SPECIFICITY OF CYTOTOXIC T CELLS FROM ATHYMIC MICE*

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The peripheral T cell pool is generated in the thymus from precursor cells, which, in turn, are derived from hematopoietic stem cells. During their development in the thymus, T lymphocytes are influenced not only in terms of functional maturation, but also in their receptor specificity. Thus, T cells specific for conventional antigens have been shown to learn restriction to a given major histocompatibility complex (MHC)[1] type during their differentiation in the thymus (1–3). More recently, we have found that alloreactive T cells, as well, can be influenced in their specificity by the MHC type of their environment during ontogeny (4). Whereas it seems clear that the end product of thymic T cell differentiation is a population of T cells with a self-preference in MHC-restricted responses, we have no knowledge of the prethymic T cell receptor repertoire and how it may become biased toward restriction to self-MHC antigens.

In this context, the induction of alloreactive cytotoxic T lymphocytes (CTL) from athymic nude mice, recently reported by Gillis et al. (5), is of great interest. They demonstrated that Thy-1$^+$ cytotoxic effector cells can be raised from nude lymphoid cells in vitro, provided that T cell growth factor (TCGF), an activity present in the culture supernate of concanavalin A (Con A)-activated T cells (6), is included in the culture medium. Although the presence of Thy-1$^+$ cells in nude mice had been described as early as 1973 by Raff (7) and was later confirmed by several laboratories (8–10), this report was the first demonstration of functional nude T cells. It is, however, not self-evident that the Thy-1$^+$ effector cells generated in vitro in the presence of TCGF are derived from Thy-1$^+$ cells present in the animal, because the in vitro conversion of Thy-1$^-$ to Thy-1$^+$ cells has been demonstrated by employing soluble factors from various sources (11–13). In any case, CTL derived from nude mice may provide a useful system to compare the T cell repertoire generated in the absence and in the presence of the thymus.

We hypothesized that nude CTL (whether they were induced from Thy-1$^-$ or Thy-1$^+$ precursors) would express the germ-line-encoded repertoire of receptors that has not been influenced by self-H-2 antigens of the thymic environment. In the absence of this self-learning, we questioned whether this repertoire would show the phenomena

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; BSS, balanced salt solution; C, complement; CASUP, concanavalin A-induced supernate; Con A, concanavalin A; CTL, cytotoxic T lymphocyte(s); FCS, fetal calf serum; LPS, lipopolysaccharide; MHC, major histocompatibility complex; MLC, mixed lymphocyte culture(s); PHA, phytohemagglutinin; TCGF, T cell growth factor; TNP, trinitrophenyl.

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of (*a*) alloreactivity, that is, the abnormally strong response to foreign H-2 antigens, and (*b*) a preferential restriction to self-H-2 antigens in the response to conventional antigens.

In the present report, we first provide evidence that the precursors of CTL generated from nude cells in MLC are, themselves, Thy-1^+; we then compare the CTL responses of nude and normal mice to allogeneic stimulator cells and to H-2-identical cells differing at minor H loci or modified with trinitrophenyl (TNP). The results indicate that although in several respects the specificity of nude CTL responses is similar to that observed with CTL from normal mice, there seems to be a distinct difference in the T cell receptor repertoire generated in the absence and in the presence of a thymus.

Materials and Methods

Mice. BALB/c nu/nu and BALB/c nu/+ control mice (fifth backcross generation) were obtained from GIBCO Animal Resources Laboratories, Madison, Wis. Lymphocytes from animals employed in the experiments reported here were found to be specifically reactive with anti-H-2^d serum and complement (C) when tested against a panel of anti-H-2 sera. CBA nu/nu and nu/+, (C57BL/6 × CBA)F_1 nu/nu and nu/+ control mice were a gift of Dr. Henry Wortis, Tufts University Medical School, Boston, Mass. NIH Swiss nu/nu and nu/+, and B10 nu/nu mice were a gift of Dr. Vicki Sato, Harvard University, Cambridge, Mass. (BALB/c × CBA)F_1 nu/nu and nu/+ mice as well as normal BALB/c, BALB.B, BALB.K, A.TL, and (BALB/c × CBA)F_1 mice were bred at our facilities at MIT (Cambridge, Mass.). A/J, C3H-H-2°/SfSn, B6/By, B6.C-H-2^{ba}/By, and C57BL/10Sn mice were obtained from The Jackson Laboratory (Bar Harbor, Maine).

Preparation of Con A-induced Supernate (CASUP). 5×10^6 spleen cells/ml from BALB.B or BALB.K mice were incubated for 22 h with 2 μg/ml Con A in RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 5% fetal calf serum (FCS), 5×10^{-5} M 2-mercaptoethanol, 5 mM Hepes, and 0.02% glutamine. Cells and debris were removed by spinning the culture fluid for 10 min at 200 *g*, and then for 10 min at 1,800 *g*. The supernate was sterilized by filtration, aliquoted, and stored at −80°C. Each batch was used at the concentration that best supported the growth of a long-term CTL line (14).

Mixed Lymphocyte Culture(s) (MLC). MLC were set up in upright Falcon 3013 flasks (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) in a total vol of 20 ml, with RPMI-1640 supplemented as above. 2.5×10^7 spleen and/or lymph node cells/flask as responder cells were stimulated either with an equal number of irradiated (1,000 rad from a ^{137}Cs source) allogeneic or with 10^7 TNP-modified, irradiated syngeneic cells. Alpha-methylmannoside (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.) was added to a final concentration of 50 mM to block residual Con A in the CASUP. Primary CTL responses were assayed on days 5 or 6 of MLC. For secondary MLC, cultures were harvested on day 10 and restimulated with the original number of stimulator cells but only 5×10^6 responder cells. Secondary MLC were assayed 5 d after restimulation.

Con A induction of CTL. 2.5×10^7 spleen and/or lymph node cells were cultured in 20 ml of supplemented RPMI-1640 medium in upright Falcon 3013 tissue culture flasks in the presence of 2 μg of Con A/ml. Alternatively, 5×10^6 cells were cultured in 2 ml of medium that contained 2 μg of Con A/ml in tissue culture wells (Cluster 24; Costar, Data Packaging, Cambridge, Mass.). The cytotoxicity assay was performed on day 3 of culture with ^{51}Cr-labeled P815 target cells in the presence of 10 μg of phytohemagglutinin (PHA)/ml (Difco Laboratories, Detroit, Mich.) There is evidence that this assay technique detects all cytotoxic effector T cells regardless of their specificity (15, 16).

Target Cells. P815 tumor cells were propagated in Dulbecco's minimum essential medium that contained 10% FCS. Con A-induced blast cells were spleen cells cultured for 2 d at 2×10^6 cells/ml in supplemented RPMI-1640 in the presence of 2 μg of Con A/ml; for lipopolysaccharide (LPS)-induced blasts, 10 μg of *Salmonella typhosa* LPS/ml (Difco Laboratories) was used.

Treatment with Antibody and C. Antibodies used were: Hybridoma-produced anti-Thy-1.2

(clone 13.4 [17]); (C3H × DBA/2)F_1 anti-BALB.B (anti-H-2^b) antiserum; (BALB.B × B10.BR) anti-B10.D2 (anti-H-2^d) antiserum; and (C57BL/6 × DBA/2)F_1 anti-BALB.K (anti-H-2^k) antiserum. These anti-H-2 sera were raised by multiple intraperitoneal injections of spleen cells and had been pretested for specificity. For characterization of cytotoxic effector cells, 10×10^6 viable cells/ml were kept for 30 min on ice in a 1:100 dilution of the monoclonal anti-Thy-1.2 in balanced salt solution (BSS) with 0.2% bovine serum albumin (BSA) or in a 1:4 dilution of the anti-H-2 sera. Cells were washed and then incubated for 45 min at 37°C in twice the original volume of rabbit C diluted 1:15 in BSS/BSA. For pretreatment of cells before culture, the monoclonal anti-Thy-1.2 was used at a 1:100 dilution in BSS/BSA followed by incubation in guinea pig C at a 1:10 dilution for 45 min at 37°C.

Haptenation of Stimulator and Target Cells. TNP modification was performed according to Shearer (18) by incubating the washed cells for 10 min at 37°C in 10 mM of trinitrobenzene sulfonate (Eastman Kodak Co., Rochester, N. Y.) in phosphate-buffered saline, pH 7.3, followed by three washes in serum-containing medium. Target cells were haptenated after labeling with ^{51}Cr-sodium chromate (New England Nuclear, Boston, Mass.).

Cytotoxicity Assay. Serial threefold dilutions of cells harvested from MLC were added to 4×10^4 ^{51}Cr-labeled target cells in a final vol of 1 ml of medium (19). After 4 h of incubation, specific lysis was assessed according to the following formula:

$$\text{percent specific lysis} = 100 \times \frac{\text{counts per minute released in experimental group} - \text{counts per minute spontaneously released}}{\text{detergent releasable counts per minute} - \text{counts per minute spontaneously released}}$$

Results

Polyclonal and Antigen-specific Induction of CTL from Nude Mice. Induction of cytotoxic activity from cells of nude mice was first attempted with the T cell mitogen Con A, which is known to polyclonally induce CTL from normal mice (15, 16). In the experiment shown in Fig. 1, nude lymph node cells were stimulated with Con A in the presence of increasing concentrations of CASUP. The cytotoxic activity was measured 3 d later by gluing the effector cells to ^{51}Cr-labeled P815 targets with PHA (16). The results show that a little cytotoxic activity was generated in the absence of exogenously added CASUP, and a marked cytotoxic response was observed when the stimulation was performed with as little as 10% CASUP in the culture medium. This apparent dependence of the induction of nude CTL on the presence of factor(s) provided with the CASUP is in agreement with the requirements reported by Gillis et al. (5) for the induction of alloreactive CTL from nude mice.

The induction of nude CTL specific for allogeneic stimulators was also readily achieved if CASUP was present in MLC. In this system, no antigen-specific cytotoxic response is observed unless residual Con A in the CASUP is blocked with alpha-methylmannoside (data not shown). Primary MLC were routinely assayed on days 5 or 6 of culture (Table I; Fig. 2), secondary MLC were assayed on day 5 after restimulation (Figs. 3–5).

Expression of Thy-1 on Nude CTL and Their Precursors. The following experiments were designed to investigate the Thy-1 phenotype of nude cytotoxic effectors and their precursors. Spleen and lymph node cells from nude mice were treated before the onset of cell culture either with monoclonal anti-Thy-1.2 and C, or with C alone, and subsequently stimulated either polyclonally with Con A, or in an antigen-specific manner with allogeneic stimulator cells. Table I shows that while C-treated nude cells generated good cytotoxic activity, pretreatment with anti-Thy-1.2 and C abolished the ability of nude cells to respond with the production of CTL.

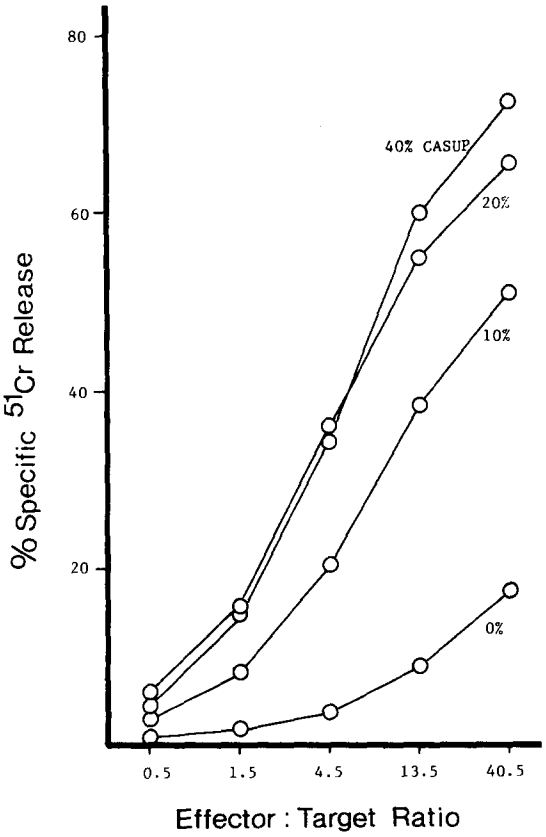

FIG. 1. Titration of CASUP in the polyclonal induction of BALB/c nu/nu CTL. Lymph node cells from a BALB/c nu/nu mouse were stimulated for 3 d with Con A in the presence of various concentrations of CASUP as indicated in the figure. ^{51}Cr-labeled P815 tumor cells in the presence of PHA were used as target cells; spontaneous release: 6.9%. Specific ^{51}Cr release in the absence of PHA did not exceed 6.7%.

Treatment of nude cells after MLC with anti-Thy-1.2 and C also completely destroyed their cytotoxic activity, indicating, as found by others (5), that the effector cells express the Thy-1 antigen (Table I).

Nude CTL Are Not Maternal Cells. Nude mice are commonly bred by mating a nu/+ female with a nu/nu male. If nude CTL are to be used as a model system for T cells that matured in the absence of a thymus, it is important to test whether the T cells were transmitted from the phenotypically normal mother during pregnancy. This question was investigated by examining the expression of both parental H-2 haplotypes on CTL generated from an H-2 heterozygous nude mouse. Fig. 2 shows that anti-B10.D2 CTL generated from spleen and lymph node cells of a (C57BL/6 nu/+ × CBA nu/nu)F_1 nude expressed both H-2^b and H-2^k antigens as evidenced by the elimination of cytotoxic activity with the appropriate anti-H-2 sera and C. Thus, nude CTL are derived from the nude's own stem cells.

Recognition of H-2K and H-2D Gene Products by Alloreactive, Nude CTL. In normal mice, alloreactive CTL, as well as those specific for conventional antigens in the context of self-H-2, are known to recognize primarily products of the K and D loci of

TABLE I

Treatment of Spleen and Lymph Node Cells from Nude Mice with Anti-Thy-1.2 and C before and after In Vitro Induction of CTL

Responder cells*	Treatment		Stimulus‡	Target cells§	E:T ratio‖	Percent specific ^{51}Cr release¶
	Before culture	Before assay				
						%
NIH Swiss nu/ nu	GPC**		Con A	P815 + PHA	36	71.3
					12	64.0
					4	46.7
	Anti-Thy-1.2 + GPC		Con A	P815 + PHA	36	1.6
					12	0.5
					4	0.1
BALB/c nu/nu	GPC		BALB.B	BALB.B	9	76.8
					3	68.4
					1	51.0
	Anti-Thy-1.2 + GPC		BALB.B	BALB.B	9	0.8
					3	−1.1
					1	−1.1
CBA nu/nu		RC**	B10.D2	P815	100	58.4
					33	41.1
					11	24.1
		Anti-Thy-1.2 + RC	B10.D2	P815	100	4.3
					33	6.0
					11	4.7

* 2.5×10^7 pooled spleen and lymph node cells cultured with 40% CASUP.
‡ 2 μg of Con A/ml or 2.5×10^7 irradiated spleen cells/flask.
§ P815 tumor cells or Con A-induced blast cells.
‖ Effector:target ratio.
¶ Spontaneous release was <6% for P815 and 22.2% for BALB.B target cells.
** GPC, guinea pig C; RC, rabbit C.

H-2 in killer-target cell interaction. In the following experiment, it was investigated if this holds true for alloreactive, nude CTL as well. Spleen and lymph node cells from a BALB/c nu/nu and a BALB/c nu/+ control mouse were stimulated twice, on day 0 and on day 10 of culture, with BALB.K cells and assayed on day 15 against target cells expressing the whole H-2^k haplotype (BALB.K), K^k and I^k antigens only (A/J), D^k antigens only (C3H-H-2^o), or I^k antigens only (A.TL). The results presented in Fig. 3 show that both populations of CTL lysed targets expressing K^k and/or D^k antigens, whereas little or no activity was observed against targets sharing only the I-region antigens with the stimulator cells. Thus, nude CTL, like those from normal mice, recognize H-2K- and H-2D-region gene products as target antigens.

Generation of CTL from an H-2^b Nude Mouse against H-$2K^b$ Mutant Cells. A remarkable example of alloreactivity is the response of H-2^b mice to H-$2K^b$ mutant strains, whose H-2K gene product differs in only one or two amino acids from the wild type (20). In the following experiment we investigated whether H-2^b nude mice could also respond to these mutant stimulator cells (Table II). Spleen and lymph node cells from a B10 nu/nu and a normal B10 control mouse were stimulated with B6.C-H-2^{ba}/By mutant cells. Both mice generated cytotoxic responses specific for the mutant strain. B10 nu/

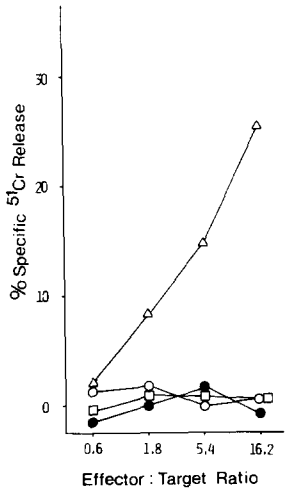

FIG. 2. Expression of H-2^b and H-2^k alloantigens on (C57BL/6 nu/+ × CBA nu/nu)F_1 nude CTL. Spleen and lymph node cells were stimulated for 6 d with irradiated B10.D2 (H-2^d) spleen cells in the presence of 40% CASUP. Effector cells were treated with antisera and rabbit C as described in Materials and Methods. ^{51}Cr-labeled targets were B10.D2 Con A blasts (spontaneous release: 27.2%). △, normal mouse serum; □, anti-H-2^b; ●, anti-H-2^k; ○, anti-Thy-1.2.

nu T cells, therefore, express receptors capable of distinguishing the product of the H-$2K^b$ mutant from the wild type.

Lack of Cross-Reactivity of Alloreactive, Nude CTL. One way to compare the fine specificity of CTL generated by various responder populations with a given allogeneic stimulator strain is to compare their cross-reactivity on additional H-2 disparate target cells (21, 22). When CTL generated from nude and control mice are compared with this parameter, a lack of cross-reactivity in the nude CTL population is observed, as illustrated by the experiment shown in Fig. 4. CBA (H-2^k) nu/nu and normal CBA spleen and lymph node cells were stimulated twice in MLC with either BALB/c (H-2^d) or with BALB.B (H-2^b) cells. The cytotoxic activity of all four populations was measured on day 5 of secondary MLC against target cells derived from both stimulator strains. CTL from the normal CBA mouse activated against BALB/c cross-reacted against BALB.B targets, and, similarly, normal anti-BALB.B CTL also lysed BALB/c target cells. CTL derived from the nude mouse, on the other hand, lysed only target cells of the type used for stimulation. In a series of six experiments, this difference in the cross-reactivity of nude and normal CTL was a consistent finding. Only rarely, and in an irreproducible fashion, was some cross-reactive lysis was also detected in nude CTL populations.

In Vitro Responses of Nude and Normal Mice to Major and Minor H Antigens. The lack of cross-reactivity observed in CTL responses of nude mice against allogeneic stimulator cells might be a result of a lack of thymic processed T cells with specificity for conventional antigens in the context of self. Indeed, the influence of self-H-2 antigens on the specificity of alloreactive T cells in thymus-bearing mice does suggest the participation of such cells in the alloreactive CTL response of normal mice (4). We chose minor H antigens as an example of conventional antigens recognized in the context of self-H-2. Our recent observation that unprimed normal mice generate anti-minor H responses in vitro if CASUP is included in the medium has enabled us to

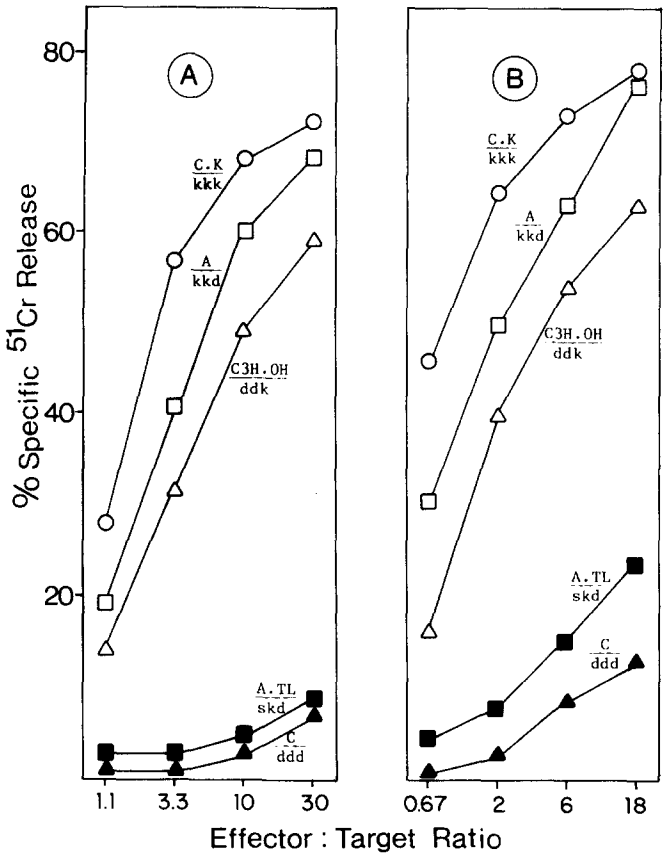

FIG. 3. Mapping of antigens recognized by anti-BALB.K CTL from nu/+ and nu/nu BALB/c mice. Spleen and lymph node cells from a BALB/c nu/+ (A) and nu/nu mouse (B) were stimulated twice in MLC with BALB.K cells in the presence of 20% CASUP and assayed on day 5 of secondary MLC. ^{51}Cr-labeled target cells were BALB.K (○, Con A blasts; spontaneous release 25.4%); A/J (□, Con A blasts; spontaneous release 17.7%); C3H-H-2^o (△, Con A blasts; spontaneous release 25.6%); A.TL (■, LPS blasts, spontaneous release 20.8%); and BALB/c (▲, Con A blasts, spontaneous release 11.9%). Expression of H-2 antigens in these target cells is indicated below the strain designations in the figure.

compare the responses of unprimed nude and normal mice to major and minor H antigens in vitro. Fig. 5 shows an experiment in which BALB/c nu/nu and nu/+ spleen and lymph node cells were stimulated twice in MLC with either B10 cells to induce alloreactive (anti-H-2^b) CTL, or with B10.D2 cells to induce a CTL response specific for the minor H antigens of the B10 series in the context of H-2^d. Both animals mounted excellent anti-H-2^b responses; on the other hand, only the cells from the phenotypically normal mouse gave a measurable self-H-2-restricted response to minor H antigens.

Induction of Nude CTL Specific for TNP-Modified Syngeneic Cells. With responder cells from normal mice, the induction of CTL to TNP-modified syngeneic cells is as readily achieved in vitro as the induction of alloreactive CTL. This property sets TNP apart from other conventional antigens, such as viral or minor H antigens. Because nude mice had been found responsive to allogeneic stimulator cells, it seemed possible that

TABLE II

CTL Response of B10 and B10 nu/nu Cells to an H-2K^b Mutant

Responder cells*	Stimulator cells‡	^{51}Cr-labeled target cells§	E:T ratio‖	Percent specific ^{51}Cr release¶
				%
B10	B6.C-H-2^{ba}/By	B10	4.5	4.7
			1.5	3.5
		B6/By	4.5	4.4
			1.5	1.6
		B6.C-H-2^{ba}/By	4.5	81.3
			1.5	76.0
			0.5	54.4
B10 nu/nu	B6.C-H-2^{ba}/By	B10	2.1	15.3
			0.7	7.2
		B6/By	2.1	12.5
			0.7	3.6
		B6.C-H-2^{ba}/By	2.1	80.3
			0.7	69.3
			0.23	52.0

* 2.5 × 10^7 pooled spleen and lymph node cells cultured for two cycles of MLC in the presence of 40% CASUP.
‡ 2.5 × 10^7 irradiated spleen cells.
§ Con A-induced blast cells.
‖ Effector:target ratio.
¶ Spontaneous release was 22.2% (B10), 19.0% (B6/By), and 16.3% (B6.C-H-2^{ba}/By).

they could also generate anti-TNP CTL in vitro. In the experiment shown in Fig. 6, spleen cells from a BALB/c (H-2^d) nu/nu mouse were stimulated with TNP-modified syngeneic cells for two cycles of MLC. It was found that the CTL generated in this fashion lysed TNP-modified, but not unmodified, BALB/c target cells and were thus TNP specific; they lysed BALB.K (H-2^k) TNP target cells with much lower efficiency, thus indicating that they were restricted to H-2^d. Similarly, B10 (H-2^b) nu/nu cells responded to TNP-modified syngeneic cells with TNP-specific CTL that lysed B10-TNP target cells threefold more efficiently than B10.D2 (H-2^d) TNP target cells (data not shown).

Absence of the Immunodominance of H-2^k over H-2^d in the Anti-TNP Response of (H-2^d × H-2^k)F$_1$ nu/nu Mice. Levy and Shearer (23) have reported that (H-2^d × H-2^k)F$_1$ mice respond to TNP-modified syngeneic stimulator cells with mostly H-2^k-restricted anti-TNP CTL. Experiments with [F$_1$ → parent] bone marrow radiation chimeras have indicated that self-H-2^k antigens on radioresistant cells, presumably in the thymus, play a role in the establishment of this immunodominance phenomenon (4). It was therefore of interest to compare the response of (H-2^d × H-2^k)F$_1$ nude and heterozygous control mice with TNP-modified syngeneic cells. A BALB/c nu/+ female was mated with a CBA nu/nu male, and spleen and lymph node cells from the nude offspring and their phenotypically normal littermates were stimulated in vitro with TNP-modified (BALB/c × CBA)F$_1$ cells. Both groups were cultured in the presence of CASUP, and, after 6 d, the cytotoxic activity against TNP-modified BALB/c and CBA targets was measured. As shown in Fig. 7, CTL from the nu/+ control mouse lysed CBA TNP targets about sixfold more efficiently than BALB/c TNP targets; the

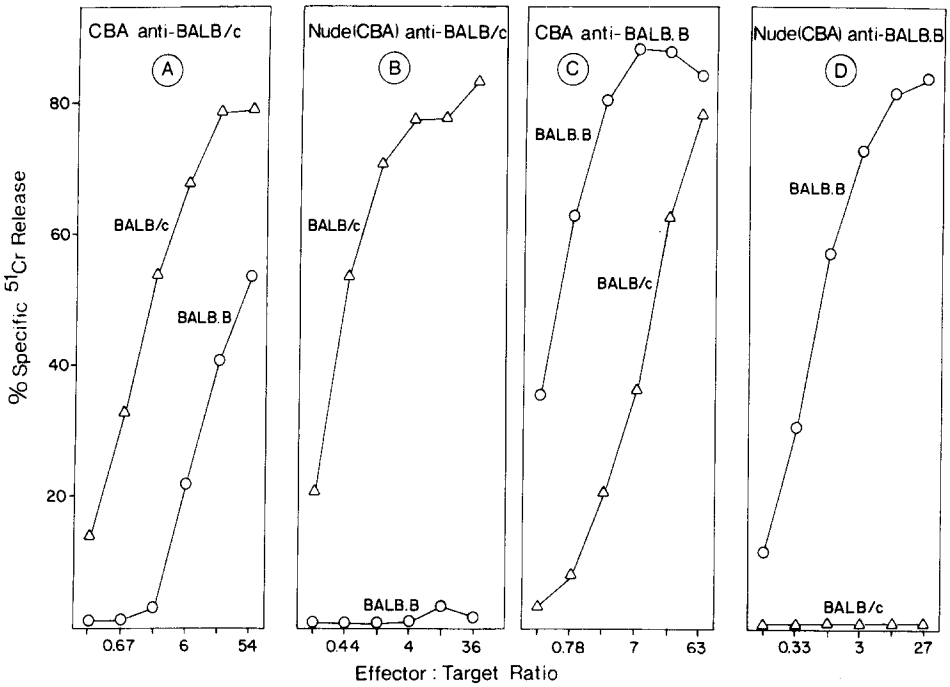

FIG. 4. Lack of cross-reactivity in alloreactive CTL populations from nude mice. Spleen and lymph node cells from a normal (A and C) and a nu/nu (B and D) CBA mouse were stimulated with BALB/c (A and B) or with BALB.B (C and D) spleen cells for two cycles of MLC in the presence of 20% CASUP, and assayed on day 5 of secondary MLC against the following ^{51}Cr-labeled Con A blasts: BALB/c (Δ, spontaneous release 19.7%), and BALB.B (○, spontaneous release 25.6%).

nude CTL, on the other hand, were equally active against both types of parental cells.

This result has been observed in three independent experiments. It strongly suggests that the haplotype preference in the anti-TNP response of (H-2^d × H-2^k)F$_1$ mice is a result of a population of T cells that is dependent on thymic maturation. Additional evidence is thus provided for the notion that the T cell receptor repertoire expressed in athymic mice is different from that in normal mice.

Discussion

We have used the in vitro induction of nude CTL in the system first described by Gillis et al. (5) to compare the CTL receptor repertoire generated in the absence and in the presence of a functional thymus.

To make such a comparison more meaningful, we initially characterized the responding cells in more detail. Immature prethymic precursor cells are believed to be Thy-1$^-$ (24). Our results demonstrate that both the polyclonal and the antigen-driven CTL responses of nude lymphoid cells are sensitive to treatment with anti-Thy-1.2 and C before culture (Table I). This strongly suggests that nude effector CTL are derived from Thy-1$^+$ precursors. The in vitro conversion of Thy-1$^-$ to Thy-1$^+$ cells, which has been observed with thymus extracts (11, 12), or through cocultivation with thymic reticuloepithelial cells (25), therefore, does not seem to play a role here. The

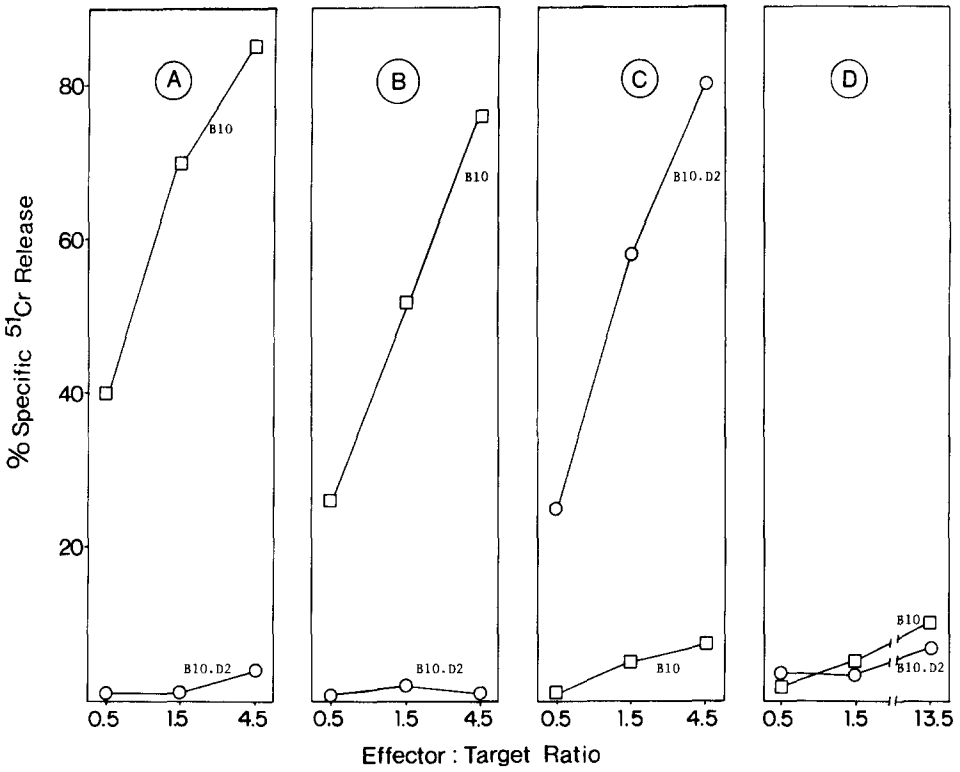

FIG. 5. In vitro response of unprimed nu/+ and nu/nu mice to major and minor H antigens. Spleen and lymph node cells from a BALB/c nu/+ (A and C) and a BALB/c nu/nu (B and D) mouse were stimulated for two cycles of MLC with B10 (A and B) or with B10.D2 (C and D) spleen cells in the presence of 20% CASUP, and assayed on day 5 of secondary MLC. ^{51}Cr-labeled targets were Con A blasts from B10 (□, spontaneous release 15.7%), and B10.D2 (○, spontaneous release 16.3%).

requirement for exogenously added TCGF in the induction of nude CTL responses has been taken as evidence that nude mice are selectively devoid of the mature T cell subpopulation that is capable of producing these factor(s) (5). In our hands, however, the induction of nude CTL was usually not fully dependent upon TCGF added in the form of CASUP (Fig. 1) (T. Huüng and M. J. Bevan. Unpublished results.). We therefore suggest that the overall low frequency of nude T cells, rather than a selective deficiency in helper T cells, is responsible for the dependence of nude CTL induction upon the addition of TCGF. This interpretation is in line with the recent demonstration of nude mouse-derived helper T cells for the humoral immune response (26).

Because it appeared that nude mice possess mature functional T cells, we deemed it necessary to prove that these cells were derived from the nude mouse itself and not from its thymus-bearing mother. Placental passage of lymphocytes has been described in humans (27) and could be the source of functional T cells in nude mice. The expression of paternal H-2 antigens on CTL from an H-2 heterozygous nude mouse (Fig. 2) clearly eliminates this possibility. It remains to be investigated, however, whether the humoral influence of the maternal thymus during pregnancy is required for the generation of functional nude T cells.

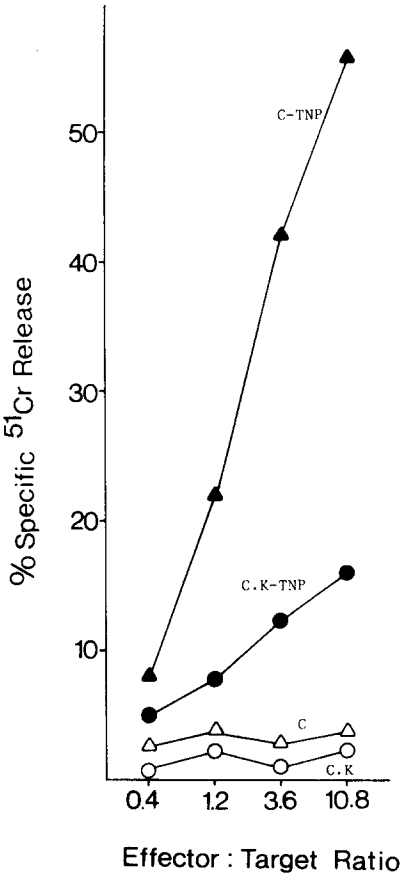

FIG. 6. Anti-TNP-self CTL response of BALB/c nu/nu spleen cells. Cells from a BALB/c nu/nu mouse were stimulated with TNP-modified BALB/c spleen cells for two cycles of MLC in the presence of 20% CASUP and assayed on day 5 of secondary MLC. ^{51}Cr-labeled target cells were Con A blasts from BALB/c (△, spontaneous release 19.0%); BALB.K (○, spontaneous release 26.1%); or TNP-modified Con A blasts from BALB/c (▲, spontaneous release 20.5%), and BALB.K (●, spontaneous release 23.9%).

Given the results outlined so far, it seems justified to state that nude mice contain mature peripheral T cells that have differentiated from their own hematopoietic stem cells. The comparison of nude and normal CTL responses, therefore, provides us with a useful system to study the contribution of the thymus in the generation of T cell diversity. In particular, it is of interest whether the phenomena of alloreactivity and restriction to self-H-2 antigens characteristic of normal peripheral T cells can even develop in the absence of a thymus.

Lymphoid cells from nude mice, like those from normal mice, respond to H-2 different stimulator cells (5) (Figs. 2–5; Table I), including cells that differ only by a mutation in H-2K (Table II), and to TNP-modified syngeneic cells (Figs. 6 and 7). These observations put the following constraint on the hypothesis that the germ-line-encoded T cell receptor repertoire is specific for the MHC-encoded antigens of the species (28): Unless extrathymic diversification is permitted as a pathway for T cell differentiation, the germ-line-encoded T cell repertoire must, as a minimum, also

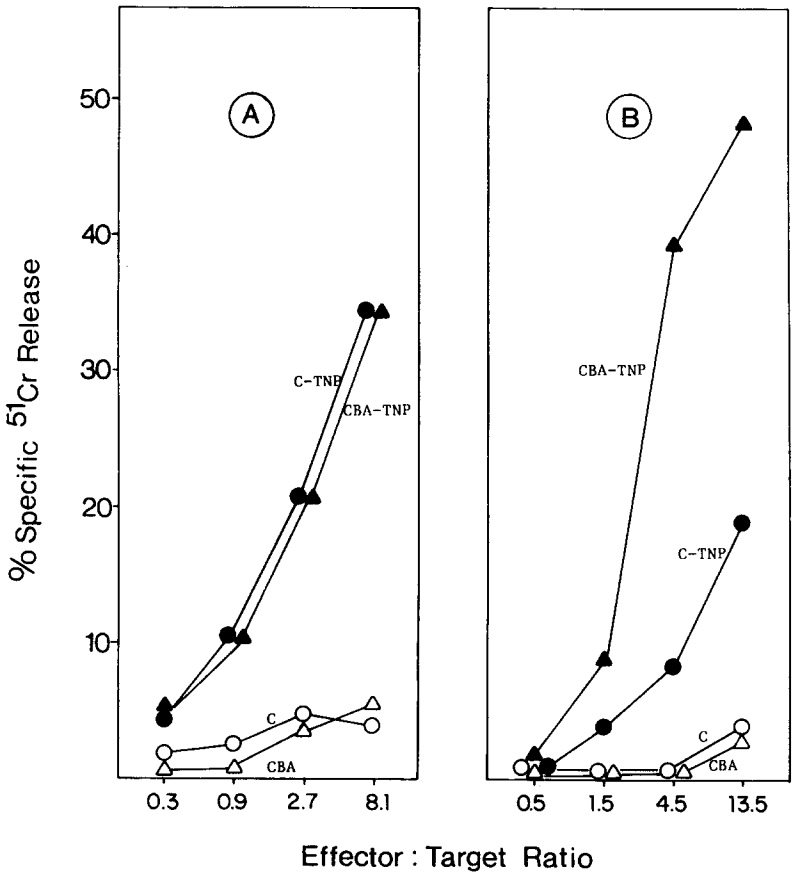

FIG. 7. Anti-TNP-self CTL response of (H-2^d × H-2^k)F_1 nu/nu and nu/+ mice. Spleen and lymph node cells from a (BALB/c × CBA)F_1 nu/nu (A) and a nu/+ control mouse (B) were stimulated for 6 d with TNP-modified (BALB/c × CBA)F_1 cells in the presence of 40% CASUP. Lymph node cells of both animals were typed with anti-H-2 sera and found to be specifically reactive with anti-H-2^d and anti-H-2^k sera. ^{51}Cr-labeled target cells were Con A blasts from BALB/c (○, spontaneous release 11.3%); CBA (△, spontaneous release 18.8%); or TNP-modified Con A blasts from BALB/c (●, spontaneous release 11.5%) and CBA (▲, spontaneous release 15.7%).

include receptors specific for the determinants created through a recent mutation in H-2 or by artificial chemical modification.

We have attempted to induce self-H-2-restricted CTL responses with nude lymphoid cells against TNP and against minor H antigens. Although stimulation with TNP-self resulted in a self-H-2-restricted CTL response (Fig. 6), no cytotoxic activity could be induced to minor H antigens in the context to self (Fig. 5). It should be noted that the in vitro response of normal mice to TNP-self is similar to responses against H-2 disparate stimulator cells, both in terms of magnitude and inductive requirements. This may be because of TNP modification of the H-2 antigens themselves (29) in the haptenation procedure employed. The CTL response to minor H antigens can be more stringently defined as being directed against conventional antigens in the context of self-H-2. Although the failure of nude lymphoid cells to respond in this system may be attributed to the overall lower number of T cells in

nude mice, it should be pointed out that an estimated 30% of the total lymphocytes of each individual nude mouse were used as responder cells in these experiments without any detectable response. The direct comparison of the response of nude cells to minor and major H antigens (Fig. 5) does, at least, show that the induction of nude CTL to an H-2 difference is much more easily achieved than the response to conventional antigens; in this sense, the phenomenon of alloreactivity is also observed in the absence of a functional thymus.

The specificity of nude CTL was studied in more detail on the effector level. It was found that, like normal CTL, alloreactive nude CTL recognize H-2K- and H-2-D-region-encoded antigens in killer-target cell interaction (Fig. 3). This finding is further proof of the T cell nature of the cytotoxic activity studied in the present report, as opposed to natural killer cell-mediated cytotoxicity. Two differences were observed in the specificity of nude and normal CTL populations: (*a*) Although alloreactive CTL from normal mice are known to be cross-reactive on third-party target cells (21, 22) as also shown here (Fig. 4), nude CTL failed to lyse targets of H-2 types other than the one used for stimulation. (*b*) In the response of ($H\text{-}2^d \times H\text{-}2^k$)$F_1$ mice to TNP-self, the dominance of $H\text{-}2^k$-restricted over $H\text{-}2^d$-restricted CTL, which is always observed in normal mice (23), was not found in nude CTL populations (Fig. 7).

H-2 antigens of the thymus have been shown to influence the specificity of T cells specific for conventional antigens (1–3), and we have recently found (4) that the bulk of alloreactive CTL from thymus-bearing mice is also influenced by self-H-2 antigens. This was shown by comparing the cross-reactive lysis of allo-induced CTL from [F_1 → parent] bone marrow radiation chimeras on TNP-modified parental target cells; it was found that haptenated targets of the chimeric host type were preferentially lysed. The same study also showed that the immunodominance of $H\text{-}2^k$- over $H\text{-}2^d$-restricted anti-TNP CTL in ($H\text{-}2^d \times H\text{-}2^k$)$F_1$ mice is dependent upon maturation in an $H\text{-}2^k$ environment. We therefore suggest that the differences in specificity between nude and normal CTL populations may be a result of a lack of thymic education in nude mice. According to this view, the cross-reactivity observed in normal alloreactive CTL populations is mediated by cells with a self-preference. The immunodominance of $H\text{-}2^k$ over $H\text{-}2^d$ in the anti-TNP response would be dependent upon the generation of a relatively larger pool of $H\text{-}2^k$-restricted TNP-specific CTL precursors in a thymus expressing $H\text{-}2^k$ antigens. This implies that confrontation of maturing T cells with self-H-2 antigens in the periphery does not have the same profound influence on T cell specificity as the confrontation with self-H-2 antigens in the thymus.

Summary

In normal mice, self-H-2 antigens in the thymus have a profound influence on T cell specificity. We have therefore investigated the properties of cytotoxic T lymphocyte (CTL) precursors from athymic nude mice (5) with the notion that they may provide a model system for the study of T cells whose receptor specificity is closer to the germ-line-encoded repertoire. It was found that the precursors of nude CTL are, themselves, $Thy\text{-}1^+$ cells. The possibility that these nude T cells were derived from the phenotypically normal mother by placental transfer was ruled out. In the presence of T cell growth factor, nude CTL can be induced by polyclonal activation with concanavalin A or by stimulation with allogeneic or trinitrophenyl (TNP)-modified

syngeneic stimulator cells, but not by stimulation with minor H antigens in the context of self-H-2.

Alloreactive, nude CTL—like those from normal mice—recognize H-2K- and H-2D-region-encoded antigens in killer-target cell interaction, but, unlike normal CTL, do not cross-react on third-party target cells. Whereas the anti-TNP response of nude mice is H-2 restricted, it does not seem to be influenced by self-H-2 antigens in the same manner as in normal mice. This is suggested by the finding that the immunodominance of $H\text{-}2^k$ over $H\text{-}2^d$ in the anti-TNP-self response of normal $(H\text{-}2^d \times H\text{-}2^k)F_1$ mice is absent in $(H\text{-}2^d \times H\text{-}2^k)F_1$ nude mice. These observations are discussed in relation to the role of the thymus in the generation of the normal mature T cell receptor repertoire.

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