ON THE THYMUS IN THE DIFFERENTIATION OF "H-2 SELF-RECOGNITION" BY T CELLS: EVIDENCE FOR DUAL RECOGNITION?

By R. M. ZINKERNAGEL,* G. N. CALLAHAN, A. ALTHAGE, S. COOPER, P. A. KLEIN, AND J. KLEIN

(From the Departments of Cellular and Developmental Immunology and Molecular Immunology of the Research Institute of Scripps Clinic, La Jolla, California 92037, Tumor Biology Unit, the Department of Pathology, University of Florida, Gainesville, Florida 32610, and the Department of Microbiology, Southwestern Medical School, Dallas, Texas 75235)

The dual specificity of thymus-derived lymphocytes (T cells) for structures coded by the major histocompatibility gene complex (MHC)[1] and for foreign antigens (X) appears to be a general finding in mice and is likely to be a more universal phenomenon in higher vertebrates. Thus, all T-cell functions that have been tested in mice i.e., T cells involved in nonlytic helper, delayed type hypersensitivity, and macrophage activation functions are specific for the murine MHC (*H-2*) coded structure mapping to the *I* region (1–5), whereas cytotoxic T-cell activity is specific for *H-2K* or *D* (6–11). Similarly, cytotoxicity appears to be MHC-restricted in the rat (12), in humans (13), and in chickens (14). This restriction of T cells by MHC determinants contrasts with the apparently complete absence of H-2 restrictedness of B-cell functions or of their antibody products.

Although the phenomenology is clear and well accepted, the explanation of it is controversial and therefore many hypotheses and speculations have attempted to catch the elusive nature of the T-cell receptor; because it is generally felt that this dual specificity of T cells reflects and may therefore reveal unique properties of the T-cell receptor(s). Two models of associative T-cell recognition have been proposed: first the dual recognition model (1–9, 14, 15) where T cells recognize two distinct antigenic entities i.e., self-H-2 structures and foreign antigen X with two separate receptors. Second, the single receptor model, which proposes that T cells possess one single receptor specificity that recognizes a neoantigenic determinant (NAD) formed either by a complex of self and X, by an antigen-specific modification of the self-H-2 structure, by host-specific, or by

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[1] Abbreviations used in this paper: ATxBM, adult thymectomized irradiated and bone marrow reconstituted mice; C, complement; GVHR, graft-versus-host reaction; H-2, murine MHC; HLA i.v., human MHC; MHC, major histocompatibility gene complex; NAD, neoantigenic determinant; PFU, plaque-forming unit.

virus-specific modification of the foreign antigen X (6–11).

Experiments that apparently contradicted the rule of *H-2* restriction with chimeric mice of various combinations demonstrated that *H-2* incompatible T and B cells could cooperate (16, 17). Katz and Benacerraf have interpreted these results (18, 19) to indicate that in chimeras lymphocytes "learn to get along with each other." This proposition of lymphocytes differentiating cell interaction molecules for the *H-2* type of the cells with which they cohabitate during immunological reconstitution was called "adaptive differentiation"; our results support this as a general idea.

This paper and the following report aims at analyzing the question whether virus-specific cytotoxic T cells act via one or two receptors. The approach we chose was to determine whether *mere tolerance to alloantigen* was sufficient to allow generation of killer T cells that were specific for the tolerated alloantigen and virus or whether *additional* and *what kind* of conditions must be fulfilled for chimeras to produce *H-2*-restricted T cells that were specific for infected tolerated allogeneic targets. If T cells possess two receptor qualities, then the prediction is that the receptors for the anti-self-H-2 structures differentiate and are selected for independently for recognition of a foreign antigen. In contrast if T cells have one receptor quality then the specificity for the self-H-2 structure and for foreign antigen must be selected for simultaneously and restriction is imposed solely by the stimulating antigenic entity.

In designing experiments to probe this puzzle we started from two contrasting sets of results. Parent A $\rightarrow$ (A $\times$ B)F_1 chimeric T cells lyse trinitrophenol-modified targets of both parental *H-2* types and virus-infected parent A $\rightarrow$ (A $\times$ B)F_1 chimeras generated cytotoxic T cells that lyse virus-infected targets of the parent A type and/or (less efficiently) of the other incompatible parental B *H-2* type (20–22). In contrast mice made tolerant to *B* during neonatal life that were infected with virus generated virus-specific cytotoxic T cells exclusively for their own *H-2* type, but failed to react to virus-infected targets of the tolerated *H-2* (23). One could explain that in neonatally tolerant mice, unlike the chimeras, the tolerated haplotype is present in such trace quantities that virus plus tolerated *H-2* will be a very minor antigenic determinant that fails to trigger measurable cytotoxic activity; or that the mechanism of tolerance in parent $\rightarrow$ F_1 and neonatally tolerant mice are simply different. Recently, Bevan demonstrated that in F_1 $\rightarrow$ parent irradiation bone marrow chimeras H-2-restricted cytotoxic T-cell specificity was preferentially associated with the recipient H-2 type, thus the recipient determined the specificity of H-2 restriction (24). Independently we used a similar experimental approach.

In this report we analyze the specificity for self-H-2 plus virus of cytotoxic T cells generated in various types of irradiation bone marrow chimeras of F_1 $\rightarrow$ parent, parent $\rightarrow$ F_1, partially H-2 incompatible F_1 $\rightarrow$ F_1 or recombinant $\rightarrow$ recombinant and completely H-2 incompatible chimeras; in addition adult thymectomized irradiated and bone marrow reconstituted mice reconstituted with a semiallogeneic thymus were tested. We demonstrate that lymphopoetic stem cells differentiate in the thymus to mature T cells and during this process acquire a recognition structure with specificity for H-2 as it is expressed on the thymic epithelium; self-recognition is thus selected for thymus H-2, is independ-

ent of the lymphocytes' own H-2, and differentiates independently (with respect to time, i.e., ontogenetically, and with respect to site) of recognition of a foreign antigen.

Materials and Methods

Animals. C57BL/10, B10.D2, B10.BR, B10.A, B10.A(4R), B10.A(5R) (C57BL/6 × A)F_1, and (BALB/c × A)F_1 mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. BALB/c, C3H, A and (BALB/c × C57BL/6)F_1 mice came from the Strong Foundation, San Diego, Calif. Some C3H and C3H × C57BL/6 mice were purchased from the Cumberland Farm, Clinnton, Tenn. 37761. Mice were usually 6–14 wk of age when used and whenever possible were matched for sex and age in any given experiment.

Chimeras. Chimeras were produced using the general protocol of Sprent et al. (25). The mice were irradiated with a supralethal dose of 900–950 rads from a cesium source and on the same day were reconstituted with 1.5–2.5 × 10^7 anti-θ plus C-treated (in part a gift from Doctors D. H. Katz and B. S. Skidmore, Scripps Clinic, La Jolla, Calif.) bone marrow cells or fetal (14–16 day) liver cells injected intravenously (i.v.). All chimeras were analyzed individually 6–12 wk later.

Thymic chimeras were made as follows. Several days to 2 wk after adult mice were thymectomized, they were irradiated with 900–950 rads and reconstituted with anti-θ plus C-treated bone marrow cells (ATxBM). The same day thymus lobes (six per recipient) were prepared from 6- to 8-wk-old mouse donors, irradiated with 875 rads, and transferred to the thymectomized and irradiated recipients subcutaneously. Thymuses were irradiated to prevent proliferating thymus cells from emigrating into the periphery, as several investigators have described (26–27). Thymic chimeras were tested for thymic function 2–3 mo after reconstitution and controlled to ensure that no rests of the original thymus were functional.

Virus and Immunization. Mice were infected i.v. with about 10^7 plaque-forming units (PFU) of vaccinia virus and sacrificed 6 days later as described elsewhere (7, 22, 29).

Cell Preparations and H-2 Typing. All cells were prepared and used in minimal essential medium supplemented with nonessential amino acids, pyruvate, bicarbonate, antibiotics, and 10% heat inactivated fetal calf serum. All these ingradients were from Flow Laboratories, Inc. (Inglewood, Calif. 90025). Spleen cells from the virus-infected mice were processed and typed for H-2 as described previously (7, 22, 23, 29). The antisera used for typing were from the NIH collection (prefix D) or from Dr. J. Klein (prefix K). In each typing assay, positive and negative control cells were included to confirm the specificity of the antisera treatment and for C activity (28).

Target Cells and Cytotoxicity Assay. The methods used and the target cells we used have been described (22, 28). L929 (*H-2K*) originate from C3H mice, MC57G (*H-2*^{b}) from C57BL/6, mice, and D2 (*H-2*^{d}), a methylcholanthrene-induced cell line is of B10.D2 origin. The D2-line spontaneously released the most ^{51}Cr (20–30% for 6 h, 30–45% for 16 h), whereas MC57G released 8–12% during a 6 h test and 12–25% over 16 h, and L929 15–25% and 20–40%, respectively. The percent of ^{51}Cr release was calculated as percent of water-release (100%) and was not corrected for medium release. Water usually released about 80–85% of the ^{51}Cr.

Statistical Methods. Means and SEM of triplicates were determined and compared by Student's *t* test. SEM was usually smaller than 3%, and always smaller than 5%.

Results

Specificity for Self-H-2 of Cytotoxic T Cells from F_1 → P Bone Marrow Chimeras. Various combinations of fully reconstituted F_1 → parent chimeras were tested for virus-specific cytotoxicity against vaccinia-infected targets (Table I). Reconstitution was assessed in each mouse individually and was complete within the limits of the serological H-2 typing method which leaves an uncertainty factor of about 3–5%. The results show that F_1 → parent irradiation *bone marrow* chimeras generated virus-specific activity associated with the donor compatible parental H-2 type, but not with the parental H-2 type that was incompatible (Table I, exp. 1).

TABLE I

*Virus-Specific Cytotoxic Activity in F_1 → Parent Irradiation Chimeras**

Donor → Recipient (H-2 types)‡	Spleen cell to target cell ratio	% ^{51}Cr Release from vaccinia infected target cells§		
		D2(*d*)	MC57G(*b*)	L(*k*)
Experiment 1—bone marrow chimeras:				
1. BALB/c × C57BL/6 → BALB/c	40:1	[71] ‖	9	12
(*d* × *b*) (*d*)	13:1	[66]	9	14
	4:1	[44]	8	12
2. B10.D2 × C57BL/10 → C57BL/6	40:1	28	[36]	13
(*d* × *b*) (*b*)	13:1	27	[27]	13
	4:1	24	11	11
BALB/c × C57BL/6 (*d* × *b*)	40:1	[92]	[43]	17
C3H (*k*)	40:1	28	10	[90]
Medium		30	11	18
Experiment 2—adult spleen cell chimeras:				
3. BALB/c × C3H → C3H (36 wk)	40:1	[88]	23	[96]
(*d* × *k*) (*k*)	13:1	[79]	21	[63]
4. BALB/c × C57BL/6 → BALB/c	40:1	[85]	[50]	45
(*d* × *b*) (*d*)	13:1	[80]	[30]	43
Medium		36	22	46

* Mice of 6–10 wk of age were irradiated with 925 rads and reconstituted with 2×10^7 anti-θ + C-treated bone marrow cells (exp. 1) or untreated adult spleen cells (exp. 2); chimeras were infected with about 10^7 PFU of vaccinia virus 10 or 14 wk (exp. 1), or 36 wk (exp. 2) after reconstitution.

‡ H-2 typing was performed as described in Materials and Methods. Positive and negative controls were included for each antiserum to control specificity. Typing results were for:

Chimera 1		Chimera 2	
Anti-K^d (D-31)	>95%	Anti-K^d (D-31)	>90%
Anti-K^b (D-33)	>95%	Anti-K^b (D-33)	>95%
Control	<5%	Control	<5%

Similarly chimeras 3 and 4 were fully reconstituted by F_1 cells.

§ Uncorrected values are given as means of triplicate determination; SEM were all smaller than 3%. Duration of test was 6 h (exp. 1) and 16 h (exp. 2). The release from uninfected targets was tested for all cell populations and was in no case significantly different from medium controls.

‖ Statistically significant values ($P < 0.01$) are boxed.

Thus, these results show that the presence of tolerated H-2 antigens in the reconstituted lymphoreticular compartment was insufficient for this *H-2* type to be treated as self-marker. F_1 → parent chimeras obviously differed in at least two ways from normal F_1 animals that generated virus-specific activity always associated with both parental haplotypes: (*a*) F_1 → parent chimeras have >95% of the somatic cells of the recipient parental *H-2* type and (*b*) F_1 → parent chimeras possess the thymus and the stroma of the lymphatic organs of recipient parental H-2 type exclusively.

Irradiation Chimeras Reconstituted with F_1 Adult Spleen Cells. In Table I,

TABLE II

*Vaccinia-Specific Cytotoxicity Generated in Semisyngeneic-Semiallogeneic Chimeras**

Donor ——→ Recipient	Spleen cell to target cell ratio	^{51}Cr Release from vaccinia infected target cells‡		
		D2(d)	L(k)	MC57G(b)
BALB/c × C57BL/6 → BALB/c × C3H ($d \times b$) ($d \times k$)§	40:1	[67] ‖	20	9
	13:1	[59]	17	10
	4:1	[37]	16	10
BALB/c	40:1	[76]	14	8
C3H	40:1	26	[90]	11
C57BL/6	40:1	28	17	[74]
Medium		28	15	10

* Recipient mice were irradiated with 925 rads and reconstituted with 2×10^7 anti-θ + C-treated bone marrow cells 24 h later. Chimeras were infected 6 wk later and killed 6 days after infection. Spleen cells were tested for *H-2* type and for virus-specific cytotoxicity.

‡ Results are uncorrected means of triplicate determination; the SEM was below 3.5%. The test duration was 6 h. Release from uninfected target cells was not significant.

§ The antiserum treatment was controlled for specificity by included positive and negative control cells. The typing results were: anti-K^k (K-603) <10%, anti-K^b (D-33) >90%, controls <10%.

‖ The results were compared statistically with the highest values of medium release or release by immune spleen cells from H-2 incompatible donor mice. Significant values ($P < 0.01$) are boxed.

exp. 2, parental A or B recipient mice were reconstituted with adult spleen cells from F_1 donors instead of anti-θ plus C-treated bone marrow cells or fetal liver cells. Quite different from the previous experiments, infected chimeras generated cytotoxic activity that was associated with both parental haplotypes. Furthermore, the cytotoxic activity could be elicited in these chimeras very rapidly after transfer, i.e., within 2–3 wk (data not shown); this contrasts with the irradiation bone marrow chimeras in which immunocompetence appeared only 4 wk after reconstitution.

These results indicate, that if mature, differentiated T cells are transferred under conditions in which no dramatic graft-versus-host reactions occur (such as in F_1 → parent chimeras) these T cells express their immunocompetence without further differentiation in the thymus.

Self-Specificity of Virus-Specific Cytotoxic T Cells from Reciprocally Semiallogeneic (A × B) → (A × C) Chimeras. Chimeras that were reciprocally semisyngeneic were made by transfusing irradiated (BALB/c × C3H)F_1 ($H\text{-}2^d \times H\text{-}2^k$) with anti-$\theta$ plus C-treated bone marrow cells from (BABL/c × C57BL/6)F_1 ($H\text{-}2^d \times H\text{-}2^b$) mice (Table II). When infected with vaccinia virus these chimeras generated virus-specific cytotoxic T cells that lysed infected $H\text{-}2^d$ targets only. This occurred despite the fact that the chimeras were reconstituted fully with (BALB/c × C57BL/6)F_1 cells and that the host's nonlymphoid somatic cells expressed $H\text{-}2^k$.

Virus-Specific Cytotoxicity Generated in K, I Compatible, K, I Incompatible, or H-2 Incompatible Chimeras. Chimeras were made by combining recombinant inbred strains of mice that were compatible for the K, I or S, D regions of

H-2. The construction of some of these chimeras was difficult, particularly those with *I* incompatibility. The survival rate of *K, I* compatible combinations was about 70%, which is comparable with the survival of parent → F_1 or F_1 → parent chimeras (50–90%); *I* incompatible chimera survived to about 20%.

The three kinds of chimeras in Exp. I in Table III were made on the same day under identical conditions. The (*K, I, S, D*) compatible semisyngeneic B10.D2 → B10.BR × B10.D2 chimera and the (*K, IA*) compatible B10.A(4R) → B10.A chimera both generated measurable cytotoxic activity when injected with virus (Table III, exp. 1). However, in parent → F_1 chimeras activity was specific for the reconstituting parental haplotype; in (*K, IA*) compatible chimeras activity was generated against infected targets of the *K* haplotype only, but neither against target of donor nor of the recipient *D* haplotype. In contrast the (*IC, S, D*) compatible B10.D2 → B10.A chimeras failed to generate a measurable response. Since the other two types of chimeras responded well, the 10 wk time between irradiation plus stem cell reconstitution and infection were sufficient for the differentiation of immunocompetence.

The capacity of (*K, IA*) and (*D*) compatible chimeras to generate virus-specific cytotoxic T cells is given in exp. 2 in Table III. Included is one example (C57BL/10 → B10.A(2R) in which the *D* region is the only one of compatibility. Again no measurable activity was associated with either compatible D^b, with donor K^b nor with recipient K^k. Similarly, completely H-2 incompatible chimeras of the C3H ($H\text{-}2^k$) → C57BL/10 ($H\text{-}2^b$) did not generate measurable virus-specific cytotoxic activity (Table IV) associated with either $H\text{-}2^k$ or $H\text{-}2^b$ 3 mo after reconstitution. The experiments in Tables I–III demonstrate that in chimeras specific cytotoxicity was generated against virus and only the *H-2* haplotype or the *K* haplotype that was shared between recipient and donor. No measurable activity was found when *K* or *D* specificities were expressed only on the donor or the recipient cells alone. In these examples (A × B)F_1 → A, (A × B)F_1 → (A × C)F_1, recombinant A|B → recombinant A|C or recombinant B|A → recombinant C|A there was plenty of *B*-haplotype expressed by the lymphoid cells in the chimeras besides the abundance of a haplotype *C* expressed by the somatic cells of the recipient, therefore it is not simply lack of expression of the *K* or *D* haplotypes that explained unresponsiveness. However, the significant result is that whenever donor stem cells and recipient shared the *I* region plus *K* and/or *D* region(s) virus-specific activity was always generated. Since (A × B)F_1 → A chimeras generated virus-specific T cells against infected A targets only these results suggested that the H-2 of the host was probably determining the specificity for self of T cells. Since no activity was generated in irradiated thymectomized recipients that were reconstituted with stem cells under otherwise comparable conditions, these results and those from irradiated F_1 → P spleen cell chimeras suggested that it may be the thymus of the recipient that not only promotes differentiation of precursor T cells to mature T cells but that the thymus determines also what *K, I,* or *D* region could be eventually recognized together with a foreign viral antigen.

The H-2 Type of Transplanted Thymuses Determine the Self-Specificity of Virus-Immune Cytotoxic T Cells. In view of our results we analyzed how the *H-2* haplotype of the thymus influences the differentiation of immunocompetent

TABLE III

*Virus-Specific Cytotoxicity Generated in K, I, or S, D Compatible Irradiation Bone Marrow Chimeras**

Donor ——→ Recipient	H-2 Compatibility	Spleen cell to target cell ratio	^{51}Cr Release from vaccinia infected target cells‡		
			D2(*d*)	L(*k*)	MC57G(*b*)
		Experiment 1:			
1. B10.D2 → B10.A	*IC,S,D*	40:1	38	34	23
(*d*) (*k*|*d*)§		13:1	35	35	23
2. B10.A(4R) → B10.A	*K,IA*	40:1	29	[76]‖	22
(*k*|*b*) (*k*|*d*)		13:1	29	[41]	23
		4:1	28	37	20
3. B10.D2 → B10.BR × B10.D2	*K,I,S,D*	40:1	[73]	35	24
(*d*) (*k* × *d*)		13:1	[53]	30	22
		4:1	37	31	23
Controls:					
B10.D2		40:1	[80]	38	32
B10.A(4R)		40:1	33	[73]	[60]
		Experiment 2:			
4. B10.A(4R) → B10.A	*K,IA*	40:1	28	[61]	15
(*k*|*b*) (*k*|*d*)		13:1	23	[43]	13
		4:1	23	[26]	11
5. C57/BL.10 → B10.A(2R)		40:1	23	12	14
(*b*) (*k*|*b*)		13:1	22	13	12
Controls:					
B10.D2(*d*)		40:1	[97]	13	15
B10.BR × C57/BL.10 (*k* × *b*)		40:1	26	[82]	[57]

* Recipient mice were irradiated with 925 rads 1 day before reconstitution with anti-θ + C-treated bone marrow cells from donors that were compatible for various parts of H-2. The recipients of exp. 1 were all reconstituted on the same day and infected with virus 7 wk later. Recipients were infected at the following times after reconstitution in exp. 1: 10 wk, exp. 2: 12 wk. 6 days after infection, mice were sacrificed and spleen cells tested for H-2 types and for cytotoxicity.

‡ Results are expressed as uncorrected means of triplicate determinations; SEM was smaller than 2.7%. The test duration was 6 h for exp. 1; 16 h for exp. 2. ^{51}Cr release from respective uninfected targets was measured for all lymphocyte populations but no significant lysis was found.

§ Antisera treatment were controlled by positive and negative cell preparations; the anti-D^b (D-2) antiserum was also low (>80) on positive H-2^b controls in exp. 1. The typing results were for:

Chimera 1		*Chimera 2*		*Chimera 3*	
Anti-K^k (K-603)	<5%	Anti-D^d (D-4)	<10%	Anti-K^k (K-603)	>95%
Anti-K^d (D-31)	>90%	Anti-D^b (D-2)	>75%	Anti-K^d (D-31)	<5%
Control	<5%	Control	<10%	Control	<5%

Chimera 4		*Chimera 5*	
AntiD^b (D-2)	>95%	Anti-K^b (D-33)	>95%
Anti-K^k (K-603)	>95%	Anti-K^k (K-603)	<5%
Anti-D^d (D-4)	<5%	Control	<5%

‖ Results were compared statistically with the highest value of medium control, immune cells on H-2 incompatible infected targets and immune cells on uninfected targets; statistically significant values ($P < 0.01$) are boxed.

TABLE IV

*Failure of 3 Mo Old H-2 Incompatible Chimeras to Generate Measurable Virus-Specific Cytotoxic T Cells**

Donor —(H-2 typing)‡→ Recipient		Spleen cell to target cell ratio	^{51}Cr Release from vaccinia infected target cells§	
			L(*k*)	MC57G *b*)
C3H	C57BL/10	40:1	20	9
(*k*)	(*b*)	13:1	11	8
		4:1	11	8
C3H		40:1	**69**	13
(*k*)		13:1	**46**	12
C57BL/6		40:1	16	**68**
(*b*)		13:1	13	**50**
Medium			14	10

* Recipient mice were irradiated with 950 rads and reconstituted with 2×10^7 anti-θ + C-treated bone marrow cells. The chimera was infected 3 mo after reconstitution with about 10^7 PFU of vaccinia virus and its spleen cells tested for cytotoxicity 6 days later.

‡ See Materials and Methods. Positive and negative controls were included for each antiserum. The type results were: Anti-K^b (D-33) <20%, Anti-K^k (K-603) >95%, Control <20%.

§ Uncorrected means of triplicate determinations, the SEM was smaller than 5%; statistically significant results ($P < 0.01$) are boxed. Cytotoxicity was also tested on uninfected target cells; no significant lysis occurred.

T cells and their specificity spectrum with respect to self-H-2. (BALB/c × A)F_1 and (C57BL/6 × A)F_1 animals were thymectomized at the age of about 8 wk. 2 wk later they were irradiated and reconstituted with anti-θ-treated bone marrow cells or fetal liver cells from appropriate F_1 donors. These ATxBM mice were implanted six irradiated (875 rads) adult thymus lobes from BALB/c, C57BL/6, or alternatively from A donors. Of 35 such thymic chimeras only about 20% were able to generate virus-specific cytotoxic T cells 3–5 mo later (Table V). All these responder animals were the only ones that possessed functional transplanted thymus tissue as assessed macroscopically and microscopically and had no remaining functional original thymus tissue. Reconstitution of (A × B)F_1 ATxBM mice with irradiated thymuses of parent A generated virus specific cytotoxic T cells against infected A targets only, whereas B thymus reconstituted animals responded to B plus virus only. These animals differed from normal F_1 mice only in that their thymus bore just one parental *H-2* type, not both. Therefore, the results are compatible with the interpretation that the *H-2* type of the radioresistant portion of the thymus determines the differentiation of self-recognition in maturing T cells.

Discussion

Our results establishing the role of a host and its thymus in determining the H-2-restricted specificity of cytotoxic T cells are summarized in Table VI and as follows: (*a*) Chimeras in which the F_1 cells are used to reconstitute an irradiated recipient of the same haplotype as one of the hybrid's parents ($F_1 \rightarrow$ P) generate virus-specific cytotoxic T cells that kill only those targets which carry the recipient parental H-2 type. This is the case when F_1 stem cells are used,

TABLE V

*Influence of Transplanted Irradiated Parental Thymus on Virus-Specific Cytotoxicity Generated in Adult Thymectomized with Syngeneic Bone Marrow Reconstituted F_1 Recipients**

Fetal liver or bone marrow donor	Thymus donor	Thymectomized recipient	Spleen cell to target cell ratio	^{51}Cr Release from vaccinia infected target cells‡		
				D2(d)	L(k)	MC57G(b)
			Experiment 1:			
BALB/c × A ($d \times k \mid d$) (Fetal liver)	None	BALB/c × A ($d \times k \mid d$)	40:1	35	39	21
			13:1	33	38	17
BALB/c × A ($d \times k \mid d$)	BALB/c (d)	BALB/c × A ($d \times k \mid d$)	40:1	[85] §	40	18
			13:1	[76]	39	16
			4:1	[63]	39	15
C57BL/6 × A ($b \times k \mid d$) (Bone marrow)	A ($k \mid d$)	C57BL/6 × A ($b \times k \mid d$)	40:1	[75]	[80]	17
			13:1	[79]	[67]	18
			4:1	37	[54]	16
	Control:‡	C57BL/10	40:1	39	40	[79]
			13:1	33	38	[87]
			Experiment 2:			
BALB/c × A ($d \times k \mid d$) (Fetal liver)	BALB/c (d)	BALB/c × A ($d \times k \mid d$)	40:1	[74] §	30	34
			13:1	[67]	27	29
			4:1	44	26	28
C57BL/6 × A ($b \times k \mid d$) (Bone Marrow)	C57BL/6 (b)	C57BL/6 × A ($b \times k \mid d$)	40:1	39	33	[100]
			13:1	39	32	[66]
			4:1	32	29	[45]
	Controls:‡	BALB/c × A	40:1	[72]	[59]	33
			13:1	[57]	[46]	29
		C57BL/6 × A	40:1	[74]	[72]	[100]
			13:1	[62]	[59]	[93]
		Medium		35	30	32

* Recipient mice were thymectomized 1 to 2 wk before irradiation (BALB/c × A: 875 rads; C57BL/6 × A: 925 rads and transfused with 1.5×10^7 anti-θ + C-treated bone marrow cells. The mice were transplanted subcutaneously with the equivalent of three (875 rads) thymuses of one parental haplotype. These recipients were infected with about 10^7 PFU of vaccinia virus 10 wk (exp. 1) or 13 wk (exp 2) later. The mice were killed 6 days after infection and their spleen cells tested for *H-2* type and cytotoxicity. The test duration was for exp. 1: 16 h; exp. 2: 6 h. All spleen cells carried both parental *H-2* haplotypes. The thymectomy was controlled macroscopically and microscopically and the transplanted thymuses were examined by histology.

‡ Results are uncorrected means of triplicate determinations; the SEM was smaller than 3%. The results were compared statistically with the higher of the values for medium release or release by immune *H-2* incompatible spleen cells. The lymphocytes were also tested on uninfected target cells; no significant lysis was detected; control mice were normal mice immunized with vaccinia virus.

§ Boxed results are statistically significantly different from controls ($P < 0.01$).

TABLE VI
Summary of the Experiments Described in Tables I through V

Table	Donor cells	Recipient	Thymus of chimera	Lysis of virus-infected target cells A, B, C
I	A × B (BM)	A	A	A
I	A × B (BM)	B	B	B
I	A × B (Adult)	A	A	A,B
I	A × B (Adult)	B	B	A,B
II	A × C (BM)	A × B	A × B	A
III	$K^AI^A\|D^C$ (BM)	$K^AI^A\|D^B$	$K^AI^A\|D^B$	A
III	$K^CI^C\|D^B$ (BM)	$K^AI^A\|D^B$	$K^AI^A\|D^B$	None
IV	B (BM)	A	A	None
V	A × B (FL)	A × B	A	A
V	A × B (FL)	A × B	B	B

BM, anti-θ plus C-treated bone marrow cells; adult: adult spleen cells; FL: fetal liver from 14- to 17-day-old fetal mice.

however, if the stem cells are contaminated with mature F_1 T cells or adult F_1 spleen cells are used for reconstitution, the chimera generates T cells that kill targets of both parental H-2 types. The F_1 spleen cell → parent chimeras were immunologically active at 2 wk (the earliest time tested) whereas F_1 stem cell → parent chimeras took 4–6 wk to become immunocompetent. (*b*) Chimeras analogous to the F_1 → P type can also be generated by grafting a parental thymus into an F_1 that has been thymectomized, irradiated, and protected with F_1 stem cells. (*c*) Chimeras of the type F_1 (A × B) → F_1 (A × C) generate T killer cells capable of lysing targets that carry H-2 antigens of the A type only. (*d*) Chimeras made from H-2 recombinants instead of F_1 mice were of the general type recombinant (A|C) → recombinant (A|B). *H-2K* and *H-2I* region compatible chimeras generated killer T cells restricted for the shared H-2K antigens only. However, when the *H-2D* region was kept constant and the *H-2K* and *H-2I* regions were varied, i.e. ($K^CI^CD^B$) → ($K^AI^AD^B$) chimeras, no cytotoxic activity was detectable on any of the targets tested. Similarly, the fully H-2 incompatible chimera A → B did not generate killer cells either.

The general rule emerges from these results that the radio-resistant portion of the thymus determines which H-2 antigens will participate in restricting virus-specific killer T-cell precursors, and the H-2 antigens carried by cells of lympho-hemopoietic origin in the chimera determine which specificities of H-2 restricted precursors will be expressed by effector T killer cells (Zinkernagel et al. *J. Exp. Med.* 147:897).

Two further important conclusions can be drawn from the data. First, if T cells are selected in the thymus for which H-2 structures are to be treated as "self", then differentiation of restriction of cytotoxic T-cell activity to certain self-H-2 antigens must be unrelated to confrontation with viral or other non-self antigens. Thus, recognition of antigens by the T-cell receptor complex may involve two independent recognition sites, which may be on either one or two membrane molecules. In addition, as demonstrated earlier and here, both recognition structures are clonally distributed (9, 22, 29, 31). Furthermore,

recognition of self-H-2 markers is most likely via an antibody-antigen-like and not a self-self-like interaction. Second, *H-2I* region compatibility must exist between the antigens of the lymphoid cells donated to the chimeric host and the antigens on that host's thymic epithelium, in addition to K or D compatibility.

Two experimental factors were critical for constructing chimeras that produced clearcut results: the use of a supralethal dose of irradiation to guarantee elimination of host cells and full reconstitution by donor stem cells and the use of fetal liver cells or bone marrow cells twice treated with anti-θ to eliminate all immunocompetent T cell in the reconstituting inoculum. The difference between the results here, e.g., that A × B → A × C chimeras generate A-specific activity only, and our and other's earlier results (20, 22) showing that P → F_1 chimeras were able to generate cytotoxic T cells mainly against the compatible but also against the incompatible parental targets are probably best explained by the use of relatively, with respect to the mouse strain used, lower doses of irradiation in the earlier experiments which may have allowed enough stimulating cells of host origin to survive. This will be analyzed further in the subsequent paper.

Vaccinia virus was chosen because it is not pathogenic for mice, and its proliferative capacity in mice seems to be limited. Otherwise, these chimeras might have died from virus infection. A noteworthy exception were the *H-2,K, I,* or *H-2* incompatible chimeras; of which some died from the vaccinia infection. This is compatible with the finding that, in contrast to all others, these chimeras did not generate measurable anti-viral cytotoxicity.

Experimentally, it is not yet possible to rule out alternative explanations for the lack of $H\text{-}2^B$ type restriction seen in (A × B)F_1 → A chimeras. Theoretically, anti-idiotypic antibodies could arise as described in the classical Ramseier-Lindenmann experiment where (A × B)F_1 lymphocytes are sensitized against anti-B receptors present in irradiated A recipients (30). However, this interpretation also requires that H-2 restricted T-cell specificity functions through a dual recognition mechanism. Furthermore, other H-2-specific suppressor mechanisms cannot be fully excluded as yet, but are under investigation.

Which is the best interpretation: T cells possess one or two recognition sites? Our and Bevan's (24) experiments might be explained along the lines of a single receptor model for an NAD. Accordingly, precursor T cells may gain their maturity by differentiating receptors for foreign antigens (NAD) in the thymus as proposed by Jerne (31). Thus in a mouse of $H\text{-}2^A$ type, T cells with specificity for A will, during ontogeny, proliferate in the thymus upon contact with self-$H\text{-}2^A$ structures; some of these proliferating T lymphocytes will mutate to express a receptor not any longer specific for A but for "slightly different from A". Through a "filter mechanism", T cells with specificity for A will not be able to leave the thymus and would be pushed to proliferate further, or alternatively are inactivated. Only T cells having a mutated recognition specificity for "different from A" will be released from the thymus. This could explain the preference of T cells of (A × B)F_1 → A chimeras for "altered A". Since these chimeric T cells cannot react to "altered B" we must introduce the rule that the specificity spectrum anti-foreign antigens X that can be generated in a thymus A cannot overlap with that generated in a thymus B. When applied to a model of single receptor specificity for NAD this rule is unpredicted and seems

unlikely, but cannot be formally excluded as yet. In the light of the data presented here, which requires the T cell's receptor quality for anti-self-H-2 structures to be specifically learned, i.e., selected for and expressed independently of the receptor quality for anti-foreign antigen X, the dual recognition model provides a simple interpretation. One can reduce the constraints these results impose on a one receptor model for the T-cell receptor to requiring that the receptor specifically recognizes self-H-2 structures, as defined by the H-2 antigens present on thymic epithelium, independent of recognition of foreign antigen X. However, to account for specificity against foreign antigen X in the lytic effector reactions, the receptor must recognize this antigen also. To argue that one receptor has two independent recognition sites is tantamount to accepting two independent recognition sites i.e., dual recognition. The distinction between models with one and two receptors may, therefore, be reduced to deciding whether signals generating by antigen binding to the anti-self-H-2 structure and anti-foreign antigen X sites are transmitted intracellularly via one or two molecules (7, 18, 32–35).

What are the Practical Implications of these Results for Reconstituting Humans with Immunodeficiency Diseases? Thymus or lymphoid stem cell deficiencies alone or combined have been treated by transplanting such patients with thymus and/or stem cell-grafts. To avoid graft versus-host reactions (GVHR), stem cell grafts usually consist of optimally HLA matched and mixed lymphocyte reaction negative bone marrow cells, or, alternatively, of liver cells from 6- to 10-wk-old fetuses. Thymic-deficient patients are usually transplanted with fetal thymus tissue. Since transplantation of these immunoincompetent fetal tissues does not cause a GVHR, these grafts are not usually HLA matched (36–38). However, many such attempts at reconstitution fail; this may perhaps be explained in part by our results. If a patient, A, with a thymic epithelial defect, is transplanted with a fetal thymus of incompatible *HLA-A^X-B^X-D^X* (the *HLA-D* region corresponds to the murine *H-2I* region, which was shown both in this and the companion report to be crucial for reconstitution of cellular immunocompetence) then the differentiating stem cells learn to recognize the donor's *HLA-A^X-B^X-D^X* markers as self, (but not his own *HLA-A^A-$B^A$$D^A$* markers). Therefore, these donor-derived T cells will be unable to interact with their own cell surface structures of A type to provide help for the lymphocytes or to get involved in any of the complex cellular interaction or communication processes. A similar paradox arises when a patient with a stem cell defect but a potentially functional thymus is transplanted with fetal liver cells that are *HLA-A-B-D* incompatible with the host's HLA markers expressed by thymic epithelium.

Obviously these examples are extremes and assume that, the thymic epithelium of the first host, or the stem cells of the second recipient, do not function at all. However, we propose that in addition to the well recognized need to transplant immunoincompetent tissues to avoid GVHR, the following minimal requirements should be fulfilled to reconstitute immunocompetence: (*a*) thymus epithelium and lymphoreticular stem cells must share one *HLA-D* haplotype, (*b*) thymus epithelium, stem cells, and the rest of the somatic cells must share at least one of the possible four *HLA-A* or *HLA-B* antigens. For reconstitution of pure thymus hormone deficiency these rules may not apply necessarily.

Furthermore, the experimental results and the interpretations presented here suggest that to assess cellular immunocompetence of reconstituted patients or mice the measurement of only alloreactivity or activation by T-cell mitogens may *not be meaningful*; these tests probably do not reflect the capacity of T cells to recognize and interact with HLA-self structures. Therefore, to assess syngeneic immunologic competence (i.e., the one which is crucial for the host's immune protection) after reconstitution it is necessary to assess the potential for antigen-specific and HLA-restricted T-cell functions.

Summary

In the thymus, precursor T cells differentiate recognition structures for self that are specific for the *H-2K*, *D*, and *I* markers expressed by the thymic epithelium. Thus recognition of self-H-2 differentiates independently of the T cells H-2 type and independently of recognition of nonself antigen X. This is readily compatible with dual recognition by T cells but does not formally exclude a single recognition model. These conclusions derive from experiments with bone marrow and thymic chimeras. Irradiated mice reconstituted with bone marrow to form chimeras of (A × B)F_1 → A type generate virus-specific cytotoxic T cells for infected targets A only. Therefore, the H-2 type of the host determines the H-2-restricted activity of killer T cells alone. In contrast, chimeras made by reconstituting irradiated A mice with adult spleen cells of (A × B)F_1 origin generate virus-specific cytotoxic activity for infected A and B targets, suggesting that mature T cells do not change their self-specificity readily. (A × B)F_1 → (A × C)F_1 and ($K^AI^A|D^C$) → ($K^AI^A|D^B$) irradiation bone marrow chimeras responded against infected A but not B or C targets. This suggests that cytotoxicity is not generated against D^C because it is absent from the host's thymus epithelium and not against D^B because it is not expressed by the reconstituting lymphoreticular system. ($K^BI^B|D^A$) → ($K^CI^C|D^A$) *K*, *I* incompatible, or completely H-2 incompatible A → B chimeras fail to generate any measurable virus specific cytotoxicity, indicating the necessity for *I*-specific helper T cells for the generation of killer T cells. Finally adult thymectomized, irradiated and bone marrow reconstituted (A × B)F_1 mice, transplanted with an irradiated thymus of A origin, generate virus-specific cytotoxic T cells specific for infected A targets but not for B targets; this result formally demonstrates the crucial role of thymic epithelial cells in the differentiation of anti-self-H-2 specificities of T cells.

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