ANTIGEN-INDUCIBLE, H-2-RESTRICTED, INTERLEUKIN-2-PRODUCING T CELL HYBRIDOMAS

Lack of Independent Antigen and H-2 Recognition*

By JOHN W. KAPPLER, BARRY SKIDMORE, JANICE WHITE,
AND PHILIPPA MARRACK‡

From the Department of Medicine, National Jewish Hospital and Research Center, Denver, Colorado 80206

The characterization of the antigen-recognizing structure on the various classes of T cells has been a difficult problem for immunologists. Three aspects of this problem are particularly controversial: first, whether the genes that code for the variable portion of immunoglobulin also code for the antigen-recognizing portion of the receptor on all classes of T cells (1–4); second, whether T cell receptors specific for allo-major histocompatibility complex (MHC)[1] products are unique or are included within the population of receptors for conventional antigens (5–10); and third, how T cells, particularly those of the cytotoxic and helper classes, are able to demonstrate an apparent dual specificity for both a conventional antigen and a product of the MHC such that they function only when confronted with both the appropriate antigen and MHC product (11–16). Various models have been proposed to explain this phenomenon. Some have proposed that antigen and MHC are recognized independently via two receptors on the T cell (17–23). Others have proposed various forms of dependent recognition, involving, for example, a single receptor on the T cell, and/or a physical interaction between antigen and MHC products in antigen-presenting cells (24–26). A number of experimental results have been used by various investigators to argue for one or the other of these models, but there is little evidence that distinguishes the various possibilities.

A direct attack on these questions has been hampered by the lack of suitable sources of clonal T cell lines analogous to the B cell myelomas. Recently, two techniques have offered promise in this regard. The first is the establishment of long-term cultures of normal antigen-specific T cells through the use of repeated in vitro antigen stimulation and/or the growth factor, interleukin 2 (IL-2; formerly, T cell growth factor) (27–30). The second is the production of immortal T cell hybrids between normal antigen-specific T cells and T cell tumor lines (31–36). In the present study, we combined these two methods to produce inducible, antigen-specific, H-2-restricted T cell hy-

[1] *Abbreviations used in this paper:* AG, 8-azaguanine; Con A, concanavalin A; cyto c, beef heart cytochrome c; HGG, human gammaglobulin; HAT, hypoxanthine, aminopterin, and thymidine; IL-2, interleukin-2; KLH, keyhole limpet hemocyanin; MHC, major histocompatibility complex; NMS, normal mouse serum; OU, ouabain; OVA, ovalbumin; PEG, polyethylene glycol; SN, supernate.

bridomas. Four cloned hybridoma cell lines were produced. Each grew rapidly in culture without stimulation but produced IL-2 in response to a challenge with specific antigen and splenic antigen-presenting cells of the appropriate *H-2* haplotype. In each case, the *H-2*-restricting element mapped to the *I* region. One of the hybrid lines responded to an allo-*H-2* determinant as well.

In addition, we used this fusion method to produce hybrids between cells that had different antigen/*H-2* specificities. These "double" hybrids often maintained the ability to recognize both parental antigen/*H-2* combinations, but no hybrids were detected with either of the "cross-specificities", i.e., the *H-2* specificity of one parent and the antigen specificity of the other. This result offers conclusive evidence against any models for *H-2* restriction that predict the independent recognition of antigen and *H-2*.

Materials and Methods

Mice. B6D2F$_1$, B6AF$_1$, C57BL/10.SgSn (B10), B10.A, B10.D2, and B10.BR mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. The following mice were bred in our facility from breeding triplets purchased from The Jackson Laboratory or kindly supplied by various individuals, as indicated: BALB/cJ, CBA/J, D1.LP, DBA/2, B10.A(5R) (The Jackson Laboratory); BALB.B (Dr. M. Bevan, Massachusetts Institute of Technology, Boston, Mass.); B10.A(4R), B10.S (Dr. J. Stimpfling, McLaughlin Research Institute, Great Falls, Mont.); B10.M (Dr. M. Cherry, The Jackson Laboratory); B10.AQR, B10.LG (Dr. C. David, The Mayo Clinic, Rochester, Minn.); B10.TL, B10.S(7R) (Dr. D. Shreffler, Washington University, St. Louis, Mo.); B10.A(3R), D2.GD, B10.HTT (Dr. D. Sachs, National Institutes of Health, Bethesda, Md.).

Antigens and Other Reagents. The following antigens were purchased from Sigma Chemical Co., St. Louis, Mo.: chicken ovalbumin (OVA), pigeon OVA, turkey OVA, and beef heart cytochrome c (cyto c). The apo form of cyto c was prepared according to a modification of the silver sulfate method (37), and its 1-65 peptide was derived by cyanogen bromide cleavage and gel filtration (38). Duck OVA was obtained from Pentex, Division of Miles Corp., Kankakee, Ill. Keyhole limpet hemocyanin (KLH) was purchased from Calbiochem-Behring Corp., American Hoeschst Corp., San Diego, Calif. Human gammaglobulin (HGG) was purified from American Red Cross Cohn Fraction II by DEAE cellulose chromatography. Concanavalin A (Con A), hypoxanthine, aminopterin, and thymidine (HAT), ouabain (OU), and 8-azaguanine (AG) were also obtained from Sigma Chemical Co. Polyethylene glycol (PEG) 1540 and 6000 were obtained from the J. T. Baker Chemical Co., Philipsburg, Pa. PEG-1540 was used at 50% wt/wt and PEG-6000 was used at 40% wt/wt.

Immunizations. Mice were immunized with 100 µg of antigen emulsified in complete Freund's adjuvant at the base of the tail, except in the case of apo cyto c, when 50 µg/mouse was used (39). Para-aortic and inguinal lymph nodes were taken 7 d later.

Tissue Culture Media. Primary lymph node cultures were established in Click's medium containing 1% syngeneic normal mouse serum (NMS) (40). Otherwise, cells were cultured in medium prepared according to Mishell and Dutton (41), but modified to contain 5×10^{-5} M 2-mercaptoethanol, 10% fetal calf serum (pre-selected to support primary spleen cell responses to sheep erythrocytes in Mishell-Dutton cultures), 50–100 µg/ml gentamicin (Shering Corp., Kenilworth, N. J.), and 10% Mishell-Dutton nutrient cocktail. This medium is referred to as complete medium.

IL-2 Production. Bulk IL-2 containing supernatant fluids (SN) was prepared using the Con A-inducible hybridoma, FS6-14.13 (42). Hybrid cells were cultured at 5×10^5–10^6/ml in complete medium containing 4 µg/ml Con A. After 24 h, the cells were removed by centrifugation, and the SN were passed through a sterile 0.2-µ filter and stored at −20°C. Occasionally, Con A-induced SN of rat spleen cell cultures were used as a source of IL-2 (40).

To test hybrids for the antigen-specific stimulation of IL-2, production cultures were prepared containing 10^6 hybrid cells/ml, antigen at the appropriate concentration, and 5×10^6 irradiated

(4,000 rad) spleen cells/ml of the appropriate *H-2* type. After 24 h, the SN were tested for the presence of IL-2.

Assay for IL-2. The T cell growth-stimulating activity of IL-2 was assayed with a semi-quantitative method using the IL-2-dependent T cell line HT-2 (kindly provided by Dr. James Watson, University of California, Irvine, Calif.). As originally described, this was a cloned helper T cell line that grew continuously in IL-2 (30). The subline used in these experiments had lost its helper activity, but maintained its absolute requirement for IL-2 for viability and growth. Within 24 h of removal from IL-2, the HT-2 cells were >95% dead. In the continuous presence of IL-2, this cell line had a viability of >95%. Therefore, to test an SN for the presence of IL-2, 0.1-ml cultures were prepared containing various dilutions of the SN or of a known IL-2-containing standard SN. These cultures received 0.01 ml of Mishell-Dutton nutrient cocktail (41) and 4,000 viable HT-2 cells. Control cultures contained no IL-2. After 24 h, the cultures were examined with an inverted phase microscope. Cultures containing <5% viable cells were scored negative (−). Cultures with 90–100% viable cells were scored positive (+). Cultures with 5–90% viable cells were scored intermediate (±).

The data are presented as the results obtained with 80% SN; however, titrations were performed in most experiments, especially in the characterizations of the cloned hybrids. In these cases, nearly all positive SN had titers >1:10. Negative SN were negative at all concentrations tested.

This assay for IL-2 has the advantage of being extremely rapid because cultures can be screened visually for IL-2 presence only 24 h after plating. We have compared it on many occasions with more routine methods of measuring IL-2 concentrations, such as stimulation of tritiated thymidine incorporation by IL-2-dependent T cell blasts. The two techniques give very similar results.

Hybridoma Antibody Reagents. To aid in the *I* region mapping of specificity of our T cell hybridomas, we used a number of hybridoma anti-*I-A* reagents produced by this and other laboratories (43). These are listed with their relevant properties in Table I. We are extremely grateful to Drs. L. Herzenberg and L. Herzenberg and their colleagues for making their hybridomas available to the scientific community via The Salk Institute Cell Distribution Center, La Jolla, Calif. The hybridoma antibodies produced in this laboratory were mapped to *I-A* using B cells from the appropriate *H-2* recombinant mice. Other laboratories using these reagents have confirmed the specificities.

Parent T Cell Tumor Lines for Fusions. Four related parental T cell tumor lines were used in these studies. The first two were derived from the Con A-inducible T cell hybridoma, FS6-14.13 (42). FS6-14.13 cells were grown in 10^{-4} M AG. Most of the hybrid cells died within the next few days, but over a period of weeks the cultures were overgrown by surviving cells resistant to the drug. These cells were cloned at limiting dilution and the resultant clones were tested both for sensitivity to HAT and for IL-2 production after Con A stimulation. One clone, FS6-14.13.AG2, was selected for use in fusions. In one case, a subclone of FS6-14.13.AG2, which had been selected for resistance to both AG and 10^{-3} M OU, was used as the tumor parent. This subclone was called FS6-14.13.AG2.OU8.

In one of the fusions described in the results, the hybrid AO-40 was produced; this hybrid could be stimulated to produce IL-2 by either Con A, *H-2^b* cells, or OVA plus *H-2^a* cells. Two parental lines suitable for use in fusions were derived from AO-40. The first was produced by

TABLE I
I-A Specific Hybridoma Antibodies Used in These Studies

| Hybrid | Tumor parent | Normal parent | | Specificity | Class | Reference |
		Strain	Immuno-gen			
11-5.2	NS-1	BALB/c	CKB	*I-A^k*	γ2b, κ	Oi et al. (43)
10-3.6	NS-1	CWB	C3H	*I-A^{k,s,f}*	γ2a, κ	Oi et al. (43)
MK-D6	P3X63-AG8.653	(B6 × A/J)F₁	B10.D2	*I-A^d*	Unknown	This paper
MK-S4	45.6TG1.7	(D1.LP × B10.A)F₁	B10.S	*I-A^{s,f}*	Unknown	This paper

reintroducing AG resistance into AO-40 before cloning. These cells (AO-40/AG) rapidly lost their ability to respond to H-2^b or to OVA plus H-2^a, but not to Con A. This uncloned line was used as the T cell tumor parent in one fusion. Another parental line was established from AO-40.10, a clone of AO-40. AG resistance was reintroduced into this line and the resultant cells were cloned to yield AO-40.10.AG1. This line continued to respond to H-2^b, to OVA plus H-2^a, and to Con A. It was used in fusions aimed at producing hybrids with two antigen/H-2 specificities.

Parental Normal T Cells for Fusions.   A population of normal T cell blasts highly enriched in antigen-specific cells was prepared by the method of Schrier et al. (40). Briefly, lymph node cells were taken from immunized mice and cultured at 4×10^6/ml for 4 d with specific antigen (125 μg/ml OVA, 10 μg/ml KLH, or 50 μg/ml apo cyto c 1-65 peptide). These cultures contained a high concentration of T cell blasts. Viable cells were isolated on Ficoll-Hypaque gradients and were recultured for 3–4 d at an initial concentration of 10^5 cells/ml in medium containing 50% IL-2 SN. The viable T cell blasts were then reisolated and used in fusions. Previous experiments had shown that this procedure resulted in a T cell preparation ~100-fold enriched over the initial lymph node cells in specific T cell helper activity or proliferative ability (40).

Fusion Protocol.   Cells were fused essentially by the method of Kontiainen et al. (33). Both PEG-1540 and PEG-6000 were used. In these experiments, ~4×10^7 T cell blasts were fused to 1–2×10^7 FS6-14.13.AG2, FS6-14.13.AG2.OU8, AO-40/AG, or AO-40.10.AG1 cells. Cells were plated in ~300–400 microculture wells in 0.1 ml of medium. HAT was added at 24 h and the medium was changed about every 5 d. Hybrid growth was first apparent at about 10 d and the last wells were scored positive for growth at about 3 wk. Hybrids were transferred to larger vessels to obtain enough cells for testing and were eventually "weaned" off HAT in HT-containing medium.

Cloning of Hybrid Cells.   Hybridomas were cloned at limiting dilution in four 96-well microculture plates containing, on the average, 0.125, 0.25, 0.5, and 1.0 cells/well. When possible, functional clones were selected from the plate having the fewest clones. At no time were clones selected from a plate containing more than 20 clones (2.4% chance of more than one clone/well). Cloning efficiencies varied considerably.

Results

Production of Hybrids with an Antigen/I Region Specificity.   To produce antigen-specific, H-2-restricted T cell hybridomas, we fused the Con A-inducible, IL-2-producing hybridoma FS6-.13.AG2 (or its derivatives, FS6-14.13.AG2.OU8 and AO-40/AG) to a population of normal T cell blasts highly enriched in antigen-specific, H-2-restricted cells. We used this approach for two reasons. First, the fact that the tumor cell partner in the fusions already carried a function meant that it had to acquire only an antigen specificity from the normal T cell parent. Second, we greatly increased the probability of a successful hybrid by enrichment of the normal T cell parents for antigen-reactive cells. This approach has been highly successful. The four cloned T cell hybridomas that we have produced thus far with specificity for antigen and an I region gene product are described below.

AO-40.10.   T cell blasts enriched in chicken OVA-specific T cells were prepared from B10.A (H-2^a) mice and fused to FS6-14.13.AG2 using PEG-6000. Eventually, 11 hybrids were recovered and tested for IL-2 production in response to a challenge with OVA and irradiated B10.A spleen cells. Only one responsive hybrid, AO-40, was found. This hybrid was cloned at limiting dilution, and 12 of the 13 clones picked and retested were positive for IL-2 production in response to chicken OVA plus B10.A cells. One clone, AO-40.10, was chosen for further characterization. AO-40.10 has been in existence for more than 6 mo, and has been recloned twice. In each recloning, the majority of subclones were responsive to OVA plus B10.A but some unresponsive

subclones were also found. We therefore established a large frozen stock of the hybrid immediately after cloning and routinely replaced the laboratory stock once a month. AO-40.10 has a doubling time in culure of ~12 h and a cloning efficiency approaching 100%.

The fine antigen specificity of AO-40.10 was determined using a series of related OVA from chicken, duck, pigeon, and turkey and B10.A antigen-presenting cells. The results, shown in Table II, were somewhat surprising, in that AO-40.10 responded only to chicken OVA despite the fact that in the lymph node proliferation assay, chicken OVA-primed B10.A cells responded equally well to chicken, duck, and turkey OVA (Table III). Apparently AO-40.10 is specific for a determinant unique to chicken OVA. AO-40.10 has also been tested for reactivity against a panel of 30 other antigens in the presence of B10.A antigen-presenting cells. Antigens tested included KLH, human and other species' gammaglobulin, cytochrome c, etc. In no case was IL-2 production induced (results not shown).

The H-2 specificity of AO-40.10 was established using chicken OVA and irradiated antigen-presenting cells from a panel of H-2 congenic and recombinant mice. The results are shown in Table IV. When tested with H-2 congenic mice, the surprising result was obtained that AO-40.10 responded to all mice of the H-2^b haplotype regardless of background and without the need for the specific antigen, OVA. Among the recombinant mice, this OVA-independent response was seen with B10.A(3R) and B10.A(5R), but not with B10.A(4R) or D2.GD, mapping this unexpected allo-H-2 response to the K-I-A region of H-2^b.

TABLE II

Antigen Specificity of Cloned T Cell Hybrids

Hybrid	Irradiated spleen cells	Antigen	IL-2 production
AO-40.10	B10.A	Chicken OVA	+
		Duck OVA	−
		Turkey OVA	−
		Pigeon OVA	−
		KLH	−
		HGG	−
AODK-1.16	B10.D2	KLH	+
		Chicken OVA	−
		HGG	−
AODK-10.4	B10.D2	KLH	+
		Chicken OVA	−
		HGG	−
DC-1.18.3	BALB/c	Apo beef cyto c	+
		Native beef cyto c	−
		KLH	−
		Chicken OVA	−
		HGG	−

All SN were tested at 80% with HT-2 cells. In tests with AO-40.10, the various OVA were used at 250 μg/ml. In other cases, chicken OVA was used at 1,250 μg/ml. KLH was used at 1,000 μg/ml. Apo beef cyto c, native beef cyto c, and HGG were used at 500 μg/ml.

TABLE III

Specificity of the Proliferative Response of Chicken OVA-primed Lymph Node
Cells from B10.A Mice

Priming antigen	Challenge antigen	Lymph node cell [³H]-TdR incorporation on day 5
		cpm/culture × 10⁻³
Chicken OVA	Chicken OVA	162 ± 2
Chicken OVA	Turkey OVA	174 ± 5
Chicken OVA	Duck OVA	154 ± 4
Chicken OVA	Pigeon OVA	10 ± 2
Chicken OVA	Human IgG	2 ± 1
Turkey OVA	Turkey OVA	176 ± 13
Duck OVA	Duck OVA	163 ± 2
Pigeon OVA	Pigeon OVA	217 ± 4

Mice were immunized as described in Materials and Methods. Lymph node cells were challenged in vitro with antigen and their proliferation was measured by [³H]TdR incorporation as described (39, 44). Antigens were used at 250 μg/ml. Incorporation shown is experimental value minus no antigen control value.

As expected, AO-40.10 mounted an OVA-dependent response with cells carrying the I region from $H-2^k$. No OVA-dependent cross-reactions were seen with other $H-2$ haplotypes. Among the recombinant mice, the response with B10.A, B10.A(4R), B10.TL, and B10.AQR, but not with B10.S(7R), B10.HTT, or D2.GD, mapped the relevant I region gene(s) to $I-A^k$.

The I region molecules important in the response of AO-40.10 were further identified in blocking experiments with anti-$I-A$ hybridoma antibodies (Table V). The response to OVA plus $H-2^a$ was inhibited by $I-A^k$-specific hybridoma antibodies but not by antibodies to $I-A^{sf}$ or $I-A^d$. That these reagents inhibited by specific blocking rather than by killing the antigen-presenting cells was shown using F_1 antigen-presenting cells. In this case, antibodies directed against the inappropriate $I-A$ molecule on the F_1 cells had no effect on the response. Therefore, the combination of genetic mapping and anti-Ia inhibition mapped the $H-2$ region that is important in the OVA-dependent response to $I-A^k$.

The responses of AO-40.10 to $H-2^b$ and to OVA plus $I-A^k$ are closely associated. In every cloning of this hybrid, each clone has had either both responses (36 clones) or neither (15 clones). We consider this point further in the Discussion.

DC-1.18.3. T cell blasts enriched in apo beef cyto c-specific T cells were prepared from BALB/c ($H-2^d$) mice and fused to FS6-14.13.AG2.OU8 using PEG-1540. Eventually, 10 hybrids were recovered and tested for IL-2 production in response to a challenge with apo beef cyto c and irradiated BALB/c spleen cells. All 10 hybrids were responsive. The hybrids were frozen and two were picked for cloning attempts. One failed to yield functional clones. However, of the 16 clones obtained from the other hybrid (cloning efficiency, 24%), one of these, DC-1.18, was found to be positive. DC-1.18 was recloned (cloning efficiency, 80%) and ~60% of the clones were positive. One subclone, DC-1.18.3, was selected for further characterization. The antigen specificity of DC-1.18.3 was tested with a number of antigens and BALB/c irradiated spleen cells (Table II). The hybrid responded to apo beef cyto c, but not to the native

TABLE IV

H-2 Specificity of Cloned T Cell Hybrids

		Irradiated spleen cells										IL-2 production							
			H-2 Subregions									AO-40.10		DC-1.18.3		AODK-1.16		AODK-10.4	
					I														
Strain	H-2	K	A	B	J	E	C	S	G	D	Alone	Plus chicken OVA	Alone	Plus apo cyto c	Alone	Plus KLH	Alone	Plus KLH
B10.BR	k	k	k	k	k	k	k	k	k	k	−	+	NT*	NT	−	−	−	−
CBA/J	k	k	k	k	k	k	k	k	k	k	−	+	−	−	−	−	−	−
B10	b	b	b	b	b	b	b	b	b	b	+	+	−	−	−	−	−	−
D1.LP	b	b	b	b	b	b	b	b	b	b	+	+	NT	NT	−	−	−	−
BALB.B	b	b	b	b	b	b	b	b	b	b	+	+	NT	NT	−	−	−	−
B10.D2	d	d	d	d	d	d	d	d	d	d	−	−	−	+	+	+	+	+
DBA/2	d	d	d	d	d	d	d	d	d	d	−	−	−	+	+	+	+	+
BALB/c	d	d	d	d	d	d	d	d	d	d	−	−	−	+	+	+	+	+
B10.S	s	s	s	s	s	s	s	s	s	s	−	−	−	−	−	−	−	−
B10.M	f	f	f	f	f	f	f	f	f	f	−	−	−	−	−	−	−	−
B10.A	a	k	k	k	k	k	d	d	d	d	−	+	NT	NT	−	−	−	−
AQR	y1	q	k	k	k	k	d	d	d	d	−	+	NT	NT	−	−	−	−
B10.TL	t1	s	s	s	s	k	d	d	d	d	−	+	NT	NT	−	−	−	−
B10.A(4R)	h4	k	k	b	b	b	b	b	b	b	−	+	NT	NT	−	−	−	−
B10.A(3R)	i3	b	b	b	b	k	d	d	d	d	+	+	NT	NT	−	−	−	−
B10.A(5R)	i5	b	b	b	k	k	d	d	d	d	+	+	NT	NT	−	−	−	−
D2.GD	g2	d	d	d	b	b	b	b	b	b	−	−	NT	+	−	−	+	+
B10.LG	ar1	d	f	f	f	f	f	f	f	?	NT	NT	−	−	−	−	−	−
B10.S(7R)	t2	s	s	s	s	s	s	s	k	d	−	−	NT	−	−	−	−	−
B10.HTT	t3	s	s	k	k	k	k	k	k	d	−	−	NT	NT	−	−	−	−
None											−	−	−	−	−	−	−	−

All SN were tested at 80% with HT-2 cells. Chicken OVA was used at 1,250 µg/ml, KLH was used at 1,000 µg/ml, and apo cyto c was used at 500 µg/ml. H-2 subregion assignments were taken from Klein et al. (47).
* Not tested.

TABLE V

Anti-I-A Inhibition of the Response of Cloned T Cell Hybrids

Hybrid	Stimulus	IL-2 production				
		No Inhibitor	Hybridoma antibody inhibitor			
			11-5.2 (I-A^k)	10-3.6 (I-$A^{k,f,s}$)	MK-D6 (I-A^d)	MK-S4 (I-$A^{s,f}$)
AO-40.10	B10.A + OVA	+	−	−	+	+
	B10	+	+	+	+	+
	B6D2F₁	+	+	+	+	+
	(B10.A × D2)F₁ + OVA	+	−	−	+	+
AODK-1.16	B10.D2 + KLH	+	+	+	+	+
	B6D2F₁ + KLH	+	+	+	+	+
	(B10.A × D2)F₁ + KLH	+	+	+	+	+
AODK-10.4	B10.D2 + KLH	+	+	+	−	+
	B6D2F₁ + KLH	+	+	+	−	+
	(B10.A × D2)F₁ + KLH	+	+	+	−	+
AOFK-11.11.1	B10.A + OVA	+	−	−	+	+
	B10.M + KLH	+	+	−	+	−
	B10	+	+	+	+	+
	(B10 × B10.M)F₁	+	+	+	+	+
	(B10.A × B10.LG)F₁ + OVA	+	−	−	+	+

All SN were tested at 80% with HT-2 cells. Chicken OVA was used at 1,250 μg/ml. KLH was used at 1,000 μg/ml. Hybridoma antibodies were added to irradiated stimulator cells and antigen 1 h before the addition of the hybridoma cells. Antibodies were added in the form of 1% ascitic fluid taken from hybridoma-containing mice.

form of the protein. The fine specificity of this hybrid correlated with that of apo beef cyto c primed lymph node cells in proliferative in vitro responses (45). As expected, the hybrid failed to respond to chicken OVA, HGG, or KLH.

The fine *H-2* specificity of DC-1.18.3 was determined using a number of *H-2* congenic and recombinant mice (Table IV). No response was seen with any *H-2* haplotype in the absence of apo cyto c. There was an apo cyto c-dependent response with all *H-2^d* mice tested. This reactivity was mapped to *I-A^d* by the response seen with D2.GD, and the lack of response with B10.LG and B10.A.

AODK-10.4 and AODK-1.16.   T cell blasts were prepared from KLH-primed DBA/2 mice (H-2^d) and were fused to AO-40/AG using PEG-1540. Eventually, 13 hybrids were recovered and tested for IL-2 production in response to a challenge with KLH and B10.D2 spleen cells. Three reactive hybrids were found and cloned at limiting dilution. The first, AODK-9, failed to yield any functional clones and the uncloned line lost reactivity over a period of weeks. All clones of the second hybrid, AODK-10, (cloning efficiency, 14%) responded to KLH with B10.D2 cells. One clone, AODK-10.4, was selected for further study. The third hybrid, AODK-1, had a poorer cloning efficiency, (~7%) and yielded only one responsive clone of the five tested. This clone, AODK-1.16, was used in these studies.

Both AODK-10.4 and AODK-1.16 were tested with B10.D2 cells and several antigens. Both responded to KLH, but no response was seen with OVA or HGG

(Table II). The H-2 specificities of these hybrids were determined as above with H-2 congenic and recombinant mice. No antigen-independent response was seen with any H-2 haplotype. KLH-specific responses were seen with all three H-2^d strains, but not with any other tested H-2 haplotype. Using H-2 recombinant mice, neither hybrid responded to KLH with B10.A or B10.LG mice, mapping their specificity to the I region. However, AODK-10.4 responded to KLH with D2.GD cells, whereas AODK-1.16 did not. This mapped the response of AODK-10.4 to I-A^d, but left the subregion specificity of AODK-1.16 in doubt. Although the response pattern apparently mapped the specificity to I-J/I-E^d, this pattern was also consistent with specificity for a composite molecule with elements from I-A^d and I-E/C^d (46). We attempted to confirm the specificity of these hybrids again using specific hybridoma anti-I-A antibodies to inhibit their recognition (Table V). Whereas AODK-10.4 was inhibited in its response to KLH plus H-2^d by an anti-I-A^d antibody, the response of AODK-1.16 was unaffected. Again, the specificities of these inhibitions were established using F_1 antigen-presenting irradiated spleen cells. Therefore, the combination of the genetic mapping and anti-Ia inhibition identified a product of I-A^d as the structure recognized by AODK-10.4 and left open the possibilities that AODK-1.16 recognized either a product of the I-J/I-E region or a molecule with elements from both I-A and I-E/C.

Production of Hybrids with Two Antigen/I Region Specificities. Next, we attempted to produce hybrids with two antigen/H-2 specificities. Using PEG-1540, AO-40.10.AG1 was fused to normal T cell blasts of a different antigen/H-2 specificity. The resultant hybrid lines were tested for IL-2 production in response to the four combinations of OVA, the second antigen, H-2^a, and the second H-2 type.

The results of three fusions of this type are summarized in Table VI. On initial screening, a large proportion of the hybrids retained reactivities to both OVA plus H-2^a and to the normal T cell parent antigen/H-2 combination. For example, in the AOFK fusion, five out of seven hybrid lines tested responded to H-2^a plus OVA, and H-2^f plus KLH. However, in no case were any hybrids responsive either to OVA plus the normal T cell H-2 type or to H-2^a plus the antigen for which the normal T cell parent was specific. This result argued strongly against the independent recognition

TABLE VI

T Cell Hybrids with Two Antigen/H-2 Specificities (Initial Screening)

Fusion	Tumor cell parent	Normal T cell parent			Number of hybrids	IL-2 production			
		Strain	H-2	Antigen		Reactivity pattern with H-2 + antigen combinations			
						H-2ᵃ + OVA	H-2ᵈ + HGG	H-2ᵃ + HGG	H-2ᵈ + OVA
AODH	AO-40.10.AG1	DBA/2	d	HGG	2	−	−	−	−
					1	+	−	−	−
					1	−	+	−	−
					3	+	+	−	−
						H-2ᵃ + OVA	H-2ᶠ + KLH	H-2ᵃ + KLH	H-2ᶠ + OVA
AOFK	AO-40.10.AG1	B10.M	f	KLH	2	−	+	−	−
					5	+	+	−	−
						H-2ᵃ + OVA	H-2ᵈ + KLH	H-2ᵃ + KLH	H-2ᵈ + OVA
DKAO	AO.40.10.AG1	B10.D2	d	KLH	2	+	−	−	−
					1	−	+	−	−
					12	+	+	−	−

H-2^a, H-2^f, and H-2^d cells used in IL-2 production were from B10.A, B10.M, and B10.D2 mice, respectively. SN were tested at 80% on HT-2 cells. HGG concentration: 500 μg/ml. KLH concentration: 500 μg/ml. OVA concentration: 1,250 μg/ml.

of *H-2* and antigen in these hybrids; however, the isolation of clones from the doubly reactive hybrids was an essential requirement for this conclusion. This proved to be a formidable task.

As might be expected, because these hybrids resulted from multiple fusions, they were unstable. Of the 20 hybrid lines with double specificity listed in Table VI, we attempted to clone six. Five of these hybrids yielded clones that had lost the response to the antigen/*H-2* combination of the normal T cell parent, whereas most of the clones retained the OVA plus $H-2^a$ reactivity. However, a few doubly specific clones were obtained from the sixth hybrid, AOFK-11. One clone, AOFK-11.11, was selected and then recloned, yielding many doubly specific clones. Because the establishment of clonality is essential to our conclusion concerning the lack of independent *H-2* and antigen recognition, the details of the cloning records of AOFK-11 are listed in Table VII. None of the clones or subclones of AOFK-11 showed any reactivity to OVA with $H-2^f$ or to KLH with $H-2^a$.

Subclone AOFK-11.11.1 was selected for further characterization. The reactivity of this hybrid was tested with OVA and KLH and a bank of *H-2* congenic and recombinant mice. The results are listed in Table VIII. AOFK-11.11.1 retained the fine *H-2* specificity of the AO-40.10 parent. An antigen-independent response was obtained with $H-2^b$. An OVA-dependent response was seen only with strains bearing $I-A^k$. A KLH-dependent response was seen only with $H-2^f$ cells. Surprisingly, no response was seen with KLH and B10.LG cells. Although the exact origin of the *I* region of B10.LG is obscure, it is thought to be very similar, if not identical (at least serologically), to the *I* region of $H-2^f$ (Dr. C. David, personal communication). This would apparently map the $H-2^f$-restricting specificity of AOFK-11.11.1 outside the *I* region. However, this conclusion was not supported by the results of anti-*I-A* inhibition studies. To confirm the mapping studies shown in Table III, we used hybridoma antisera directed at various *I-A* haplotypes to inhibit antigen presentation to AOFK 11.11.1. Our results are shown in Table V. As was the case with AO-40.10, the response of AOFK-11.11.1 to OVA plus *H-2* was specifically inhibited by hybridoma anti-*I-A* antibodies directed against $I-A^k$ (43). The response of AOFK-11.11.1 to KLH plus $H-2^f$ was inhibited by two anti-$I-A^f$ hybridomas, 10-3.6 (43) and MK-S4. The MK-S4 hybridoma was prepared in our laboratory from mice immunized with $H-2^s$ cells. Its specificity was mapped to $I-A^s$ using a panel of the appropriate *H-2* recombinant mice. It was found to cross-react with B10.M ($H-2^f$) and with B10.LG. In both B10.S and B10.M, it reacted with 50–60% of spleen cells and 75–80% of B cells, but not with splenic T cells. Its inhibition of the response of AOFK-11.11.1 to KLH plus B10.M strongly implicated the $I-A^f$ molecule as the recognition structure. A control anti-$I-A^d$ hybridoma, MK-D6, failed to inhibit $H-2^a$ plus OVA, $H-2^b$, or $H-2^f$ plus KLH presentation.

Controls using F_1 antigen-presenting cells with this and other hybrids showed that inhibitions caused by the antibodies were due to specific *I-A* blocking rather than, for example, cytotoxic effects on the antigen-presenting cells, because the antibodies had no effect when directed against inappropriate *I-A* molecules on F_1 antigen-presenting cells.

Discussion

We developed a method for producing T cell hybridomas that responded to a challenge with specific antigen by producing the lymphokine, IL-2. In each case, the

TABLE VII

Cloning of AOFK-11

Hybrid	Tested clone number	Number of cells plated/well	Number of wells	Number of wells with growth	IL-2 production reactivity patterns			
					$H\text{-}2^a$ + OVA	$H\text{-}2^f$ + KLH	$H\text{-}2^a$ + KLH	$H\text{-}2^f$ + OVA
AOFK-11	.1	0.125	96	1	+	−	−	−
	.2	0.25	96	5	+	NT*	−	−
	.3				−	−	−	−
	.5				+	−	−	−
	.6				+	−	NT	NT
	.7	0.50	96	18	−	−	−	−
	.9				−	−	−	−
	.10				+	±	−	−
	.11				+	+	−	−
	.12				+	±	−	−
	.13				+	−	−	−
	.15				NT	−	NT	NT
	.16				−	−	−	NT
	.17	1.00	96	18	+	+	−	−
	.19				−	−	−	−
	.20				−	−	−	−
	.21				+	−	−	−
	.23				+	−	−	−
AOFK-11.11	.1	0.125	96	7	+	+	−	−
	.2				+	+	−	−
	.4				+	+	−	−
	.7				+	+	−	−
	.8	0.25	96	6	+	+	−	−
	.10				+	±	−	−
	.11				+	+	−	−
	.12				−	−	−	−
	.13				+	+	−	−
	.14	0.5	96	15	+	−	−	−
	.15				+	±	−	−
	.17				+	+	−	−
	.20				+	±	−	−
	.23				+	+	−	−
	.24				+	+	−	−
	.25				+	+	−	−
	.26				+	−	−	−
	.27				+	±	−	−

$H\text{-}2^a$ and $H\text{-}2^b$ cells used in IL-2 production were from B10.A and B10.M mice, respectively. SN were tested at 80% on HT-2 cells. KLH concentration: 500 μg/ml. OVA concentration: 1,250 μg/ml.

* Not tested.

hybrids responded to antigen only when it was presented by irradiated splenic antigen-presenting cells of the appropriate *H-2* haplotype. In three cases, the relevant *H-2* molecule mapped to the *I-A* region. In the fourth, the mapping of the relevant *I* region molecules was ambiguous, although the data are consistent with either an *I-J-*

TABLE VIII

Mapping the H-2 Specificities of AOFK-11.11.1

Strain of antigen-presenting cells	H-2	H-2 subregions I									IL-2 production		
		K	A	B	J	E	C	S	G	D	Alone	+ OVA	+ KLH
B10.BR	k	k	k	k	k	k	k	k	k	k	−	+	−
B10	b	b	b	b	b	b	b	b	b	b	+	+	+
B10.D2	d	d	d	d	d	d	d	d	d	d	−	−	−
B10.S	s	s	s	s	s	s	s	s	s	s	−	−	−
B10.M	f	f	f	f	f	f	f	f	f	f	−	−	+
B10.A	a	k	k	k	k	k	d	d	d	d	−	+	−
B10.A(4R)	h4	k	k	b	b	b	b	b	b	b	−	+	−
B10.AQR	y1	q	k	k	k	k	d	d	d	d	−	+	−
B10.TL	t1	s	k	k	k	k	k	k	k	d	−	+	−
B10.A(3R)	i3	b	b	b	b	k	d	d	d	d	+	+	+
B10.A(5R)	i5	b	b	b	k	k	d	d	d	d	+	+	+
B.10S(7R)	t2	s	s	s	s	s	s	s	s	d	−	−	−
B10.HTT	t3	s	s	s	s	k	k	k	k	d	−	−	−
B10.LG	ar1	d	f	f	f	f	f	f	f	?	−	−	−

SN were tested at 80% with HT-2. OVA concentration: 1,250 μg/ml. KLH concentration: 1,000 μg/ml. H-2 subregion assignments are from Klein et al. (47).

I-E molecule or a molecule with one chain from *I-A* and one from *I-E/C* as the actual restricting element (46). Future experiments testing complementation in appropriate F_1 mice [e.g., (D2.GD × B10.A)F_1] should confirm or reject this possibility.

These hybrids and others produced similarly should prove extremely useful in determining the nature of the receptor(s) on *H-2*-recognizing T cells. In this regard, they offer a number of advantages over continuous lines of normal T cells propagated using repeated antigenic stimulation and/or IL-2 as a growth factor (27–30). These hybrids grow constitutively without antigenic stimulation or growth factors. They have doubling times of about 12 h and grow in a variety of media. Preliminary experiments have shown that they can be adapted for ascitic growth in vivo. These properties should make it possible to produce easily large numbers of the hybrid cells for biochemical characterization. Also, the ease with which these cells can be grown and manipulated in vitro has made them particularly useful in genetic experiments in which the behavior of their receptor can be examined after multiple clonings or subsequent fusions to other cells.

The reactivity of the hybrid, AO-40.10, both for OVA plus self *I-A*[k] and for allo-*H-2*[b] is of particular interest. There is considerable controversy over the question of the origin of T cells that recognize allo-*H-2* antigens. Some have suggested that T cells with these specificities arise as a fortuitous cross-reaction in which the combination of self *H-2* and a particular antigen is mimicked by an allo-*H-2* antigen (6–8). Others have suggested that these two types of recognition are for the most part mutually exclusive, arising from different germline genes (5, 9, 48). There are indeed a few reports supporting in part the former hypothesis, in which T cell lines or clones selected for a particular self *H-2* plus antigen specificity have been found to have an

allo-H-2 reaction as well (6–8, 29, 49). AO-40.10 appears to be another example of this type. In this case, one would postulate that the OVA plus I-A^k-specific receptor(s) obtained from the normal B10.A parent in the fusion was also capable of recognizing a product of H-2^b. In support of this view is the finding that all subclones of AO-40.10 that have been examined so far demonstrate both specificities or neither specificity, i.e., the genes controlling the two specificities apparently segregate together. However, it should be kept in mind that the T cell tumor parent, FS6-14.13.AG2, was derived from a fusion between BW5147 (an AKR thymoma) and BDF$_1$ (H-2^b × H-2^d) normal T cells (42). Although FS6-14.13.AG2 does not react with H-2^b itself, it may have inherited a receptor from its normal T cell parent, with specificity for H-2^b plus some unknown antigen (X). It is therefore possible that part of the anti-H-2^b receptor of AO40.10 is derived from this, H-2^b plus X, receptor. Our future experiments will test this possibility.

In this report we also demonstrated that T cell hybrids could be constructed from two parents with different antigen/H-2 specificities. Many of these hybrids responded to both parental antigen/H-2 combinations, but no hybrids were detected that displayed a new specificity constructed from the antigen specificity of one parent and the H-2 specificity of the other. These results are clearly inconsistent with the two receptor models for dual recognition in which the recognition of antigen is independent of the recognition of H-2 (17–19, 20, 22, 23). On the other hand, these results are consistent with most models for dual recognition in which the recognition of antigen and H-2 are dependent on one another. For example, any single receptor model in which a complex T cell receptor recognizes the combination of antigen and H-2 would predict that the specificities for antigen and H-2 would not mix in these doubly specific hybrids (24–26). However, also consistent with these results would be models for dual recognition in which an interaction between antigen and an H-2 molecule in antigen-presenting cells determines the actual antigenic unit presented to the T cell, because one could not predict that a given antigen would interact with the products of one H-2 haplotype in the same way as with a second H-2 haplotype (23, 25). Distinguishing among these various models will have to await a more direct biochemical characterization of the receptor on these T cells.

The cloned doubly specific hybrid AOFK-11.11.1 responded to Con A (data not shown), H-2^b, OVA + H-$2^{a,k}$, and KLH plus H-2^f. The response to OVA was mapped to I-A^k by the combination of genetic mapping with H-2 recombinant mice and inhibition of the response with I-A hybridoma antibodies. The response to KLH was mapped to I-A^f using hybridoma antibody inhibition, but surprisingly, no response was seen with cells from B10.LG mice, a strain thought to have an I region derived from H-2^f. This finding is perhaps explicable if one considers the origins of the B10.LG mouse (C. David, personal communication). Originally, the LG/J mouse was an inbred strain developed for large body size. It was typed as H-2^d. Subsequently, Dr. Chai of The Jackson Laboratory crossed this strain to an unknown stock to develop the H-2 recombinant strain, LG/ckJ, whose H-2 genes (H-2^{ar1}) were then placed on the B10 background to produce the B10.LG strain. We obtained this strain from Dr. C. David. The assignment of the I region of H-2^f to H-2^{ar1} was done on the basis of serological typing with anti-Ia antibodies rather than by its known derivation from a H-2^f mouse. An additional disquieting feature about the H-2 haplotype of B10.LG animals is the fact that its D region has yet to be assigned and its type remains

unknown. Therefore, it is quite possible that, although they are similar, the I-A regions of H-2^{arl} and H-2^f differ somewhat, including a difference in the restricting determinant recognized by AOFK-11.11.1. A second possibility is that the I-A molecules from H-2^f and H-2^{arl} are identical, but that the availability of the determinant recognized by AOFK-11.11.1 is influenced by D and/or K region products expressed on the surface of the antigen-presenting cells. This possibility is not unlikely in light of the recent work by Emerson et al. (50) on the influence of one H-2 product on the relative expression of other H-2 products.

Summary

We developed a method for production of antigen-specific, H-2-restricted T cell hybrids. The tumor cell partner in the fusions was itself a T cell hybrid, FS6-14.13.AG2 (or its derivatives), which could be induced to produce the growth factor, interleukin-2 (IL-2), in response to a challenge with concanavalin A, but had no known antigen specificity. The normal T cell partner in the fusions was a population of lymph node T cell blasts that had been highly enriched in antigen-specific, H-2-restricted T cells by in vivo immunization, followed by in vitro challenge with antigen and clonal expansion in IL-2-containing medium. These fusions produced hybrids that grew constitutively in culture. A sizable proportion of the hybrids demonstrated the ability to produce IL-2 in response to a challenge with specific antigen presented by irradiated spleen cells of the appropriate H-2 type.

Four cloned antigen/H-2-specific hybrid lines were produced. AO-40.10 responded to chicken ovalbumin (OVA) when presented by I-A^k-bearing cells. DC1.18.3 responded to the apo form of beef cytochrome c when presented with I-A^d. AODK-10.4 responded to keyhole limpet hemocyanin (KLH) presented with I-A^d. AODK-1.16 also responded to KLH presented by a product of the I region of H-2^d, but the data were consistent with either a product of the I-J-I-E^d region or a combinatorial molecule with elements from both I-A^d and I-E^d/I-C^d. Coincidentally, AO-40.10 was shown to have an unexpected alloreactivity with a product of H-2^b mapping to the K-I-A region. These hybrids should prove invaluable as sources of monoclonal material for the study of the receptor(s) on T cells with H-2-restricted antigen specificities.

We also generated T cell hybrids with two antigen/H-2 specificities by fusing an azaguanine-resistant clone of AO-40.10 to normal T cells with a different antigen/H-2 specificity. Many of the hybrids retained reactivity to OVA plus H-2^a and to the second antigen/H-2 combination. None reacted to either OVA plus the second H-2 type or to the second antigen plus H-2^a.

One of these hybrids was successfully cloned to produce the line AOFK-11.11.1. It retained the ability to recognize OVA plus I-A^k inherited from one parent, and KLH plus I-A^f inherited from the other. It did not recognize OVA plus I-A^f or KLH plus I-A^k.

These results have some bearing on models describing the nature of T cell receptors for antigen recognized in association with H-2 products. They do not support models in which antigen and H-2 are recognized separately by two independent T cell receptors.

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