T cells to parental and hybrid MHC determinants. The very high frequency of helper T cells restricted to hybrid determinants suggests that this may be an important mechanism for increasing the antigen-specific repertoire in heterozygotes.

Summary

Antigen-specific major histocompatibility complex (MHC)-restricted helper T cell precursors were induced to proliferate in cultures of keyhole limpet hemocyanin-primed lymph node cells. Clones of F_1 hybrid helper T cells were isolated in limiting-dilution cultures. Each positive culture at a limiting-dilution of lymph node cells gave rise to >10 helper T cells with a single MHC-restricted specificity. This made it possible to independently assay specific helper activity of isolated clones in secondary cultures with B cells of diverse origin. Different clones with helper activity restricted to either parental or unique F_1 hybrid MHC determinants were found to occur at approximately equal frequency. The results are discussed in relation to hybrid Ia specificities and dual-complementing MHC-linked *Ir* genes.

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