RESTRICTION SPECIFICITIES, ALLOREACTIVITY, AND ALLOTOLERANCE EXPRESSED BY T CELLS FROM NUDE MICE RECONSTITUTED WITH H-2-COMPATIBLE OR -INCOMPATIBLE THYMUS GRAFTS*

By R. M. ZINKERNAGEL, A. ALTHAGE, E. WATERFIELD, B. KINDRED, R. M. WELSH, G. CALLAHAN, and P. PINCETL

From the Departments of Immunopathology and Molecular Immunology, Scripps Clinic and Research Foundation, La Jolla, California 92037; and the Deutsches Krebs Forschungs Institute, Heidelberg, Federal Republic of Germany

Thymus-derived lymphocytes (T cells) have two outstanding characteristics: they depend on a differentiation step occurring in the thymus to gain immunocompetence (1–4) and they express two specificities, one for foreign antigenic determinants and one for a self-cell surface antigen coded for by the major histocompatibility gene complex (MHC)[1] (*H-2* in mice), i.e., T cells are MHC restricted (5–7). One consequence of MHC restriction is that MHC genes (*Ir* genes) influence the capacity of T cells to respond to a foreign antigen; it is suspected, but still unproven, that *Ir* -gene products and restrictive elements may be identical (7–9). Whether it is the *H-2* antigens expressed by the thymus or those of the lymphohemopoietic cells that determine T-cell responsiveness was studied first by McDevitt et al. (10, 11) in a series of conceptually outstanding experiments. They deprived mice of immunocompetence with irradiation, reconstituted them with fetal liver stem cells, and found that the MHC genotype of the donor rather than that of the recipient host determined responsiveness. This result was basically confirmed by Kindred (12) when genetically thymus- and T-cell-deficient nude mice reconstituted with syngeneic or allogeneic thymus grafts, acquired T cells with the responder phenotype of the host *H-2*, not the *H-2* type of the transplanted thymus. However, researchers in some laboratories (13–16), but not others (17), later found that the thymus played a crucial role in determining both restriction specificity and *Ir* phenotype (16, 18–20). Thus, parental mice of two different strains, designated A and B, were crossed to produce F_1 hybrids without thymuses or T cells. These hybrids were transplanted with A thymuses and developed T cells that were restricted to A but only marginally to B; the cells thereby assumed the *Ir* phenotype of A mice. Because the data that support the notion that restriction is determined mainly by the MHC genotype of the nude host's lymphohemopoietic cells rather than its thymus stem from tests of T-helper-cell function (12, 20), we evaluated reconstituted nude mice with respect to the restriction specificity of virus-specific cytotoxic cells. After reconstitution with unirradiated allogenic fetal or

* Supported by grants NIAID A1-13779, A1-0273, and A1-07007. This is Publication 1910 from the Department of Immunopathology, Scripps Clinic and Research Foundation, La Jolla, Calif.

[1] *Abbreviations used in this paper:* Con A, concanavalin A; LCMV, lymphocytic choriomeningitis virus; LPS, lipopolysaccharide; MHC, major histocompatibility gene complex; MLC, mixed lymphocyte culture(s); PFC, plaque-forming cell(s); PFU, plaque-forming units; PHA, phytohemagglutinin; SRBC, sheep erythrocyte(s).

newborn thymus grafts, the nude mice became immunocompetent (21–23) and their T cells expressed the restriction specificity of the nude host irrespective of the origin of the thymus grafts as in Kindred's experiments. In contrast, irradiated adult thymus grafts reconstituted nude mice only if the graft and recipient shared an MHC haplotype.

Materials and Methods

Mice. C57BL/6 ($H\text{-}2^b$), C57BL/6J ($H\text{-}2^b$), B10.BR ($H\text{-}2^k$), C3H ($H\text{-}2^k$), BALB/c ($H\text{-}2^d$), (BALB/c × C3H)F_1 ($H\text{-}2^d \times H\text{-}2^k$), and (BALB/c × C57BL)$F_1$ ($H\text{-}2^d \times H\text{-}2^b$) were bred locally from stocks originating either from The Jackson Laboratory, Bar Harbor, Maine or the L. Strong Foundation, San Diego, Calif. The nude mice were generously donated, purchased, or bred at Scripps Clinic and Research Foundation, La Jolla, Calif. BALB/c nu/nu and littermates from the colony of Dr. G. Sato, University of California, San Diego, Calif. came from the stock of G.I. Bomholtgard Ltd. in Denmark (for more detailed information on this colony see [24]). The nude BALB/c mice of Table III, Exp. 2 were purchased directly from the Bomholtgard Ltd. The CBA nu/nu mice and littermates were from the colony of Dr. M. B. A. Oldstone at the Scripps Clinic and Research Foundation and originated from stocks of Dr. J. F. A. P. Miller, The Walter and Eliza Hall Institute, Melbourne, Australia. The C57BL nu/nu of the fourth to fifth backcross generations were from the colony of Dr. W. Weigle at the Scripps Clinic and Research Foundation (for more detailed information on these mice see [25]).

Thymus Transplantation. Nude mice were engrafted with thymuses taken from fetuses at 15–17 d of gestation, from newborn mice <24-h-old, or from adult mice that had been lethally irradiated 0–3 d before the thymuses were excised. The recipient mice were anesthetized with ether, one kidney was exposed, and three to four thymus lobes were placed under the kidney capsule. The small incision in the capsule was not closed but the peritoneum was sutured with silk, and the skin was closed with Clay Adams autoclips (Clay Adams, Inc., Div. of Becton, Dickinson & Co., Parsippany, N. J.). Some 50–80% of these mice had functional and/or histologically demonstrable grafts in their kidneys some 3–60 wk after the operation. Some thymus grafts were transplanted through a subcutaneous incision placed either dorsally between the shoulders or into the armpits. Attempts to transplant thymuses subcutaneously into the ear failed. Not until 6 wk after transplantation were the nu/nu mice judged as reconstituted, because in preliminary experiments no virus-specific T-cell-mediated cytotoxicity was measurable 2, 3, or 4 wk after successful grafting. At 5 wk the responses were variable, whereas after 6 wk or more, responses were regularly high.

Virus, Immunization, Cell Preparation, and ^{51}Cr Release Assay. Vaccinia WR virus (26) or lymphocytic choriomeningitis virus (LCMV) (27) was injected intravenously at $\sim 3 \times 10^6$ or 10^5 plaque-forming units (PFU), respectively, into recipient mice killed 6 or 8–9 days later, respectively. Their spleen cells were suspended as previously described in minimal essential medium with 10% heat-inactivated fetal calf serum, pyruvate, bicarbonate, nonessential amino acids, and streptomycin–penicillin (called medium). *H-2* typing of effector cells was performed as described (28). Effector cell activity was tested on virus-infected and -uninfected ^{51}Cr-labeled target cells as described in detail (29–31). The target cells were established, tissue cultured lines originally from C3H (fibroblast, L929, $H\text{-}2^k$), B10.D2 (fibroblast, D2, $H\text{-}2^d$), C57BL (fibroblast, MC57G, $H\text{-}2^b$), DBA/2 (mastocytoma, P815, $H\text{-}2^d$), and C57BL mice (T-cell lymphoma, EL4, $H\text{-}2^b$). Normally, 300 μl of lymphocytes (7×10^6/ml), or ⅓ or 1/9 dilutions thereof (ratio of 40:1, 13:1, and 4:1), was tested on 5×10^4 suspended ^{51}Cr-labeled and extensively washed target cells in flat-bottomed microtiter wells (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) for 6 or 16 h at 37°C. The test conditions are specified in the legend on each table.

Adoptive Immunization. Spleen plus lymph node cells from unprimed or virus-primed mice were injected into lethally irradiated (900–950 R) recipients that had been infected with vaccinia virus 1–2 h before cell transfer. 5–6 d later, these recipient mice were killed, and the cytotoxic activity was determined as described (32).

Primary Footpad Reaction to LCMV and Test of LCMV Elimination. About 10^6 PFU of LCMV (30 μl) were injected into the right hind feet of test mice. The swelling of their footpads was measured daily with a spring-loaded caliper (Kroplein GmbH, Schluchtern, Hessen, Federal

Republic of Germany) and compared with the thickness of the contralateral foot that had been injected with control medium not containing virus (33). These mice were also bled at days 10 and 15 after injection, and PFU of LCMV per 100 μl of blood were determined by the method of Pulkkinen and Pfau (34).

Anti-Sheep Erythrocyte (SRBC) Response. Mice were injected with 0.1 ml of a 10% SRBC solution and killed 6 d later. The number of plaque-forming cells (PCF) was determined in a modified Jerne plaque assay (35).

Mitogen Stimulations. Lypopolysaccharide ([LPS] *Escherichia coli* 055:B5, Difco Laboratories, Detroit, Mich.) was used at the optimal doses of 5 μg/ml, phytohemagglutinin ([PHA], Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.) at 5 μg/ml, and concanavalin A ([Con A], Difco Laboratories) at 5 μg/ml. 100 μl of cells at 2×10^6/ml plus 100 μl of mitogen in medium that contained 5×10^{-5} M β-2-mercaptoethanol were mixed in flat-bottomed microtiter plates and incubated for 48 h at 37°C in a mixture of 7% O_2, 13% CO_2 in N_2. 1 μCi of [^{3}H]thymidine 5 Ci/ml (New England Nuclear, Boston, Mass.) was added 8 h before harvest in an automatic multiple culture harvester (Model M24V, Brandel, Rockville, Md.). [^{3}H]Thymidine uptake was counted according to standard procedures.

Mixed Lymphocyte Cultures (MLC). 2×10^5 responder lymphocytes were mixed with 4×10^5 irradiated (1,000 R) stimulator cells in 200 μl of medium supplemented with 5×10^{-5} M β-2-mercaptoethanol. After incubation in flat-bottomed microtiter plates for ~80 h, the cells were labeled with [^{3}H]thymidine as described above.

In Vitro Generation of Alloreactive Cytotoxic T Cells. Standard procedures were used as described by Lafferty et al. (36). 2–4 × 10^6 responder cells were mixed with 5×10^6 irradiated (1,000 R) stimulator cells in 2.5 ml of medium combined with 5×10^{-5} β-2-mercaptoethanol in Linbro plates with 16-mm wells (Linbro Chemical Co., Hamden, Conn.). The cultures were incubated in the gas mixture described. The cells were harvested on day 5, washed, and tested on two types of target cells, undiluted or diluted ⅓ and 1/9 (37).

Statistical Methods. Means and SEM of triplicate determination were compared by the Student's *t* test. SEM were usually < 3%.

Results

Characterization of Nude Mice Used. The nude mice were tested with respect to their capacities to (*a*) generate indirect PFC against SRBC, (*b*) respond to mitogens (Fig. 1), (*c*) respond to alloantigens as assessed by [^{3}H]thymidine uptake (Table I) or in cytotoxic tests (*d*) generate virus-specific cytotoxic T cells in vivo (Table II), and (*e*) produce primary T-cell-dependent inflammatory reactions to LCMV injected into the footpads (Fig. 2) (the identical results for CBA nu/nu and C57BL nu/nu mice are not shown). Whereas homozygous (nu/nu) C57BL or BALB/c nude mice generated no indirect PFC per 10^6 spleen cells, their heterozygous (nu/+ littermates generated ~ 90, 250, and 180 PFC, respectively (data not shown). After mitogenic stimulation, the responses of nu/nu mice and nu/+ littermates were comparable for LPS; nu/nu mice failed to respond to PHA or Con A, whereas the littermates produced high proliferative responses (Fig. 1). It is noteworthy that at the concentrations of mitogens used, the C57BL mice were relatively poor responders compared with CBA or BALB/c mice. The proliferative responses by lymphocytes from BALB/c nu/nu or nu/+ mice stimulated with irradiated syngeneic or allogeneic lymphocytes are summarized in Table I. Compared with littermates, the lymphocytes from nu/nu mice failed to proliferate when stimulated with allogeneic C57BL nu/+ cells. However, lymphocytes from BALB/c nu/+ littermates stimulated with either nu/nu or other nu/+ cells, proliferated notably, which indicates that these mice are not truly inbred. Nude mice from all three strains failed to generate specific alloreactive cytotoxic T cells in MLC (data not shown).

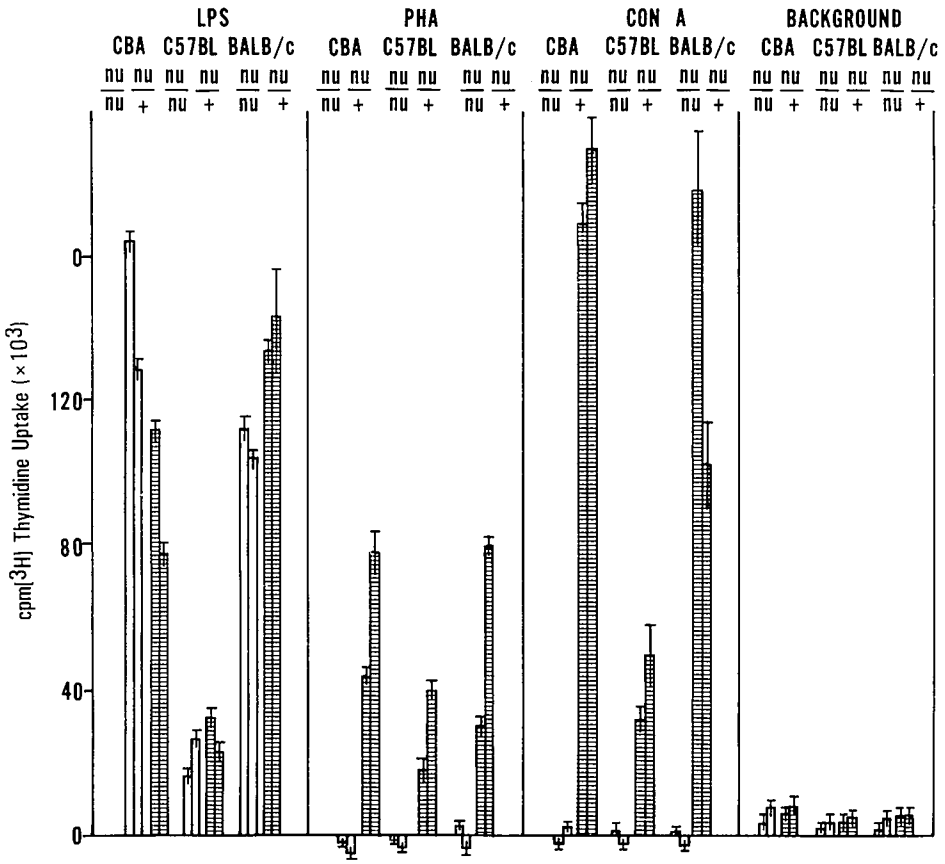

FIG. 1. Responses of lymphocytes from individual nu/nu mice (open columns) and nu/+ heterozygote littermates (striped columns) to LPS, PHA, or Con A stimulation as measured by [^{3}H]-thymidine uptake.

Serological H-2 typing of lymphocytes from these nu/nu mice revealed conventional H-2 specificities (data not shown). When infected with vaccinia virus the nu/nu mice failed to generate measurable virus-specific cytotoxic T-cell responses (Table II). However, the nu/+ littermates produced the expected virus-specific cytotoxic T cells that were restricted according to the host's H-2 type. When their footpads were injected with LCMV, nu/+, but not nu/nu, mice developed the usual primary delayed-type hypersensitivity reaction starting promptly 6 d after injection and declining rapidly after 9 d (Fig. 2). Taken together, these results indicate that the nu/nu mice studied were conventional in H-2 type and devoid of measurable T-cell immunocompetence (3, 4, 38–42).

Antiviral Reactivity of Nude Mice Reconstituted with Syngeneic or Allogeneic Fetal or Neonatal Thymus Grafts. In general, the rates of graft take (Fig. 3a) and survival of some 150 mice given thymic transplants was as follows: syngeneic or semisyngeneic thymus chimeras > 80%; recipients of histoincompatible grafts > 50%. C57BL nu/nu mice reconstituted with fetal C3H ($H\text{-}2^k$) thymus graft and infected with virus 12 wk later generated very little, if any, significant virus-specific cytotoxic activity during priming

TABLE I

Mixed Lymphocyte Reaction Reactivity of Lymphocytes from BALB/c nu/nu or BALB/c nu/+ Heterozygous Littermates

Stimulator	Δ cpm [^{3}H]thymidine uptake by responder BALB/c			
	nu/nu (1)	nu/nu (2)	nu/+ (3)	nu/+ (4)
BALB/c nu/nu (1)	2,400 ± 800	2,100 ± 300	3,200 ± 600*‡	11,500 ± 600
BALB/c nu/nu (2)	2,000 ± 100	2,400 ± 200	14,600 ± 600§	15,000 ± 300§
BALB/c nu/+ (3)	NT‖	NT	6,600 ± 1,000	34,900 ± 1,100
BALB/c nu/+ (4)	NT	NT	34,100 ± 2,000§	10,400 ± 400
C57BL nu/+ (1)	2,500 ± 200	3,300 ± 200‡	17,100 ± 800§	16,800 ± 2,000‡
C57BL nu/+ (2)	3,500 ± 200	2,900 ± 1,300	23,800 ± 1,400§	22,000 ± 1,000§

Numbers in parentheses are the mouse number.
* Significance vs. autostimulation.
‡ $P < 0.01$.
§ $P < 0.001$.
‖ NT, not tested.

TABLE II

H-2-restricted, Antiviral Cytotoxic Response of Various Strains of nu/nu and nu/+ Littermate Mice

Strain	H-2 type	Lympho-cytes: tar-get cells ratio	Percent specific ^{51}Cr release from infected target cells		
			H-2^d(D2)	H-2^k-(L929)	H-2^b-(MC57G)
BALB/c nu/nu	d	40	3	3	0
		13	1	5	0
		4	4	4	0
BALB/c littermate nu/+	d	40	65*	<1	0
		13	47	0	0
		4	12	0	0
CBA nu/nu	k	40	3	2	0
		13	0	0	0
		4	1	1	0
CBA littermate nu/+	k	40	12	65	0
		13	5	55	0
		4	0	33	0
C57BL nu/nu	b	40	4	0	0
		13	3	1	0
		4	1	0	0
C57BL littermate nu/+	b	40	5	8	51
		13	0	4	30
		4	6	4	13

Test durations were 6 h; spontaneous release from infected D2, 32%; L929, 10%; and MC57G, 13%.
* Results that are significantly different from those by normal or histoincompatible immune lymphocytes are boxed ($P < 0.01$).

in vivo, whereas C57BL nu/nu mice reconstituted with syngeneic grafts generated substantial responses that were, however, lower than those of unmanipulated control mice (Table III, Exp. 1-A). When the reactivity of lymphocytes from the C57BL nu/nu mice with C3H thymuses was boosted in irradiated and infected (C3H × C57BL)F_1 (*H-2^k* × *H-2^b*) mice, significant cytotoxic activity was detected (Table III, Exp. 1-B) on infected *H-2^b* but not on infected or uninfected *H-2^k* target cells. Thus, immuno-

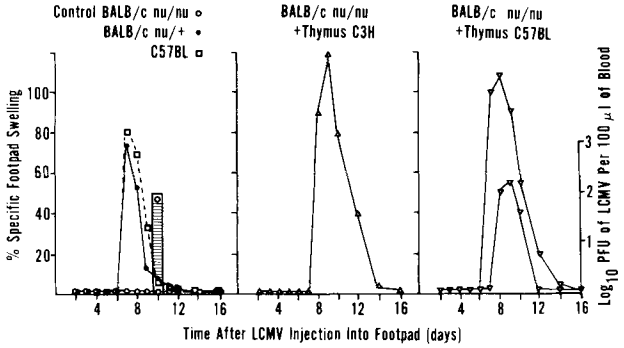

FIG. 2. Primary delayed-type hypersensitivity reaction at the site of LCMV injection in to the footpads of BALB/c (H-2^d) nu/nu or nu/+ mice or C57BL control mice (left panel), of a BALB/c nu/nu mouse with a histoincompatible (H-2^k) fetal thymus graft from a C3H donor (middle panel) or two BALB/c nu/nu mice with fetal histoincompatible thymus grafts from C57BL (H-2^b) donors (right panel). Viral titers were determined in these mice 10 d after injection; only nu/nu mice had detectable levels of LCMV in their blood.

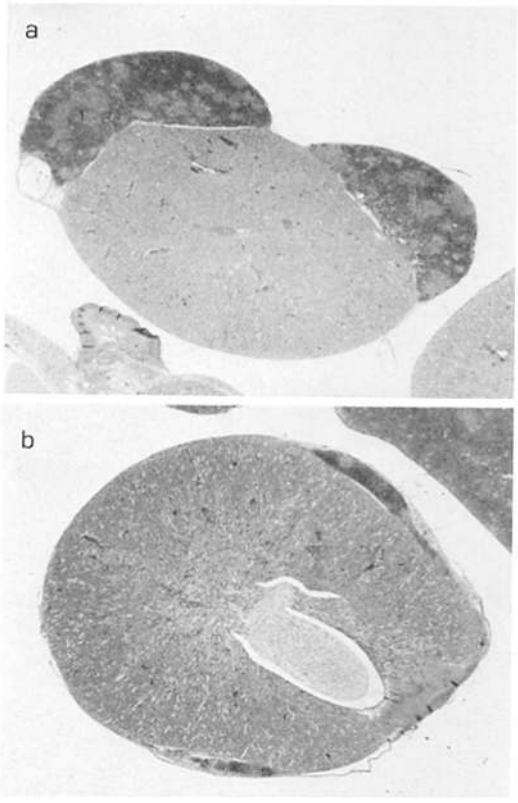

FIG. 3. Cross sections of kidneys from nu/nu mice reconstituted under the kidney capsule with unirradiated newborn thymus grafts (a) or irradiated (1,000 R) adult thymus grafts (b).

competence was observed in C57BL nu/nu mice reconstituted with allogeneic thymus grafts. Several nude mice with unsuccessful transplants and control nude mice, both infected with vaccinia virus, failed to reveal either primary or secondary antiviral reactivity under identical conditions (data not shown). In a somewhat different

TABLE III

Nude Mice Reconstituted with Syngeneic or Allogeneic Thymus Grafts: Quantitative Comparison of the Capacity to Generate Virus-Specific Cytotoxic T Cells

Experiment	Thymus chimeras*		Lymphocytes:target cells ratio	Percent specific ^{51}Cr release from infected target cells‡					
				H-2^d (D2)		H-2^k (L929)		H-2^b (MC57G)	
	Recipient (H-2)	(H-2) Thymus donor		Vaccinia virus	Normal	Vaccinia virus	Normal	Vaccinia virus	Normal
1-A	C57BL (H-2^b) nu/nu	C3H (H-2^k)	40	NT	NT	2	<1	14	10
			13			2	<1	6	4
			4			6	1	2	3
	C57BL (H-2^b) nu/nu	C57BL (H-2^b)	40	NT	NT	2	2	61§	3
			13			3	1	31	2
			4			1	1	14	1
	Control C57BL		40	NT	NT	2	4	100	5
			13			4	2	85	3
			4			1	1	41	2
	C3H		40	NT	NT	65	4	10	6
			13			29	2	8	4
			4			14	<1	2	2
1-B	Adoptive sensitization of (C57BL nu/nu + C3H thymus) in irradiated and infected F_1 recipients		40	NT	NT	6	4	33	0
			13			2	2	19	0
			4			1	2	5	
	Control C3H		40	NT	NT	36	2	4	2
			13			16	2	1	0
			4			9	2	1	0

2-A	BALB/c nu/nu (H-2^d)	BALB/c (H-2^d)	40	[70]	NT	NT	NT	0	NT
			13	[53]				0	NT
			4	[29]				0	NT
	BALB/c nu/nu (H-2^d)	C57BL (H-2^b)	40	[10]	NT	NT	NT	0	NT
			13	6				0	NT
			4	1				0	NT
	Control C57BL (H-2^b)		40	5	NT	NT	NT	[68]	NT
			13	3				[50]	NT
			4	3				[24]	NT
2-B	Adoptive sensitization of (BALB/c nu/nu + C57BL thymus) in irradiated and infected F_1 recipients		40	[67]	NT	NT	NT	1	NT
			13	[44]				0	NT
			4	[15]				1	NT
	Control C57BL		40	0	NT	NT	NT	[110]	NT
			13	0				[58]	NT
				0				[31]	NT
3	C57BL (H-2^b)	C3H(H-2^k)	40			13	NT	[47]	NT
			13			10		[21]	
			4			10		4	
	Control C57BL(H-2^b) littermates		40			18	NT	[88]	NT
			13			17		[90]	
			4			10		[70]	
	B10.BR(H-2^k)		40			[110]	NT	10	NT
			13			[89]		8	
			4			[51]		6	

NT, not tested.

* Exp. 1-A unirradiated, 15–17 d fetal thymus, 12 wk after reconstitution. Exp. 2-A unirradiated newborn thymus subcutaneously, 10 wk after reconstitution. Exp. 3 unirradiated newborn thymus in kidney capsule, 10 wk after reconstitution.

‡ Test conditions were for Exp. 1-A: duration 6 h; spontaneous release from L929 <12%; MC57G: <13%. Exp. 1-B: duration 6 h; spontaneous release L929 <21%; MC57G: < 16%. Exp. 2-B: duration 16 h; spontaneous release from D2 <31%; MC57G <22%. Exp. 3: duration 16 h; spontaneous release from L929 <23%; MC57G <25%.

§ Results that are significantly different from those by normal or histoincompatible immune lymphocytes are boxed ($P < 0.01$).

TABLE IV

Assessment of Restriction Specificity Expressed by Antivaccinia Virus Immune T Cells from the H-2-Incompatible Thymus Chimeras by Cold Target Blocking Experiments

Thymus chimeras*		Cold infected target cells added		Lymphocytes: target cells ratio	Percent specific ^{51}Cr release from infected target cells‡		
Recipient	Thymus donor	Type	Fold excess		H-2^k (L929)	H-2^d (D2)	H-2^b (MC57G)
CBA nu/nu (H-2^k)	BALB/c (H-2^d)	None		40	48	5§	<1
				13	16	4§	<1
		H-2^k	3	40	19‖	7	NT
		(L929)	6	40	12‖	2	
		H-2^d	3	40	33¶	8	NT
		(D2)	6	40	30¶	9	
		H-2^b	3	40	31¶	5	NT
		(MC57G)	6	40	35¶	7	
Control: BALB/c		None		40	1	43	<1
				13	3	14	<1

NT, not tested.

* Chimeras and experimental conditions were: newborn thymus grafts under the kidney capsule, test 10 wk after reconstitution.

‡ Test conditions: test duration 6 h; spontaneous release L929 <21%, D2 <25%, and MC57G <12%.

‖ Results are significantly ($P < 0.05$) smaller than H-2^d- or H-2^b-infected cold target blockers.

¶ Results are not significantly different when infected H-2^d cold blocking targets are compared with infected H-2^b targets.

§ No significant ^{51}Cr release.

example (Table III, Exp. 2-A), some BALB/c nu/nu mice were reconstituted with subcutaneously transplanted newborn thymuses by Kindred (23) and had been found to generate anti-SRBC antibodies. Very low, questionably significant virus-specific cytotoxic activity was found in the primary responses of BALB/c nu/nu mice reconstituted with H-2-incompatible newborn C57BL thymus grafts. Again syngeneic reconstitution of the BALB/c nu/nu mice led to excellent responsiveness. On adoptive boosting in appropriate F_1 mice, the lymphocytes from nude mice reconstituted with syngeneic or semiallogeneic (see below) grafts responded substantially better that those with allogeneic thymus grafts. However, many allogeneic thymus chimeras responded very well during the primary response in vivo (Tables III [Exp. 3] and IV). There seemed to be a direct relationship between age of the thymus when transplanted into the kidney and immunocompetence. Grafts from 15- to 17-d-old fetuses yielded relatively poor immunocompetence when compared with newborn (< 24 h) thymus grafts (Table III: Exp. 1 vs. Exp. 3 or vs. Table IV). Effector cells were sensitive to treatment with anti-Thy-1.2 plus C (data not shown).

The immunocompetence of T cells from nudes with allogeneic thymus grafts was restricted to the nudes' *H-2* type. Thus, T cells from C57BL (*H-2^b*) nu/nu mice with C3H (*H-2^k*) thymus grafts or BALB/c (*H-2^d*) nu/nu mice with C57BL (*H-2^b*) thymuses lysed infected *H-2^b* or *H-2^d* targets, respectively, but not infected targets bearing the thymic *H-2* type. When tested on infected *H-2^k* or *H-2^d* targets, the ^{51}Cr release was reduced significantly only by infected cold *H-2^k* targets (Table IV); the blocking effect of cold infected *H-2^d* targets (compatible with the thymus graft) was not significantly different from that of completely unrelated cold infected *H-2^b* cells.

TABLE V

Alloreactivity of Nude Mice Reconstituted with Syngeneic or Allogeneic Thymus Grafts

Experiment	Responder cells*	Stimulator cells	Percent specific ^{51}Cr release‡		
			H-2^k (L929)	H-2^d (D2)	H-2^b (MC57G)
1	C57BL nu/nu (b) with C3H(k) thymus	C3H (k)	<1	<1	<1
			<1	<1	<1
			<1	<1	<1
		D2 (d)	5	63§	<1
			3	32	<1
			<1	10	<1
		C57BL (b)	<1	NT	<1
			<1		<1
			2		<1
	C57BL nu/nu (b) with C57BL thymus	C3H (k)	79	NT	<1
			46		<1
			17		<1
		D2 (d)	15	96	<1
			3	44	<1
			0	28	2
		C57BL (b)	NT	<1	<1
				3	<1
				<1	<1
2	BALB/c nu/nu (d) with fetal (C3H × BALB/c)F_1 (k × d) thymus	C57BL (b)	16	NT	67
			4		56
			2		26
		B10.BR (k)	6	NT	10
			3		8
			4		7

NT, not tested.

* As in Table III. In Exp. 1, chimeras from Table VI were tested; in Exp. 2, a chimera from Table VI was tested.

‡ Test conditions: Exp. 1 test duration 6 h; spontaneous release from L929 <10%, D2 <15%, MC57G <15%. Exp. 2 test duration 6 h; spontaneous release from L929 <6%, MC57G <13%.

§ As in Table III.

Therefore, the T-cell reactivity cannot be readily explained by cross-reactivity between *H-2^b* and *H-2^k* or *H-2^d* and *H-2^b*.

One possibility was that allogeneic effects exerted by T cells from the histoincompatible thymus graft had promoted T-cell maturation. One might therefore have expected that in F_1 nu/nu mice with a parental thymus graft both restriction specificities should have been triggered because of allogeneic effects. As documented earlier, however, this has not been found (43, 44), and additional experiments with F_1 nu/nu recipients of irradiated, fetal, and neonatal parental thymuses have not revealed significant T-cell reactivity restricted to the nonthymic *H-2* type.

Lymphocytes from nude mice reconstituted with syngeneic or allogeneic fetal thymus grafts were capable of generating alloreactive cytotoxic T cells in MLC (Table V). Again, the reactivity of lymphocytes from syngeneically reconstituted C57BL nudes against D2 (*H-2^d*) stimulator cells was greater when compared with recipients of allogeneic grafts. Nudes reconstituted with allogeneic fetal or newborn thymus grafts were all unresponsive to the thymic H-2 type; for example, lymphocytes from

TABLE VI

Repopulation of the Lymphohemopoietic Compartment of nu/nu Mice by Thymus Graft-derived Lymphocytes

Experiment	Nu/nu recipient	Thymus donor	Treatment with anti-H-2 antisera	Killer:targets ratio	Percent ^{51}Cr release from infected target cells				
					H-2^b (MC57G)		H-2^d (D2)		H-2^s (S)
					Vaccinia virus	Uninfected	Vaccinia virus	Uninfected	Vaccinia virus
1	(BALB/c × C57BL)F$_1$ (H-2^d × H-2^b)	C57BL (H-2^b)	None	40	73	<1	7	19	
				13	58	<1	3	13	
				4	26	<1	2	8	
			anti-H-2^d + C	10	42	NT	NT	NT	
				3	23				
			anti-H-2^b + C	10	6	NT	NT	NT	
				3	4				
	Control: BALB/c + C57BL		None, C	40	52	<1	65	16	
				13	36	<1	78	10	
				4	17	<1	10	10	
			anti-H-2^b	40	2	NT	78	NT	
				13	5		30		
				4	6		16		
			anti-H-2^d	40	56	NT	6	NT	
				13	21		1		
				4	13		<1		
	Spontaneous release over 6 h:				10	18	17	23	
2	BALB/c (H-2^d)	SJL (H-2^s)	None, C	40	NT	NT	18	12	43
				13			11	8	33
				4			6	7	17
			anti-H-2^d	40	NT	NT	12	NT	40
				13			8		35
				4			7		18
	Control: BALB/c		None, C	40	NT	NT	43	4	3
				13			36	9	4
				4			18	8	5
			anti-H-2^d	40	NT	NT	5	NT	NT
				13			4		
				4			3		
	Spontaneous release over 6 h:						17	13	15

NT, not tested.

C57BL nu/nu with a C3H (*H-2^k*) thymus graft did not react against C3H stimulator cells assessed by cytotoxicity (Table V) or by [^{3}H]thymidine uptake (data not shown). An apparent exception were some (BALB/c × C57BL)F$_1$ (*H-2^d* × *H-2^b*) nu/nu mice with fetal or newborn C57BL thymuses that responded against *H-2^d*. *H-2* typing of these chimeras revealed that their lymphocyte type was predominantly *H-2^b* (Table VI, Exp. 1). Thus, in these few chimeras the thymus-derived lymphocytes must have rejected and repopulated the lymphohemopoietic system of the F$_1$ nudes. Similar

TABLE VII

Restriction Specificity of T Cells from P → F_1 nu/nu and F_1 → P nu/nu Thymus Chimeras

Thymus chimeras*			Ratio of lymphocytes: target cells	Percent specific ^{51}Cr release from infected targets‡	
Experiment	Recipient	Donor		H-2^d (D2)	H-2^k (L929)
A-1	BALB/c nu/nu (H-2^d)	(C3H × BALB/c)F_1 (H-2^k × H-2^d)	40	[28]	9
			13	[19]§	7
			4	[5]	0
	Control CBA/J (H-2^k)		40	1	[74]
			13	1	[82]
			4	0	[40]
A-2	Adoptive boosting in infected and irradiated (C3H × BALB/c)F_1		40	[81]	0
			13	[62]	1
			4	[24]	0
	Control (BALB/c × C3H)F_1		40	[73]	[65]
			13	[44]	44
			4	7	[7]
B-1	BALB/c nu/nu (H-2^d)	(C3H × BALB/c)F_1 (H-2^k × H-2^d)			
	Adoptive sensitization of unprimed spleen and lymph node cells in infected and irradiated (C3H × BALB/c)F_1		40	[45]	2
			13	[30]	<1
			4	[12]	<1
	Control (BALB/c × C3H)F_1		40	[87]	[75]
			13	[60]	[50]
			4	[28]	[26]

* Chimeras were formed by transplanting 19 d fetal thymus grafts under the kidney capsule of 6- to 24-wk-old nude mice. Mice were infected 12 wk after reconstitution. 6 d later mice were killed and the lymphocytes were tested for cytotoxicity. Thymus grafts were examined histologically and lymphocytes were typed for H-2 (> 90–95% of recipient nu type).

‡ ^{51}Cr release assay conditions were as follows: Exp. A-1: duration 6 h; spontaneous release from D2: <18%; L929 <10%. Exp. A-2: Duration 6 h; spontaneous release from D2: <38%, MC57G <30%. Exp. B-1: Duration 6 h, spontaneous release from D2: <20%, L929 <15%.

§ Significant results are boxed ($P < 0.01$).

observations have been made by S. Hedrick and J. Watson (University of California, Irvine, personal communication). This phenomenon has not been observed so far with F_1 nu/nu mice engrafted with BALB/c thymuses or in BALB/c nu/nu with C57BL thymuses or C57BL nu/nu with BALB/c thymuses. However, in two separate experimental groups where BALB/c nu/nu mice were grafted with SJL (*H-2^s*) 19-d fetal or neonatal thymus, the haplotype of the effector lymphocyte and their restriction specificity was for *H-2^s* only (Table VI, Exp. 2); similarly, some BALB/c nu/nu grafted with (C57BL/6 × DBA/2)F_1 neonatal thymuses were repopulated by F_1 cells from the graft (data not shown).

As stated before, nude mice carrying histoincompatible fetal or neonatal unirradiated thymus grafts have survived well for up to 1 yr after reconstitution. This contrasts with our experience with allogeneic irradiation chimeras or nude mice reconstituted with irradiated allogeneic thymus grafts, as will be discussed later. The

TABLE VIII

Immunocompetence of Nude Mice Reconstituted with Adult Irradiated Thymus Grafts: Antiviral Cytotoxicity

Experiment	Thymus chimeras*		Lymphocytes:target cells ratio	Percent specific ^{51}Cr release from targets					
				H-2^k (L929)		H-2^d (D2)		H-2^b (MC57G)	
	Recipient	Donor		Vaccinia virus	Normal	Vaccinia virus	Normal	Vaccinia virus	Normal
1-A	BALB/c nu/nu	with (BALB/c	40	4	4	27‡	2	NT	NT
		× C3H)	13	4	4	10	4		
		thymus (950 R)	4	4	4	<1	3		
	C57BL nu/nu	with C57BL		40	NT	NT	NT	18	4
		thymus (950 R)		13				5	4
				4				3	4
	Control (C3H × C57BL)F_1		40	64	2	NT	NT	44	4
			13	37	1			16	4
			4	12	1			6	4
	Control BALB/c littermate		40	1	4	57	9	<1	2
			13	4	4	33	1	<1	2
			4	4	4	11	3	<1	2
1-B	Lymphocytes (3×10^7) from BALB/c nu/nu with irradiated (BALB/c × C3H) thymus adoptively boosted in irradiated infected (BALB/c × C3H)F_1		10	19	16	31	7	NT	NT
			3	4	<1	12	8		
	Control: (BALB/c × C57BL)F_1		10	7	6	70	11	NT	NT
			3	2	<1	39	<1		

2-A	BALB/c nu/nu	with C57BL	40	NT	NT	1	3	2	<1
		thymus (950 R)	13			1	3	1	<1
			4			1	2	0	<1
	(BALB/c × C57BL)F_1 nu/nu	with C57BL	40	NT	NT	5	NT	57	NT
		thymus (1,000 R)	13			<1		40	
			4			<1		10	
	Control (BALB/c × C57BL)F_1		40	NT	NT	67	6	59	4
			13			20	3	33	2
			4			17	2	7	2
2-B	Lymphocytes (3×10^7) from BALB/c nu/nu with C57BL thymus (950 R) (Exp. 2-A) adoptively boosted in (BALB/c × C57BL)F_1 alone		40	NT	NT	11	NT	<1	NT
			13			2		<1	
			4			9		<1	
	Mixed with 3×10^7 (BALB/c × C57BL)F_1		40	NT	NT	76	NT	47	NT
			13			28		19	
			4			11		5	
	3×10^7 (BALB/c × C57BL)F_1 adoptively boosted		40	NT	NT	76	NT	46	NT
			13			29		15	
			4			6		3	
	Control: C57BL/6		40	NT	NT	9	NT	65	NT
			13			3		49	

NT, not tested.

* The nude mice were reconstituted with grafts from adult irradiated donor mice. Exp. 1, 8 and 12 wk after reconstitution; Exp. 2, 12 and 14 wk after reconstitution, respectively. Test conditions: Exp. 1: test duration 6 h; spontaneous release L929 <13%; MC57G <18%. Exp. 2-A: test duration 16 h; spontaneous release D2 <28%; MC57G <20%. Exp. 2-B: test duration 16 h; spontaneous release D2 <33%; MC57G <28%.

immune performance of nude mice reconstituted with fetal thymus grafts was excellent when assessed by their capacity to mount primary delayed-type hypersensitivity responses against LCMV and to eliminate LCMV (Fig. 2). Whereas unreconstituted nude mice failed to show any significant footpad swelling upon challenge with LCMV and could not eliminate LCMV, nude mice reconstituted with allogeneic fetal thymuses showed the same footpad reaction as nu/+ littermates or normal controls and at no time contained measurable amounts of circulating virus.

Restriction Specificity of Homozygous Nudes Reconstituted with Heterozygous F_1 Thymus Grafts. The role of the thymic H-2 in the restriction specificity of T cells from homozygous nude mice was investigated further by reconstituting them with fetal heterozygous F_1 thymus grafts. BALB/c ($H\text{-}2^d$) nu/nu mice with fetal (C3H × BALB/c) ($H\text{-}2^k \times H\text{-}2^d$)$F_1$ thymus grafts generated virus-specific cytotoxic T cells that lysed infected host-compatible, but not targets that expressed the nonhost *H-2* type of the thymus. Irrespective of whether lymphocytes from such chimeras were sensitized primarily (Table VII, Exp. B-1) or were secondarily boosted (Table VII, Exp. A-2) in irradiated and infected appropriate F_1 hosts, no significant lysis of infected target cells of the second thymic *H-2* type was observed. Comparable results have been obtained with C57BL nu/nu mice with fetal (BALB/c × C57BL)F_1 thymus grafts.

Reconstituting Capacity of Irradiated, Adult Thymus Grafts in Nude Mice. Because the results obtained in nude mice with unirradiated fetal or newborn thymus grafts differed partially from the results obtained with irradiation bone marrow chimeras and to exclude allogeneic effects, the restorative capacity of irradiated young adult thymus grafts was investigated. Similar to our experience with allogeneic irradiation chimeras, we found that nude mice reconstituted with irradiated H-2-incompatible thymus grafts were immunoincompetent and that few survived the 6–10 wk from reconstitution to testing. Of the 25 mice grafted this way, only 6 survived, and 3 of these died from infection despite the low pathogenic strain of vaccinia virus used. BALB/c ($H\text{-}2^d$) nu/nu mice were transplanted under the kidney capsule with irradiated thymus grafts of semisyngeneic (BALB/c × C3H) ($H\text{-}2^d \times H\text{-}2^k$)$F_1$ (Fig. 3, b; Table VIII, Exp. 1-A) allogeneic C57BL mice [($H\text{-}2^b$) Table VIII, Exp. 2-A]. These irradiated grafts were always much smaller (Fig. 3, b) than unirradiated fetal or neonatal ones (compare Fig. 3 a with 3 b) but showed repopulation and formation of cortex and medulla. Syngeneic combinations (~10 examples tested) expressed relatively low, but highly significant, virus-specific (Table VIII) or alloreactive (Table IX) cytotoxicity, whereas recipients of completely H-2-incompatible irradiated thymus grafts (three examples tested) revealed no measurable immunocompetence (Table VIII, Exp. 2-A). Even after the lymphocytes of this nude BALB/c with an irradiated C57BL thymus graft were adoptively boosted in appropriate F_1 stimulator mice, we could detect no virus-specific cytotoxic activity (Table VIII, Exp. 2-B). This lack of response was not readily explained by suppression, because adoptive sensitization of a mixture that contained lymphocytes from the thymus chimeras and from normal (BALB/c × C57BL)F_1 mice resulted in activation of virus-specific cytotoxicity.

Nude C57BL mice reconstituted with adult irradiated syngeneic thymus grafts under the kidney capsule were immunocompetent, as were heterozygote (BALB/c × C57BL)F_1 nu/nu mice that had received irradiated parental thymus grafts (Table VIII, Exp. 2-A). Again, as shown in Table VI for animals given fetal or newborn thymus grafts, homozygous nude mice reconstituted with irradiated heterozygous F_1 thymus grafts generated T cells that were restricted to the host H-2 only. However, in

TABLE IX

Immunocompetence of Nude Mice Reconstituted with Adult Irradiated Thymus Grafts: Alloreactivity

Experiment	Thymus Chimeras*	Alloreactivity	Dilution of lymphocytes	Percent specific ^{51}Cr release from targets‡			cpm [^{3}H]-thymidine uptake in MLR × 10^{-3}
				H-2^k (L929)	H-2^b (MC57G) (EL4)	H-2^d (P815)	
1	BALB/c(d) nu/nu with (BALB/c × C3H) (d × k) thymus (950 R)	anti-BALB/c(d)	1	NT	NT	3	17
			1/3			1	
			1/9			<1	
		anti-C3H(k)	1	71	0	NT	35
			1/3	48§	7		
			1/9	20	1		
		anti-C57BL(b)	1	2	47	NT	41
			1/3	<1	32		
			1/9	<1	10		
		None					18
2	BALB/c(d) nu/nu with C57BL (b) thymus (950 R)	anti-B10.BR(k)	1	<1	4	NT	
			1/3	<1	6		
			1/9	<1	5		
		anti-C57BL(b)	1	<1	8	NT	
			1/3	<1	6		
			1/9	<1	5		

NT, not tested.

* See Table VIII.

‡ Test conditions: Exp. 1: test duration 16 h; spontaneous release L929 < 28%; MC57G < 20%; P815 < 29%. Exp. 2: test duration 6 h; spontaneous release L929 < 14%; EL4 < 12%; P815 < 16%.

§ Significant results are boxed ($P < 0.01$).

contrast to the tolerance found in homozygous nudes with fetal heterozygous thymus grafts, the recipients of irradiated thymus grafts were not tolerant to the nonshared thymic haplotype with respect to both cytotoxicity and mixed lymphocyte reaction (Table IX, Exp. 1). After adoptive boosting of spleen cells from nudes with irradiated F_1 thymuses in irradiated and infected F_1 recipients, no virus-specific activity restricted to the nonhost thymus H-2 was detected because reactivity against infected and uninfected target cells was small but evident. This lack of tolerance to the nonhost thymic *H-2* was more striking in the MLC test (Table IX, Exp. 1). We found repeatedly that lymphocytes from BALB/c (*H-2^d*) nu/nu mice carrying irradiated (BALB/c × C3H) (*H-2^d* × *H-2^k*)F_1 grafts (but not fetal or newborn F_1 grafts, [Tables V and VI]) reacted against parental C3H (*H-2^k*) stimulator cells to lyse *H-2^k* target cells (Table IX, Exp. 1). This could not readily be explained by reactivity against differentiation antigens on lymphohemopoietic cells of the nonshared H-2 type because the cell used as target was a continuous fibroblast line (L929). Also, as we showed previously with unirradiated fetal thymus grafts, irradiated adult parental thymus grafts reconstituted F_1 heterozygote nudes to express T cells that were restricted to the thymic parental *H-2* type only (three tested) (Table VIII, Exp. 2-A).

The alloreactive potential of nude mice reconstituted with irradiated thymus grafts, as tested in MLC, confirmed the immunologic incompetence of BALB/c nu/nu mice reconstituted with irradiated histoincompatible grafts (Table IX, Exp. 2), a result

TABLE X

Search for a Functioning Nude Thymic Rudiment in BALB/c nu/nu Engrafted with Fetal C57BL Thymus

Stem cell donor	Irradiated recipient (825R)		Effector: target cells ratio	Percent specific ^{51}Cr release from target cells			
				$H\text{-}2^b$ (MC57G)		$H\text{-}2^d$ (D2)	
	Nude recipient	Thymus donor		Vaccinia virus	Uninfected	Vaccinia virus	Uninfected
(BALB/c × C57BL) $H\text{-}2^d$ × $H\text{-}2^b$	BALB/c $H\text{-}2^d$	C57BL $H\text{-}2^b$	40	54*	<1	6	2
			13	26	<1	2	1
			4	13	<1	<1	<1
Controls: BALB/c ($H\text{-}2^d$)			40	<1	<1	48	<1
			13	<1	<1	37	2
			4	<1	<1	11	<1
C57BL ($H\text{-}2^b$)			40	57	<1	<1	<1
			13	36	<1	<1	<1
			4	17	<1	<1	<1
Spontaneous release over 6 h:				12	22	17	21

* Significant results are boxed ($P < 0.01$).

that contrasts with the competence of nude mice reconstituted with irradiated grafts from donors with which one *H-2* haplotype was shared.

Search for Direct Allogeneic Effects Promoting Nude T-Cell Maturation without Reconstituting Thymus Graft. 10^8 thymocytes or 10^8 spleen and lymph node cells from C57BL mice were injected intravenously or intraperitoneally into BALB/c nu/nu mice. These recipients did not survive better than did unmanipulated nu/nu mice. Survivors were infected 6–8 wk after transfer of allogeneic cells, and in no case was immunocompetence found assessed by antiviral cytotoxicity or alloreactivity generated in a mixed lymphocyte reaction in vitro.

Search for a Nude Thymus Functioning Under the Influence of a Grafted Allogeneic Fetal Thymus. The capacity of fetal allogeneic thymus grafts to reconstit ute nude mice could be explained by a thymic rudiment in nudes that is somehow reactivated by the presence of the allogeneic thymus. This possibility was tested as follows. BALB/c ($H\text{-}2^d$) nu/nu mice possessing a functioning grafted C57BL ($H\text{-}2^d$) thymus were irradiated 10 wk after reconstitution with 825–875 rads and were then reconstituted with T-cell-deprived bone marrow cells from (BALB/c × C57BL)F_1 donor mice and left for 8 wk. If only the grafted C57BL thymus functioned we expected that only $H\text{-}2^b$-restricted antiviral cytotoxic T cells would be generated; if both the grafted thymus and the nude thymus rudiment functioned, both restriction specificities would be expected. As shown in Table X, the F_1 lymphocytes were restricted to $H\text{-}2^b$, indicating at least, that under the given conditions the grafted C57BL thymus was much more efficient in promoting maturation of restricted T cells and that no such function could be documented for the BALB/c nude thymus rudiment. Therefore, either a primitive thymus is not present, or could be functioning in these chimeras only when this rudiment is histocompatible with stem cells; it would not be used when the more efficient pathway is open to stem cells to mature in a fully functioning histocompatible thymus graft.

TABLE XI
Summary

Table	Nude stem cells	Thymus donor	Restriction		Allo-reactivity	Tolerance
			In host	In A × B sensitizing environment		
III, IV, V	A	A Unirradiated	a	NT	+	a
III, IV, V	A	B Unirradiated	a	a	+	a, b
VII	A	(A × B) Unirradiated	a	a	+	a, b
VII, VIII	A	A Irradiated	a	NT	+	a
VII, VIII	A	B Irradiated	—	—	—	NT
VII, VIII	A	(A × B) Irradiated	a	a	+	a, not b
Reference 43	A × B	A Unirradiated	a	a	+	a, b ("except.")*
Reference 43	A × B	B Unirradiated	b	b	+	a, b
Reference 43	A × B	A + B Unirradiated	a, b	NT	+	a, b
VIII	A × B	A Irradiated	a	a	+	a, b (only one tested)
Unpublished	A × B	B Irradiated	b	b	+	a, b

NT, not tested.
* Except for nu/nu repopulated by lymphocyte from grafted thymus.

Discussion

The experimental results of this study are summarized in Table XI. Genetically thymus- and T-cell-deficient nu/nu mice of strain A could be reconstituted with unirradiated fetal or newborn *H-2*-incompatible (strain B) thymus grafts, but not with irradiated thymus grafts from B adults, to express T-cell immunocompetence measured by antiviral or antialloantigen responses. Reconstitution with semisyngeneic (A × B) thymus grafts led to T-cell immunocompetence restricted to A. The A nu/nu recipient was unresponsive to the H-2 haplotype of the thymus donor if the B or (A × B) graft was fetal or neonatal and unirradiated but was not tolerant to B when receiving an adult irradiated (A × B) graft. We found no evidence that a rudiment thymus was reactivated by grafted thymuses in nude mice nor could we induce T-cell differentiation in nude mice with allogeneic thymocytes or lymph node cells without thymus grafts. The effector cells were T cells by several criteria. They were *H-2* restricted, sensitive to anti-Thy-1.2 plus C treatment (not shown), and did not lyse uninfected targets or targets that are sensitive to natural killer activity to any greater extent than did lymphocytes from control mice.

The results with allogeneic fetal or newborn thymus grafts, which reconstituted nu/nu mice, are fully compatible with Kindred's analyses of nude mice and the requirements to restore helper T-cell immunocompetence (4, 12, 21–23). The literature contains few studies assessing T-cell function in nude mice reconstituted with irradiated allogeneic thymus grafts (3, 4, 40–42). In general, these studies agree that reconstitution is limited at best.

There is no doubt that the differing results produced by nude mice given unirradiated fetal or neonatal grafts and those receiving irradiated adult thymus grafts are important to one's understanding of T-cell differentiation. Two possible explanations

are: (*a*) irradiated adult thymus grafts have an inherent low capacity to promote T-cell differentiation and (*b*) irradiated *H-2*-incompatible thymic epithelial cells alone (or with irradiated thymocytes) are not sufficient to promote T-cell maturation; alloreactive T cells from the thymus graft promote maturation of nude precursor T cells by abnormal induction.

These results show that less immunocompetence is conferred by the adult irradiated thymus than is usually observed with fetal or neonatal thymuses. From this point of view, one might argue that the lesser immunocompetence induced by irradiated semisyngeneic grafts makes that promoted by irradiated allogeneic grafts undetectably low. However, if this were so, we would expect that, upon adoptive boosting of the thymus chimeras' cells in an appropriate infected and irradiated F_1 recipient, cytotoxicity should ensue. Because this has not been found, there seems to be a great difference in the efficiency with which *H-2*-compatible irradiated thymic grafts reconstitute nude mice recipients compared with *H-2*-incompatible grafts (but this is also true to a much smaller extent for fetal or neonatal grafts [Table III] [42]), and this difference is not readily explained by difficulties in repopulation alone. The alternative explanation is that T cells or thymocytes from the transplanted allogeneic fetal or neonatal thymus grafts may exert some allogeneic effect on nude precursor cells and promote T-cell differentiation via abnormal induction (45, 46), by direct contact or by releasing T-cell growth factors, as postulated by Gillis et al. (47). Attempts to induce immunocompetence in nude mice by injecting allogeneic thymus cells or lymphocytes have failed so far. We should also have expected that in (A × B)F_1 nu/nu with a parental A thymus, the postulated allogeneic effect should have triggered the maturation of B-restricted T cells; this has not been found in earlier studies (43, 44) or here. Nevertheless, if such mechanisms alone were responsible for T-cell maturation in nu/nu mice (or in vitro [47]), then thymic selection may be only the most efficient but not the only possible differentiation pathway for T cells.

The result that homozygous nu/nu A mice grafted with an irradiated adult or unirradiated fetal or neonatal heterozygous thymus (A × B) do express T cells restricted to A but not to B, indicates that thymic selection alone is not sufficient to promote T cells to mature and express the restriction specificity for the *H-2* expressed in the thymus. This result is in contrast to the previously published findings (48–50) that lymphocytes from A → (A × B) irradiation bone marrow chimeras could be shown to be restricted mainly to A; but upon appropriate adoptive sensitization in irradiated and infected (A × B)F_1 recipients such chimeric lymphocytes reacted significantly also to B plus virus. This discrepancy is now being examined, and it appears that these earlier chimera studies were inadequate, in that most of these chimeras were not completely reconstituted (these studies will be the subject of a separate report). Our results are therefore best compared with those obtained with (A + B) → (A × B) irradiation chimeras, where subpopulations of T cells of A or B haplotype are capable to interact with A or B target cells (18, 20, 51). In these chimeras thymic *H-2* and *H-2* expressed on lymphohemopoietic and antigen-presenting cells are compatible, whereas in A nu/nu or A (ATXBM) mice (R. M. Zinkernagel. Unpublished observations.) receiving an irradiated (A × B) thymus graft no lymphohemopoietic, antigen-presenting cells of B haplotype are present. Therefore, thymic selection of the restriction specificity is necessary for T-cell maturation but not sufficient; for the latter to occur, T cells have to be exposed to lymphohemopoietic

cells with the *H-2* of the thymus. It is unclear whether this differentiation step occurs in the thymus or in the periphery as postthymic maturation (52). This differentiation step, that depends upon lymphohemopoietic, antigen-presenting cells, may be explained to amplify (or tolerize) in an *H-2*-restricted fashion the small numbers of thymically selected committed T cells. This amplification may depend in part upon multiple lymphocyte interactions that should be possible in A nude recipients of (A × B) thymus grafts but not in A nudes with a B thymus; in part, this amplification may be driven by antigen (self-debris and/or environmental antigens) that are exposed on antigen-presenting cells. Alternatively, cells presenting self-debris or environmental antigens may be essential in actually driving the diversification of the T-cell repertoire.

Because A mice without T cells, grafted with a thymus (A × B) do not have T cells restricted to B, the question arises, Is all of T-cell maturation promoted by the *H-2* of lymphohemopoietic cells rather than influenced by the thymus? Although there is no evidence available now that *H-2* restriction specificities that do not correspond to the T-cell genotype can be positively selected by the thymus alone, there is strong evidence that the possible restriction specificities corresponding to the *H-2* type differentiate only when encountered in the thymus and are influenced by the thymus in (*a*) (A × B) → A irradiation bone marrow chimeras (13, 14) and (*b*) T-cell-deficient (A × B)F_1 mice that are grafted with an A- or with a B-irradiated (13, 14) or fetal thymus (43, 44). Attempts to demonstrate formally in a two-step experiment that the restriction phenotype is determined by the thymus and that these T cells are amplified by exposure to lymphohemopoietic cells have yielded preliminary results that are compatible with the proposal (52).

Interesting results were obtained when alloreactivity and tolerance to transplantation antigens carried by thymus grafts were analyzed in this study. One unexpected finding was that F_1 (BALB/c × C57BL) nu/nu mice reconstituted with unirradiated fetal grafts of the C57BL parental type reacted against $H\text{-}2^d$ when stimulated in MLC in vitro. In several examples of the reverse combination, i.e., F_1 (BALB/c × C57BL) nu/nu reconstituted with BALB/c thymus grafts, we did not detect alloreactivity against C57BL ($H\text{-}2^b$). The fact that parental C57BL T cells have repopulated the F_1 nu/nu recipient to a great extent indicates that alloreactive T cells generated in the transplanted thymus may have eliminated the host lymphocytes during a subclinical graft-vs.-host reaction; an alternative (but unlikely) explanation for these findings is that the BALB/c nu/nu strain has undergone a gain mutation in *H-2* so as to differ from the standard BALB/c $H\text{-}2^d$.

Another notable finding was that nude mice with unirradiated allogeneic or semiallogeneic thymus grafts were tolerant to the grafts' *H-2* antigens, whereas nude mice reconstituted with semiallogeneic irradiated thymus grafts were not tolerant to the grafts' nonshared donor *H-2* type. This alloreactivity is probably not directed against lymphohemopoietic differentiation antigens other than *H-2* because the targets used were fibroblast lines. Nude mice with allogeneic-irradiated thymus grafts failed to generate significant levels of alloreactive T cells; therefore, the tolerance status of these mice could not be assessed. We would like to interpret the findings to reflect that alloantigens presented on lymphohemopoietic cells (surviving in unirradiated thymus grafts) are tolerogenic, whereas alloantigens presented on thymic epithelial cells and other radioresistant long-lived cells that are probably not of

lymphohemopoietic origin are not tolerogenic. Whether this difference is quantitative – many lymphoid cells with great concentrations of MHC products in unirradiated grafts vs. few cells with lower concentrations of MHC products in irradiated grafts – or, more likely, qualitative and related to antigen presentation, cannot be concluded unequivocally from these studies. Nevertheless, these results could indicate that antigens or alloantigens presented on nonlymphohemopoietic cells can neither tolerize nor induce an immune response. They mimic the classical experiments of Lafferty and Woolnough (53) in which histoincompatible epithelial thyroid grafts that had lost most or all of their lymphohemopoietic passenger cells were accepted by histoincompatible recipients without inducing an alloresponse. Whether the unresponsiveness to alloantigens on unirradiated thymus grafts and thymocytes is mediated by proper, tolerogenic, alloantigen presentation on lymphohemopoietic antigen-presenting cells alone and/or by some balanced suppressive mechanisms, possibly directed against the host's allorecognition receptors (54, 55), remains to be examined not only in the thymus chimeras but also in irradiation bone marrow chimeras.

Our results are relevant to hypotheses on the relationship between alloreactivity and the T-cell-receptor repertoire for foreign antigens. According to the model proposed by Jerne (56) and modified by von Boehmer et al. (18), A precursor T cells maturing in an (A × B)F_1 thymus graft should potentially express restriction specificities for A and for B and should be alloreactive against C, D, E, etc. but not against A or B. The finding that lymphocytes from A-type nude mice mature in irradiated (A × B) thymuses and express restriction specificity A, but not B, and are alloreactive against C and B but not A indicates that the thymic *H-2* cannot alone drive diversification of the T-cell repertoire nor can it alone induce and/or maintain tolerance to MHC products.

Summary

Congenitally thymusless nude mice that lacked functional T cells were reconstituted with *H-2*-compatible or -incompatible thymus grafts taken from either fetal, newborn, or adult mice and transplanted under the kidney capsule or subcutaneously. Transplantation with unirradiated fetal (15–17 d) or newborn thymus grafts reconstituted the nude mice as assessed by their subsequent generation of virus-specific cytotoxic T cells in vivo or alloreactive T cells in vitro. The restriction specificity of T cells from homozygous mice was exclusively for the nude host *H-2*, as shown by direct cytolysis or by cold target competitive inhibition assays, irrespective of whether nude mice were reconstituted with *H-2*-compatible, semiallogeneic, or *H-2*-incompatible, unirradiated newborn or fetal thymus grafts (in order of decreasing efficiency of reconstitution). The restriction specificity for the nonhost *H-2* of the thymus could not be demonstrated even after primary or secondary sensitization in an infected appropriate F_1 environment. These nude mice reconstituted with fetal or newborn grafts were tolerant to the *H-2* of the thymus donors.

Nude mice transplanted with irradiated adult thymus grafts were reconstituted functionally with syngeneic or semisyngeneic but not with allogeneic thymus grafts. In homozygous nu/nu irradiated heterozygous recipients of F_1 thymus grafts, the restriction specificity for the nonhost thymic *H-2* could not be elicited upon adoptive sensitization in irradiated and infected F_1 heterozygote stimulator mice; in fact, these chimeras' lymphocytes were not tolerant to the nonhost *H-2*. The discrepancy between

the restorative capacity of unirradiated vs. irradiated thymus grafts suggests that precursors of T cells in nude mice can acquire restriction specificity and immunocompetence independently of a conventional, functioning *H-2*-compatible thymus if exposed to an allogeneic fetal or a newborn thymus that contains functioning thymocytes of donor type but not if reconstituted with an irradiated adult allogeneic thymus.

We would like to thank Cindy Currier and Colleen Johnson for their excellent help in maintaining the mice; Dr. G. Sato, D. Mackensen, Doctors W. Weigle, E. Parks, M. B. A. Oldstone, F. Dutko, J. Watson, and S. Hedrick for making nude mice available to us or donating them generously for these studies; and Doctors Mel Cohn and Rod Langman for most helpful, clarifying discussions. We gratefully acknowledge Ms. Annette Parson's and Phyllis Minick's help in preparing this manuscript.

Received for publication 21 September 1979.

References

1. Miller, J. F. A. P., and D. Osoba. 1967. Current concepts of the immunological function of the thymus. *Physiol. Rev.* **47**:437.
2. Davies, A. J. S. 1969. The thymus and the cellular basis of immunity. *Transplant. Rev.* **1**:43.
3. Stutman, O. 1977. Two main features of T cell development: thymus traffic and post thymic maturation. *Contemp. Top. Immunobiol.* **8**:1.
4. Kindred, B. 1979. Nude mice in immunology. *Prog. Allergy.* **26**:137.
5. Paul, W. E., and B. Benacerraf. 1977. Functional specificity of thymus-dependent lymphocytes. *Science (Wash. D.C.)* **195**:1293.
6. Katz, D. H. 1977. Lymphocyte Differentiation, Recognition and Regulation. Academic Press, Inc., New York.
7. Zinkernagel, R. M., and P. C. Doherty. 1979. MHC-restricted cytotoxic T cells: studies on the biological role of polymorphic major transplantation antigens determining T cell restriction-specificity function and responsiveness. *Adv. Immunol.* **27**:51.
8. Zinkernagel, R. M., A. Althage, S. Cooper, G. Kreeb, P. A. Klein, B. Sefton, L. Flaherty, J. Stimpfling, D. Shreffler, and J. Klein. 1978. *Ir* genes in H-2 regulate generation of antiviral cytotoxic T cells. Mapping to K or D and dominance of unresponsiveness. *J. Exp. Med.* **148**:592.
9. Benacerraf, B., and R. N. Germain. 1978. The immune response genes of the major histocompatibility complex. *Immunol. Rev.* **38**:71.
10. Tynan, M. L., and H. O. McDevitt. 1970. Antibody responses to two synthetic polypeptides: the role of the thymic epithelial reticulum. *J. Immunol.* **105**:1190.
11. Chesebro, B. W., G. F. Mitchell, F. C. Grumet, L. A. Herzenberg, H. O. McDevitt, and T. G. Wegmann. 1972. Cell transfer studies in a genetically controlled immune response. *Eur. J. Immunol.* **2**:243.
12. Kindred, B. 1977. Effect of thymus donor strain on the response of reconstituted nude mice to GLT. *Dev. Comp. Immunol.* **1**:271.
13. Zinkernagel, R. M., G. N. Callahan, A. Althage, J. Cooper, P. A. Klein, and J. Klein. 1978. On the thymus in the differentiation of H-2 self-recognition by T cells. Evidence for dual recognition? *J. Exp. Med.* **147**:882.
14. Fink, P. J., and M. J. Bevan. 1978. H-2 antigens of the thymus determine lymphocyte specificity. *J. Exp. Med.* **148**:766.
15. Waldmann, H., H. Pope, C. Bettles, and A. J. S. Davies. 1979. The influence of thymus on the development of MHC restrictions exhibited by T-helper cells. *Nature (Lond.).* **277**:137.
16. Miller, J. F. A. P., J. Gamble, P. Mottram, and F. I. Smith. 1979. Influence of thymus

genotype on acquisition of responsiveness in delayed-type hypersensitivity. *Scand. J. Immunol.* **9:**29.

17. Katz, D. H., L. R. Katz, C. A. Bogowitz, and B. J. Skidmore. 1979. Adaptive differentiation of murine lymphocytes. II. The thymic microenvironment does not restrict the cooperative partner cell preference of helper T cells differentiating in $F_1 \rightarrow F_1$ thymic chimeras. *J. Exp. Med.* **149:**1360.
18. von Boehmer, H., W. Haas, and N. K. Jerne. 1978. Major histocompatibility complex-linked immune responsiveness is acquired by lymphocytes of low-responder mice differentiating in thymus of high responder mice. *Proc. Natl. Acad. Sci. U. S. A.* **75:**2439.
19. Zinkernagel, R. M., A. Althage, S. Cooper, G. Callahan, and J. Klein. 1978. In irradiation chimeras, K or D regions of the chimeric host, not of the donor lymphocytes determine immune responsiveness of antiviral cytotoxic T cells. *J. Exp. Med.* **148:**805.
20. Kappler, J. W., and P. Marrack. 1978. The role of H-2 linked genes in helper T cell function. IV. Importance of T cell genotype and host environment in I-region and Ir gene expression. *J. Exp. Med.* **148:**1510.
21. Kindred, B. 1978. Functional activity of T cells which differentiate from nude mouse precursors in a congenic or allogeneic thymus graft. *Immunol. Rev.* **42:**60.
22. Kindred, B., and F. Loor. 1974. Activity of host-derived T cells which differentiate in nude mice grafted with co-isogenic or allogeneic thymuses. *J. Exp. Med.* **139:**1215.
23. Kindred, B. 1979. Lymphocytes which differentiate in an allogeneic thymus. III. Parental T cells which differentiate in an allogeneic or semi-allogeneic thymus help only B cells of their genotype. *Cell. Immunol.* In press.
24. Reid, L. M., J. Holland, C. Jones, B. Wolf, G. Miroayana, R. Williams, N. O. Kaplan, and G. Sato. 1978. Some of the variables affecting the success of transplantation of human tumors into the athymic nude mouse. *In* Use of Athymic (Nude) Mice in Cancer Research. D. P. Houchins and A. A. Ovejera, editors. Gustav Fischer, New York, Inc., New York. 107.
25. Parks, D. E., M. V. Doyle, and W. O. Weigle. 1977. Effect of lipopolysaccharide on immunogenicity and tolerogenicity of HGG in C57BL/6J nude mice: evidence for a possible B cell deficiency. *J. Immunol.* **119:**1923.
26. Joklik, W. K. 1966. The pox virus. *Bacteriol. Rev.* **30:**33.
27. Lehmann-Grube, F. 1971. Lymphocytic choriomeningitis virus. *Virol. Mongr.* **10:**1.
28. Callahan, G. N., S. Ferrone, M. D. Poulik, R. A. Reisfeld, and J. Klein. 1976. Characterization of Ia antigens in mouse serum. *J. Immunol.* **117:**1351.
29. Doherty, P. C., J. Palmer, and R. M. Zinkernagel. 1977. Experimental analysis of effector thymus derived lymphocyte in viral infections. *In* Methods in Virology. K. Maramorosch and H. Koprowski, editors. Academic Press, Inc., New York. **6:**29.
30. Gardner, I. D., N. A. Bowern, and R. V. Blanden. 1974. Cell-mediated cytotoxicity against ectromelia virus-infected target cells. I. Specificity and kinetics. *Eur. J. Immunol.* **4:**63.
31. Koszinowski, U., and H. Ertl. 1976. Role of early viral surface antigens in cellular immune response to vaccinia virus. *Eur. J. Immunol.* **6:**679.
32. Zinkernagel, R. M., and P. C. Doherty. 1974. Activity of sensitized thymus-derived lymphocytes in lymphocytic choriomeningitis reflects immunological surveillance against altered self components. *Nature (Lond.).* **251:**547.
33. Zinkernagel, R. M. 1976. H-2 restriction of virus-specific T-cell-mediated effector functions in vivo. II. Adoptive transfer of delayed type hypersensitivity to murine lymphocytic choriomeningitis virus is restricted by the K and D regions of H-2. *J. Exp. Med.* **144:**776.
34. Pulkkinen, A. J., and C. J. Pfau. 1970. Plaque size heterogeneity: a genetic trait of lymphocytic choriomeningitis virus. *Appl. Microbiol.* **20:**123.
35. Panfili, P. R., and R. W. Dutton. 1978. Alloantigen-induced T helper activity. I. Minimal genetic differences necessary to induce a positive allogeneic effect. *J. Immunol.* **120:**187.

36. Lafferty, K. J., M. Ryan, and I. S. Misko. 1974. An improved system for the assay of stimulation in mouse mixed leukocyte cultures. *J. Immunol. Methods.* **4**:263.
37. Nabholz, M., H. Young, A. Rijnbeek, R. Boccardo, C. S. David, T. Meo, V. Miggiano, and D. C. Shreffler. 1975. I-region associated determinants: expression of mitogen stimulated lymphocytes and detection by cytotoxic T cells. *Eur. J. Immunol.* **5**:594.
38. Pritchard, H., and H. S. Micklem. 1974. The nude (nu/nu) mouse as a model of thymus and T-lymphocyte deficiency. *In* Proceedings of the First International Workshop on Nude Mice. Gustav Fischer Verlag, Stuttgart. 127.
39. Wortis, H. H. 1971. Immunological responses of "nude" mice. *Clin. Exp. Immunol.* **8**:305.
40. Loor, F., and L.-B. Hagg. 1977. The restoration of the T-lymphoid system of nude mice: lower efficiency of nonlymphoid, epithelial thymus grafts. *Cell. Immunol.* **29**:200.
41. Radov, L. A., D. H. Sussdorf, and R. L. McCann. 1975. Relationship between age of allogeneic thymus donor and immunological restoration of athymic ('nude') mice. *Immunology.* **29**:977.
42. Loor, F., B. Kindred, and L.-B. Hagg. 1976. Incomplete restoration of T-cell reactivity in nude mice after neonatal allogeneic thymus grafting. *Cell. Immunol.* **26**:29.
43. Zinkernagel, R. M., A. Althage, and G. Callahan. 1979. Thymic reconstitution of nude F_1 mice with one or both parental thymus grafts. *J. Exp. Med.* **150**:693.
44. Hedrick, S. M., and J. Watson. 1979. Genetic control of the immune response to collagen. II. Antibody responses produced in fetal liver restored radiation chimeras and thymus reconstituted F_1 hybrid nude mice. *J. Exp. Med.* **150**:646.
45. Bretscher, P., and M. Cohn. 1970. A theory of self-nonself discrimination. *Science (Wash. D. C.).* **169**:1402.
46. Katz, D. H. 1972. The allogeneic effect on immune responses. Model for regulatory influences of T lymphocytes on the immune system. *Transplant. Rev.* **12**:141.
47. Gillis, S., N. A. Union, P. E. Baker, and K. A. Smith. 1979. The in vitro generation and sustained culture of nude mouse cytolytic T lymphocytes. *J. Exp. Med.* **149**:1460.
48. Pfizenmaier, K., A. Starzinski-Powitz, H. Rodt, M. Rollinghoff, and H. Wagner. 1976. Virus and trinitrophenol hapten-specific T cell-mediated cytotoxicity against H-2 incompatible target cells. *J. Exp. Med.* **143**:999.
49. Zinkernagel, R. M. 1976. H-2 restriction of virus-specific cytotoxicity across the H-2 barrier. Separate effector T cell specificities are associated with self H-2 and with the tolerated allogeneic H-2 in chimeras. *J. Exp. Med.* **144**:933.
50. Zinkernagel, R. M., G. N. Callahan, A. Althage, S. Cooper, J. W. Streilein, and J. Klein. 1978. The lymphoreticular system in triggering virus-plus-self-specific cytotoxic T cells. Evidence for T help. *J. Exp. Med.* **147**:897.
51. von Boehmer, H., and J. Sprent. 1976. T cell function in bone marrow chimeras: absence of host-reactive T cells and cooperation of helper T cells across allogeneic barriers. *Transplant. Rev.* **29**:3.
52. Zinkernagel, R. M., A. Althage, E. M. Waterfield, P. Pincetl, G. N. Callahan, and J. Klein. 1979. Two stages of H-2 dependent T cell maturation. *In* Proceedings of the Symposium on Cell Lineage and Stem Cell Determination. Seillac, France. Excerpta Medica Elsevier, Amsterdam. In press.
53. Lafferty, K., and J. Woolnough. 1977. The origin and mechanism of allograft reaction. *Immunol. Rev.* **35**:231.
54. Ramseier, H., and J. Lindenmann. 1969. F_1 hybrid animals: reactivity against recognition structures of parental strain lymphoid cells. *Pathol. Microbiol.* **34**:379.
55. Binz, H., and H. Wigzell. 1977. Antigen-binding idiotypic T-lymphocyte receptors. *In* Contemporary Topics in Immunobiology. O. Stutman, editor. Plenum Publishing Corp., New York. **7**:113.
56. Jerne, N. K. 1971. The somatic generation of immune recognition. *Eur. J. Immunol.* **1**:1.