FREQUENCY ANALYSIS OF CYTOTOXIC T LYMPHOCYTE PRECURSORS IN CHIMERIC MICE

Evidence for Intrathymic Maturation of Clonally Distinct Self-Major Histocompatibility Complex- and Allo-Major Histocompatibility Complex-Restricted Virus-Specific T Cells*

By HERMANN WAGNER, CONNY HARDT, ROBERT BARTLETT, HUBERTUS STOCKINGER, MARTIN RÖLLINGHOFF, HANS RODT,‡ AND KLAUS PFIZENMAIER

From the Institute of Medical Microbiology, Johannes Gutenberg University, D-6500 Mainz; and the Institute of Hematology, D-8000 Munich, Federal Republic of Germany

Products of the major histocompatibility complex (MHC)[1] influence T cell immunoreactivity in various ways. H-2K- and H-2D/L-encoded membrane glycoproteins function for cytotoxic T lymphocytes (CTL) as restriction elements (1–6), as immune response gene product (7–12), and as alloantigen (13, 14). H-2I-encoded membrane glycoproteins function similarly for T regulatory cells (15–17). There are two major theories to explain these phenomena. First, it is thought that T cells express a nonlimited receptor repertoire yet recognize extrinsic antigens only when bound to MHC products. Because the strength of binding is assumed to depend on the MHC allelic product, T cell responsiveness is controlled at the level of antigen presentation (18–20). The second theory holds that MHC products influence the receptor repertoire of T cells, via selection within the thymus (1, 3, 7). Accordingly, the T cell repertoire is limited to self-MHC-restricted responses because only those T cells that are able to recognize extrinsic antigens in the context of the thymic MHC type mature in the thymus.

To discriminate between both theories, the question becomes important whether T cells of one MHC type can respond to extrinsic antigens presented in the context of an unrelated MHC glycoprotein. In analyzing in vivo the CTL immunocompetence of radiation chimeras, compelling evidence has accumulated that the T cell receptor repertoire is profoundly influenced, presumably via selection, by the thymic MHC type (3, 7, 21, 22), and that the T cell repertoire is confined to thymic self-MHC-restricted responses. On the other hand, the T cell repertoire of conventionally reared mice appears not to be greatly influenced by the thymic MHC genotype. Simple

removal of alloreactive T cells leaves T cells able to respond to viral antigens presented in the context of allogeneic MHC products (20, 23).

The latter finding suggested that the previous demonstration of allorestricted, virus-specific (24, 25), as well as minor histocompatibility complex-specific (26) T cells in splenic lymphocytes of fully allogeneic chimeras may not represent a sneaking through of a minority of allo-MHC-restricted CTL-precursors (CTL-P), but mirrors the thymic ability to allow maturation of T cells expressing a full receptor repertoire.

To evaluate the significance of allo-MHC-restricted CTL-P in radiation chimeras, an analysis of the quantitative relationship between self-MHC-restricted and allo-MHC-restricted virus-specific CTL-P was initiated. Here, we describe that irrespective of the MHC type of grafted bone marrow cells, a grafted thymus allows maturation of both self-MHC- and allo-MHC-restricted virus-specific CTL-P, respectively. Their frequencies were comparable to those in control mice. We discuss the data with respect to the role of the thymus for the generation of T cell diversity.

Materials and Methods

Mice.   CBA (H-2^k), BALB/c (H-2^d), and C57BL/6 (H-2^b) mice were obtained from G. I. Bomholtgaard, Ry, Denmark.

Preparation of Chimeric Mice.   The preparation of bone marrow- and thymus-reconstituted radiation chimeras has been described in detail (24). Briefly, the chimeras used where made as follows: (CBA × BALB/c)F$_1$ mice were first thymectomized and subsequently grafted with two neonatal thymuses of either CBA or BALB/c origin under the right kidney capsule. The mice were irradiated with 900 rad and reconstituted with bone marrow cells that had been pretreated twice with heterologous rabbit anti-mouse T cell serum plus complement. The four combinations of thymus and bone marrow graft reconstitution employed here are listed in Table I. The mortality rate of the chimeras constructed (n = 60) was 15%. The chimeras were used 3–9 mo after repopulation. Chimerism was tested in a complement-dependent cytotoxicity test: splenic lymphocytes (10^7) or cytotoxic effector cells (10^7) generated after primary mixed lymphocyte culture (MLC) in vitro, were incubated for 30 min at 4°C in either 1 ml of a 1:4 dilution of a non-cross-reactive anti-H-2^d alloantiserum [(CBA × C57BL/6)F$_1$ antiBALB/c; 50% titer on spleen cells, 1:80] or 1 ml of a 1:400 dilution of a monoclonal anti-H-2^k antibody (clone 11.4.1; 50% titer on spleen cells, 1:6,400), which was obtained through the Salk Institute Cell Distribution Center, San Diego, Calif. Thereafter, cells were washed once and resuspended in 1 ml of a 1:15 dilution of a selected batch of nontoxic rabbit complement and incubated for a further 45 min at 38°C. Viability of spleen cells was tested by the dye exclusion method.

^{51}Cr-release Cytotoxicity Assay.   This was performed as described previously (27). Target cells were labeled with sodium [^{51}Cr]chromate (Amersham-Buchler, Frankfurt, Federal Republic of Germany) and washed twice before use. Serial dilutions of the CTL were titrated against 10^3 ^{51}Cr-labeled targets in a final vol of 200 μl and incubated for 4 h. The percent specific ^{51}Cr release was calculated according to the formula described (27). The spontaneous release of the target cells did not exceed 28%.

Estimation of CTL-P Frequency.   This was carried out under limiting-dilution conditions as described (20). If not otherwise specified, 16 replicates of microcultures were set up by culturing graded numbers of responder lymphocytes (100 × 10^3–1.5 × 10^3) together with 5 × 10^5 irradiated (2,000 rad) splenic stimulator cells for 6 d in the presence of 10% (vol:vol) Interleukin-2, a soluble mediator of T helper activity (28). Thereafter each microculture was split into aliquots and both were assayed for cytotoxicity against 10^3 ^{51}Cr-labeled target cells in a 4-h cytotoxicity assay. Cultures that gave a ^{51}Cr-release exceeding the mean spontaneous release by at least 3 × SD (16 replicates) were considered positive. The percent of negative cultures was plotted against the number of responder cells plated. Single-hit kinetics were controlled by both linear regression analysis and intercept of the straight line with the ordinate. Frequencies were then calculated according to the Poisson equation (29). Additionally, from the highest

number of responder cells plated (10^5), the mean percent specific lysis obtained in 16 individual cultures was determined according to the formula described (27).

Cell Culture. CTL were induced in MLC as described (27, 30) using spleen cells as a source of responder and stimulator cells. In those experiments where thymocytes were used as responder cells, T cell-derived helper factor (Interleukin-2) was added to substitute helper cells as described (30). Before culture, stimulator cells received a dose of 2,000 rad (Philips RT200; C. H. F. Müller, Hamburg, Federal Republic of Germany) at a dose rate of 620 rad/min. For the induction of Sendai virus-specific CTL, spleen cells were incubated for 1 h with ultraviolet-light-inactivated Sendai virus preparations as described previously (27). 3×10^6 responder cells/well were cultured for 5–6 d with 2×10^6 stimulator cells in 24-well multiculture plates (Costar 3524; Costar, Data Packaging, Cambridge, Mass.) using a mixture of Click's and RPMI-1640 media (50:50) supplemented with 10 mM Hepes, 5×10^{-5} M 2-mercaptoethanol, and 10% fetal calf serum.

Target Cells. 2-d concanavalin A (Con A) (2.5 µg/ml)-stimulated lymphoblasts were used as target cells. Sendai virus infection of target cells has been described previously (27).

Results

Characterization of the Chimeric Mice Used. We have recently reported that healthy, long-lived chimeric mice of the fully allogeneic A→B type can be constructed by using, as a host, adult thymectomized (A × B)F₁ mice that were subsequently grafted with a neonatal B type thymus, followed by lethal (900 rad) irradiation and reconstitution with A type bone marrow-derived stem cells deprived of contaminating T cells (24). Such chimeras have a survival rate of 80–90% and are capable of mounting in vitro alloreactive as well as virus-specific CTL responses restricted to either thymic or bone marrow H-2 type.

Using the above protocol, we have constructed chimeras of the CBA (H-2^k) and BALB/c (H-2^d) combination. This report summarizes the data obtained in a series of seven independent experiments, where we have individually analyzed 45 chimeric mice for CTL responsiveness toward alloantigens on the one hand, and toward Sendai virus on the other hand.

All of the 45 mice tested were capable of mounting primary alloreactive CTL responses, and only two failed to elicit a significant Sendai virus-specific CTL response. H-2 typing of chimeric lymphocytes was done in a complement-dependent cytotoxicity test using both fresh splenic lymphocytes and cytotoxic effector cells generated after in vitro MLC. 32 out of 35 mice typed were shown to be fully reconstituted with donor type lymphoid cells. These mice all mounted in vitro alloreactive, as well as Sendai virus-specific, CTL responses. For a comprehensive presentation, we have chosen the data obtained with 9 chimeric mice out of this group of 32. Basically similar results were obtained with all other mice.

The four types of chimeric mice used are listed in Table I. We constructed two fully allogeneic combinations [BALB/c thymus (B$_T$)-CBA bone marrow (C$_{BM}$), (CB)F₁B$_T$-C$_{BM}$, and vice versa, i.e., CBA thymus (C$_T$)-BALB/c bone marrow (B$_{BM}$) (CB)F₁C$_T$-B$_{BM}$], and for controls, two combinations where bone marrow and thymus donor were MHC compatible [(CB)F₁C$_T$-C$_{BM}$; (CB)F₁B$_T$-B$_{BM}$]. These mice were used 3 mo (mice 1-3) and 6–9 mo (mice 4-9) after reconstitution. The chimerism was tested by (a) H-2 typing of spleen cells (Figs. 1 A and 3 A), (b) H-2 typing of cytotoxic effector cells (Figs. 1 B and 3 B), and (c) additionally, some mice were MLC typed, i.e., tested for their capacity to stimulate a CTL response using C57BL/6-derived splenic responder cells (data not shown). Applying these criteria, the chimeras used here were found to

TABLE I

Types of Chimeric Mice Used

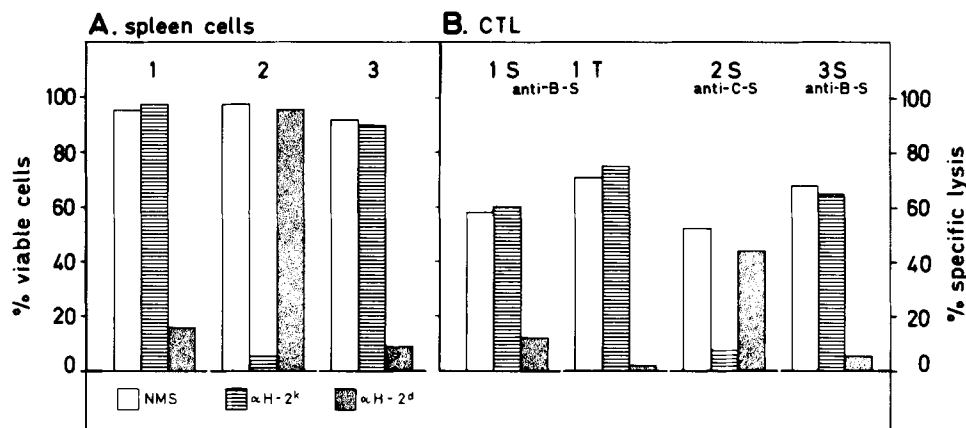| Mouse	Adult thymecto-mized, host*	Neonatal thymus graft*	Origin of bone marrow graft	Abbreviation
2, 7, 8	(CBA × BALB/c)F$_1$	CBA	CBA	(CB)F$_1$C$_T$-C$_{BM}$
1, 6	(CBA × BALB/c)F$_1$	CBA	BALB/c	(CB)F$_1$C$_T$-B$_{BM}$
5	(CBA × BALB/c)F$_1$	BALB/c	CBA	(CB)F$_1$B$_T$-C$_{BM}$
3, 4, 9	(CBA × BALB/c)F$_1$	BALB/c	BALB/c	(CB)F$_1$B$_T$-B$_{BM}$

* 900-rad-irradiated.

FIG. 1.   H-2 typing of splenic lymphocytes and cytotoxic effector cells derived from chimeras 1–3. Spleen cells (A) and in vitro induced Sendai virus-specific cytotoxic effector cells (B) (S, splenic responder; T, thymocyte responder) of mouse 1–3 (for details, see Table I) were treated with either anti-H-2^d (▨), anti-H-2^k (▤), or normal mouse serum (□) and complement as detailed in Materials and Methods. The remaining viable cells (A) and cytolytic activity against ^{51}Cr-labeled virus-infected target cells (BS, BALB/c-Sendai; CS, CBA-Sendai) (B) were determined. The data given in (B) were obtained at an initial effector:target cell ratio of 50:1. After treatment with antiserum plus complement, cells were readjusted to the original volume.

be completely (>95%) reconstituted with lymphocytes expressing the bone marrow donor H-2 type (Figs. 1 and 3). Splenic T cells from these chimeras did not display any significant MLC or CTL activity against cells of either of the two parental H-2 haplotypes involved (data not shown).

Intrathymic Differentiation of Bone Marrow-derived Stem Cells into Immunocompetent CTL-P.   The generation of virus-specific CTL from splenic lymphocytes of fully allogeneic chimeric mice (25) has raised the question of whether the CTL-P have matured and gained immunocompetence within the allogeneic thymus. If so, CTL-P should be detectable within a grafted thymus of a fully allogeneic chimera. Accordingly, we set up MLC using thymocytes and spleen cells from a given chimera as responder cells, and compared their ability to generate both alloreactive and Sendai virus-specific CTL.

Out of a group of 15 mice tested, data obtained from 3 mice are given here. Evidence is presented, that not only splenic lymphocytes, but also thymocytes that had differentiated in an allogeneic H-2^k thymus (mouse 1) were capable effectively of

mounting both alloreactive (Fig. 2 C) and virus-specific CTL responses (Fig. 2 A and B). The cytolytic activity generated was in the same order of magnitude as the activity induced in lymphocytes from chimeric mice reconstituted with bone marrow cells that were H-2 identical to the grafted thymus (Fig. 2, mice 2 and 3). Dependent on the stimulator cells used (Fig. 2 A, BALB/c-Sendai; Fig. 2 B, CBA-Sendai), thymocytes, like splenic responder cells, gave rise to either $H-2^d$- or $H-2^k$-restricted Sendai virus-specific CTL.

H-2 typing of both spleen cell (Fig. 1 B, 1 S) and thymocyte-derived CTL (Fig. 1 B, 1 T) indicated that cytolysis was entirely carried out by effector cells expressing the $H-2^d$ haplotype. Thus, these data suggested that H-2 compatibility between differentiating lymphoid cells and radioresistant thymic cells is not a prerequisite for the acquisition of CTL-P immunocompetence.

We also found no evidence for a marked selection of T cells restricted to the MHC type of the thymic epithelial cells. On the contrary, both splenic and thymocyte responder cells were preferentially stimulated by and restricted to virus-infected cells expressing their own (i.e., bone marrow donor) MHC type and not the thymic MHC type (Figure 2 A and B).

Interestingly, we noted, that control F_1 chimeric mice, which were reconstituted with parental type bone marrow cells syngeneic to the thymic graft (mouse 2 and 3), showed a similar pattern of responsiveness as the chimera reconstituted in the allogeneic combination (mouse 1). Accordingly, mouse 2 and 3 mediated preferentially a self-MHC-restricted, virus-specific CTL response. Yet, with both mice, a lower, but significant, virus-specific CTL activity was induced in the context of allogeneic MHC determinants that had not been encountered during intrathymic maturation (Fig.
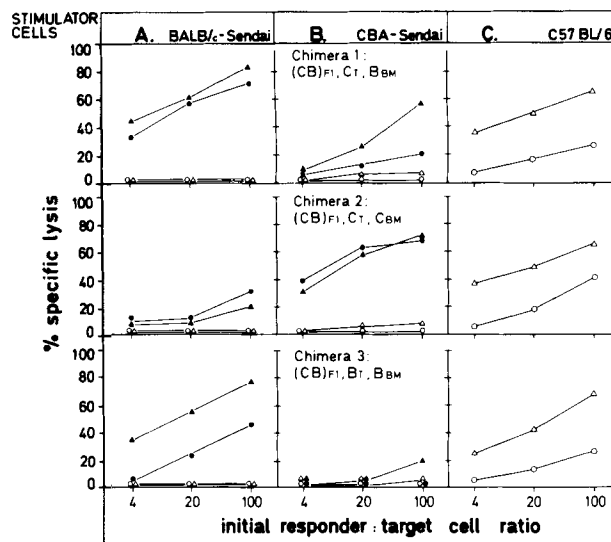

Fig. 2. Induction of alloreactive and virus-specific, self-MHC- and allo-MHC-restricted CTL within splenic and thymocyte responder cells of chimeric mice. MLC were set up as described in Materials and Methods using both splenic (△, ▲) and thymocyte (○, ●) responder cells stimulated with Sendai virus-conjugated BALB/c (A), Sendai virus-conjugated CBA (B) or C57BL/6 (C) spleen cells. Cytotoxic activity was determined at different effector:target cell ratios at day 5 with ^{51}Cr-labeled Con-A-stimulated lymphoblast target cells (A) BALB/c; (B) CBA; (C) C57BL/6) that were left untreated (△, ○) or were infected with Sendai virus before the assay (▲, ●).

2 A, mouse 2; Fig. 2 B, mouse 3). The cytolytic activity generated was clearly virus specific. Noninfected target cells, being either syngeneic or allogeneic to the CTL, were not lysed (Fig. 2 A and B). Again, the splenic effector cells of these chimeras could be characterized as of bone marrow donor type (Fig. 1 B, 2 S and 3 S).

MHC-Restriction Specificities of T Lymphocytes from Chimeric Mice. We have investigated the restriction specificity of Sendai virus-specific CTL derived from mice 1, 2, and 3, and obtained the following results: When cultured with virus-infected stimulator cells syngeneic to the bone marrow donor-derived T cells, the CTL activity generated was found to be both antigen specific and restricted to the stimulator cell MHC haplotype (Table II, lines 1, 3–5). In contrast, when H-2^d responder cells derived from chimeric mouse 1, (CB)F$_1$C$_T$-B$_{BM}$, were stimulated with allogeneic, virus-infected CBA (H-2^k) spleen cells we not only observed lysis of virus-infected target cells of the stimulator cell type (CBA-Sendai) but also of virus-infected target cells of the responder cell type (BALB/c-Sendai) (Table II, line 2). This cytolytic activity was specific for cells bearing the viral antigens and MHC antigens of either of the two parental strains involved; neither noninfected target cells nor virus-infected target cells of an unrelated MHC haplotype (C57BL/6) were lysed (Table II, line 2).

These findings can be interpreted in at least two ways. First, in the chimeric mice used here, two sets of virus-specific CTL are present, one of which is restricted to self-MHC, the other being cross-reactive with the allogeneic MHC haplotype in question. When stimulated in the context of the latter MHC determinants, only cross-reactive CTL activity will be induced. After stimulation with virus-infected syngeneic cells, the cross-reactive set would not detectably contribute to the overall lytic activity generated, resulting in exclusively self-MHC-restricted CTL activity.

Considering the particular features of the antigen used here, namely that Sendai virus is an agent that mediates cell fusion, another alternative becomes apparent. It is conceivable that Sendai virus antigen is transferred onto cells of the responding population either by cell fusion, the jumping of the antigen from one cell to another, or by antigen-processing by cells present in the splenic responder cells. If so, the data obtained here would be in accordance with the view that clonally distinct CTL exist that are exclusively restricted to syngeneic or allogeneic MHC determinants. A segregation analysis performed under limiting dilution conditions for CTL precursor cells supports the latter conclusion (vide infra).

Frequency Analysis of Alloreactive, Self-MHC-restricted and Allo-MHC-restricted, Virus-

TABLE II

Are CTL Derived from Fully Allogeneic Chimeras H-2 Restricted?

		MLC*		Percent specific lysis of target cells											
	Mouse	Responder cells (chimera type)	Stimulator cells	BALB/c 100:1‡	BALB/c-Sendai			CBA 100:1	CBA-Sendai			C57BL/6-Sendai		
					100:1	20:1	4:1		100:1	20:1	4:1	100:1	20:1	4:1
1.	1	(CB)F₁C$_T$-B$_{BM}$	BALB/c-Sendai	0	51	35	15	0	0	0	3	2	0	1
2.			CBA-Sendai	0	50	38	25	5	25	20	11	0	3	1
3.	2	(CB)F₁C$_T$-C$_{BM}$	CBA-Sendai	0	2	2	0	8	69	42	25	0	4	3
4.			C57BL/6	0	0	0	0	—	5	0	0	62	44	24
5.	3	(CB)F₁B$_T$-B$_{BM}$	BALB/c-Sendai	0	60	30	23	0	4	3	4	5	1	0

* Bulk culture MLC as described in Materials and Methods.
‡ Initial responder:target cell ratio.

specific CTL-P in Chimeric Mice. The above-described data revealed that, under conditions of tolerance to a given alloantigen, allo-MHC-restricted, virus-specific CTL are detected in chimeric mice. Such CTL are present not only among T cells that have differentiated in a thymus expressing the alloantigens in question, but are found also within T cells that matured within and were exported from a syngeneic thymus. A priori, these findings contradict the currently favored models of intrathymic generation of T cell receptor diversity (7) and thymus-dependent selection of T cell H-2-restriction specificities (21, 22, 31). Therefore, the quantitative relation of self-MHC- vs. allo-MHC-restricted, virus-specific CTL-P had to be established.

The experimental approach was a twofold: Splenic lymphocytes from individual chimeric mice, which had been previously H-2 typed (Fig. 3 A), were stimulated in bulk-culture MLC against either third-party alloantigens (C57BL/6) or BALB/c-Sendai virus- and CBA-Sendai virus-infected stimulator cells, respectively, to perform a H-2 typing of the cytotoxic T effector cells generated (Fig. 3 B). Simultaneously, using the very same responder and stimulator cells, cultures were set up under limiting-dilution conditions as previously described (20) to estimate CTL precursor frequencies and the restriction specificity of the CTL generated.

Out of 25 mice tested, data obtained from one representative experiment are given in Figs. 3 and 4 and Table III, where four (CB)F_1 chimeras, reconstituted with thymus and bone marrow in four different combinations as indicated in Tables I and III have been individually tested for in vitro CTL responsiveness. The H-2 typing of the effector cells generated (Fig. 3 B) clearly indicates that CTL were derived from the bone marrow donor. For example, lysis of BALB/c-Sendai virus-infected target cells by both mouse 4 (syngeneically reconstituted with B_T and B_{BM}) and mouse 6 (allogeneic combination, C_T and B_{BM}) was completely abolished by anti-H-2^d antiserum treatment but was virtually unimpaired by treatment of effector cells with anti-H-2^k antiserum. Similarly, cytolysis of CBA-Sendai virus-infected target cells by mouse 6-derived effector cells that had been stimulated against CBA-Sendai virus, was abrogated by anti-H-2^d and not by anti-H-2^k treatment of effector cells (Fig. 3 B). On the other hand, CTL derived from both mouse 5 (allogeneic combination, B_T and C_{BM}) and mouse 7 (syngeneic combination, C_T and C_{BM}) were only affected by anti-H-2^k treatment (Fig. 3 B).
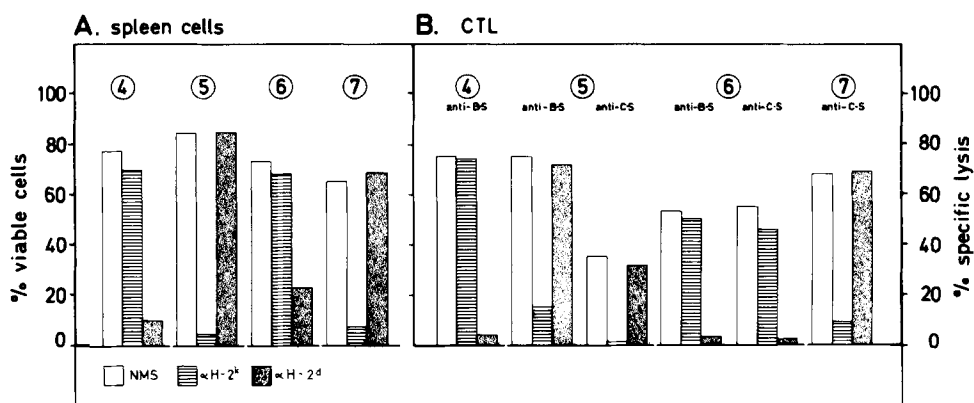

Fig. 3. H-2 typing of splenic lymphocytes and CTL derived from chimeras 4–7. The typing of the chimeras 4–7 (for details, see Table I) was done as described in legend to Fig. 1.
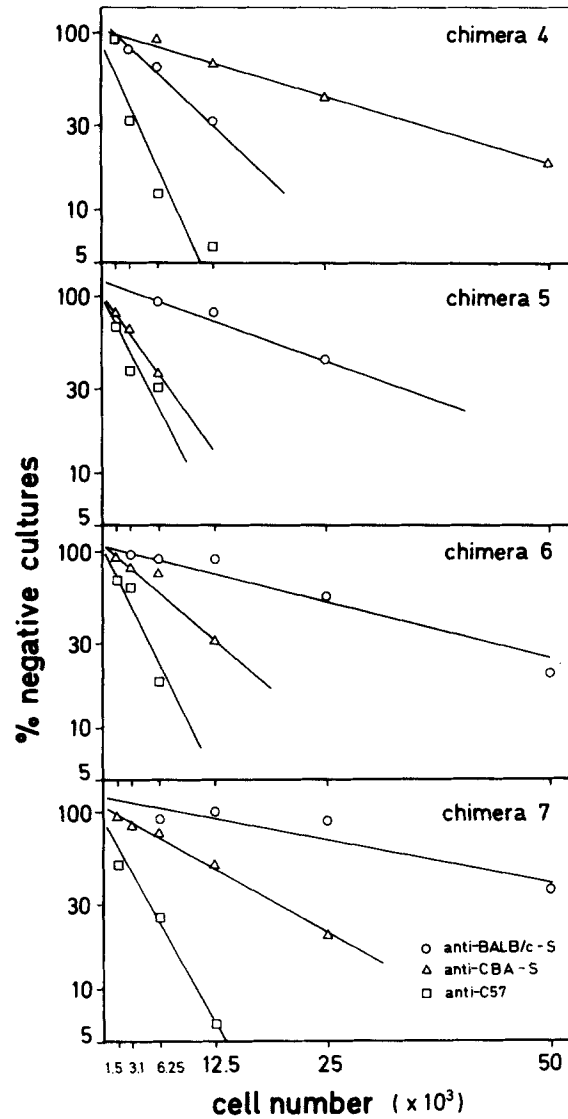
Fig. 4. Determination of alloreactive as well as virus-specific, self-MHC- and allo-MHC-restricted CTL-P frequencies in the spleen of chimeric mice. The CTL-P frequency was determined by the limiting-dilution technique as detailed in Materials and Methods using as stimulator and target cells, either C57BL/6 (□), BALB/c-Sendai (○), or CBA-Sendai (△). The percent negative cultures (^{51}Cr release <3 × SD) is plotted against the number of responder cells plated.

The alloreactive and Sendai virus-specific CTL-P frequencies in spleen cells of chimeric mice were calculated from the percentage of nonresponding cultures after plating limiting numbers of responder cells as indicated in Fig. 4. The fit of the experimental data to a straight line, determined by linear regression analysis (29, 32), and the intercept of the line with the ordinate close to 1.0 (32) verifies that the data obtained follow single-hit kinetics. The CTL-P frequencies obtained, the regression coefficient, and the intercept with the ordinate, together with the cytolytic activity

TABLE III

Frequency Analysis of CTL-P Derived from Chimeric Mice

| Mouse | | MLC | | CTL-P frequency* | | | Percent specific lysis‡ | | |
		Responder cells (chimera type)	Stimulator cells	PF	r²	a	C57	BALB/c-Sendai	CBA-Sendai
1			BALB/c-Sendai	1/10,942	0.99	1.13	—	36	3
2	4	(CB)F₁Bᴛ-Bʙᴍ	CBA-Sendai	1/30,554	0.99	1.12	—	40	35
3			C57BL/6	1/3,501	0.86	0.82	95	—	—
4			BALB/c-Sendai	1/29,535	0.97	1.27	—	31	15
5	5	(CB)F₁Bᴛ-Cʙᴍ	CBA-Sendai	1/6,444	0.97	1.10	—	—	60
6			C57BL/6	1/4,660	0.86	0.88	91	—	—
7			BALB/c-Sendai	1/38,880	0.97	1.05	—	18	2
8	6	(CB)F₁Cᴛ-Bʙᴍ	CBA-Sendai	1/11,504	0.97	1.16	—	30	60
9			C57BL/6	1/4,164	0.94	1.13	98	—	—
10			BALB/c-Sendai	1/54,780	0.95	1.21	—	15	30
11	7	(CB)F₁Cᴛ-Cʙᴍ	CBA-Sendai	1/16,000	0.96	1.12	—	0	45
12			C57BL/6	1/4,012	0.98	0.85	93	—	—

* CTL-P frequencies were calculated from the data given in Fig. 4 as detailed in Materials and Methods; PF, precursor frequency; r², regression coefficient; a, intercept with ordinate.

‡ Mean percent specific lysis of 10^3 target cells in cultures with 10^5 responder cells plated.

and MHC-restriction specificity of the CTL generated are listed in Table III. The data can be summarized as follows: Irrespective of the thymus-bone marrow combination, all chimeras contained similar frequencies of alloreactive CTL-P (Table III, lines 3, 6, 9, 12), which in turn are comparable to those found in normal, nonchimeric mice (33, 34). Moreover, the frequencies of Sendai virus-specific CTL-P were of the same order of magnitude as those of normal mice (20, 34). In all chimeras, both self-MHC-restricted (Table III, lines 1, 5, 7, 11) and allo-MHC-restricted (Table III, lines 2, 4, 8, 10) Sendai virus-specific CTL-P were found. It is our experience that, with the exception of mouse 6 (1 out of 25), the self-MHC restricted CTL-P were in a three- to fivefold excess over allo-MHC-restricted CTL-P. For example, the CTL-P frequency obtained with the allogeneic chimera 5 (Bᴛ and Cʙᴍ), after stimulation with BALB/c-Sendai (line 4) was ~1/30,000, whereas 1/6,500 cells was able to respond to Sendai virus when presented on syngeneic CBA cells (line 5). Similar CTL-P ratios were found in the syngeneic chimera 4 (Bᴛ and Bʙᴍ) (Table III, lines 1 and 2), and 7 (Cᴛ and Cʙᴍ) (Table III, lines 10, 11).

In addition to the determination of the CTL-P frequency, the limiting-dilution technique was used to test the lytic activity and MHC-restriction specificity of the CTL generated. This was achieved by splitting the microcultures into two parts, to each of which, in the case of virus-specific CTL, either BALB/c- or CBA-derived Sendai virus-infected lymphoblasts were added as target cells. Alloreactive CTL activity was determined by testing split cultures against C57BL/6 lymphoblasts and lymphoblasts syngeneic to the responder cell population. The mean percent specific lysis of 10^3 target cells, obtained in individual microcultures plated with 10^5 responder cells, indicate the high lytic efficiency of the CTL induced (Table III).

Evidence for a Clonal Segregation of Self-MHC- and Allo-MHC-restricted CTL

Activity. With respect to the MHC restriction specificity of virus-specific CTL derived from fully allogeneic chimeras, we noted that sensitization under nonlimiting conditions toward Sendai virus-infected cells of the opposite parental haplotype resulted in CTL able to lyse both BALB/c as well as CBA virus-infected targets (Table II, line 2; Table III, lines 2, 4, 8, 10). However, by decreasing the number of responder cells to concentrations where, on the average, less than one CTL-P was plated, a segregation of the self-restricted and allo-restricted Sendai virus-specific CTL activity was observed. An example is given in Table IV, where cells of chimera 5 (B$_T$ and C$_{BM}$) were stimulated against BALB/c-Sendai virus-infected cells and individual cultures assayed on both BALB/c-Sendai and CBA-Sendai target cells (Table IV). By plating ≤12,500 responder cells/well, we obtained only 17.4% (11/64) positive cultures. Under these conditions, CTL activity was specifically restricted to either BALB/c- or CBA-virus-infected target cells (Table IV). From these results, we postulated that chimeric mice do contain Sendai virus-specific CTL-P that are exclusively restricted to either self- or allo-MHC determinants. To further substantiate this reasoning, a segregation analysis was performed. Accordingly we set up large-scale MLC under limiting-dilution conditions (96 replicates of 0.5 × 10^4–2 × 10^4 cells/well) where spleen cells of five (CB)F$_1$ chimeras, reconstituted with a thymus and bone marrow cells of one parental haplotype were tested individually. Stimulation was done with Sendai virus-infected spleen cells of the opposite parental strain, and cytolytic activity assayed on day 6 by splitting the cultures into two parts and adding CBA- Sendai virus and BALB/c-Sendai virus-infected target cells, respectively. When the radioactivity released in the individual cultures from Sendai virus-infected BALB/c target cells was plotted against that released from Sendai virus-infected CBA target cells, double- and single-positive cultures could be identified. In Fig. 5, data from only 2 out of 5 chimeras tested are given; but essentially similar results were obtained with the other mice.

Out of the 288 wells plated, ~79% of the cultures were negative in both cases. Again, under these conditions, we noted a profound segregation of BALB/c- and CBA-restricted virus-specific CTL activity, with only 10% (mouse 8) and 13% (mouse 9) double-positive cultures, respectively (Fig. 5). These data therefore strongly support the view that clonally distinct CTL-P exist in thymus- and bone marrow-reconstituted chimeras that do respond to Sendai virus antigens in the context of self-MHC or

TABLE IV

Segregation of Self-MHC- vs. Allo-MHC-restricted Sendai Virus-specific CTL Activity Using Limiting Numbers of Chimeric Spleen Cells

Mouse	MLC			Number of positive wells detected[*]			
	Responder cells		Stimulator cells (5 × 10⁵)	Total	Double positive	Single positive on	
	Chimera type	Cell number				BALB/c-Sendai	CBA-Sendai
		× 10³					
5	(CB)F₁B_T-C_BM	50	BALB/c-Sendai	15 (93.7)[‡]	11	4	0
	(CB)F₁B_T-C_BM	25	BALB/c-Sendai	13 (81.3)	7	4	2
	(CB)F₁B_T-C_BM	≤12.5	BALB/c-Sendai	11 (17.4)	0	7	4

[*] Wells were scored positive if ⁵¹Cr-release exceeded 3 × SD of spontaneous release of target cells.
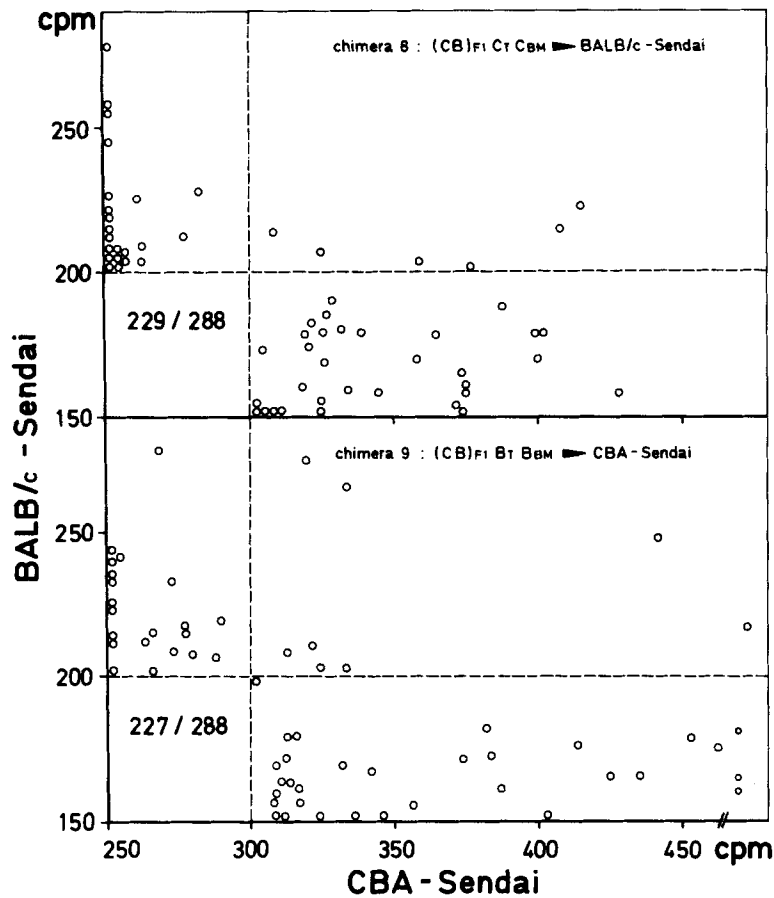[‡] Percentage positive is in parentheses.

Fig. 5. Segregation analysis reveals clonally distinct sets of self-MHC- and allo-MHC-restricted, virus-specific CTL-P. Allo-MHC-restricted, virus-specific CTL were induced in (CB)F₁C_TC_BM (mouse 8) and (CB)F₁B_TB_BM (mouse 9) chimeras and individual wells were screened for cytolytic activity on both CBA-Sendai and BALB/c-Sendai targets. Each individual culture that released radioactivity (given as cpm) >3 X the SD above spontaneous release (dotted line) of one or both of the targets as indicated (○). Data from negative cultures (mouse 8, 229/288; mouse 9, 227/288) have been omitted for simplicity.

allogeneic MHC, respectively. Therefore, we explain the induction of the self-MHC restricted, Sendai virus-specific CTL in bulk cultures of chimeric responder cells sensitized toward virus-infected allogeneic stimulator cells with antigen transfer onto cells of the responder population (Table II, line 2).

Discussion

The aim of the experiments reported here was to determine whether or not the repertoire of T cells is limited to self-MHC-restricted responses, or whether it also comprises allo-MHC-restricted responses. Experimentally, the frequency of self-MHC-restricted and allo-MHC-restricted Sendai virus-specific CTL-P was determined in lymphocytes from fully allogeneic chimeric mice. The results obtained are consistent with the view that the thymus allows maturation and export of CTL-P expressing an unlimited receptor repertoire. Dependent on antigen presentation, clonally distinct

CTL-P were shown to differentiate in vitro into either self-MHC-restricted or allo-MHC-restricted virus-specific CTL. Although the frequency analysis revealed a three- to fivefold excess of self-MHC-restricted CTL-P over allo-MHC-restricted CTL-P, this ratio was found both in H-2-compatible as well as in H-2-incompatible thymus- and bone marrow-grafted chimeric (A × B)F$_1$ mice. Because the very same ratio has also been determined in spleen cells (20) and thymocytes (25) from normal mice, we conclude that in the Sendai virus system, the thymic MHC type imposes no major constraint on the receptor repertoire of maturing CTL-P.

To bypass possible regulatory mechanisms that might influence experimental data obtained in vivo (21, 35–38), we decided to investigate CTL responsiveness not only in in vitro bulk cultures, but in addition also under single-hit conditions, where only the number of CTL-P is rate-limiting. From the results (Figs. 1 and 3) it can be inferred that all radiation chimeras tested were completely reconstituted by the bone marrow inoculum. These data in turn allowed us to conclude that not only splenocytes but also thymocytes of either MHC-compatible (mice 2–4, 7–9) or of MHC-incompatible (mice 1, 5, 6) thymus- and bone marrow-grafted chimeric (A × B)F$_1$ mice could be sensitized in bulk cultures to become virus-specific CTL (Fig. 2). Interestingly the CTL-restriction phenotype was dependent on the mode of antigen presentation. Although virus-infected syngeneic stimulator cells triggered only self-MHC-restricted, virus-specific CTL, the virus-infected allogeneic stimulator cells sensitized CTL that were able to lyse virus-infected allogeneic targets (Table II). Because lysis of syngeneic targets was also noted in the latter case (Table II), a specificity analysis of the lytic activity of CTL induced under single hit conditions became mandatory. The experimental evidence for a clear clonal segregation of self-MHC- and allo-MHC-restricted CTL (Fig. 5 and Table IV) substantiated our conclusion that (a) lymphocytes of both MHC-compatible and MHC-incompatible thymus and bone marrow-grafted (A × B)F$_1$ chimeras contained immune-reactive CTL-P, (b) that the pool of immune-reactive T cells included both self-MHC-restricted and allo-MHC-restricted CTL-P and (c) that the H-2-restriction phenotype of virus-specific CTL was dependent on the MHC type of the antigen-presenting cells. Hence the data supported the view that independent of the thymic MHC type, the maturating T cells express an unlimited receptor repertoire.

The previously reported failure (21) to observe virus-specific CTL responsiveness in allogeneic radiation chimeras could be explained by the assumption that their actual frequency is too low to be detected in vivo. Therefore we first questioned the biological significance of the in vitro data reported previously (24). However the CTL-P frequency analysis reported here evidenced not only the presence of high numbers of alloreactive CTL-P, but also high numbers of self-MHC- and of allo-MHC-restricted, virus-specific CTL-P (Table III and Fig. 4). Moreover, the frequencies of self-MHC- and allo-MHC-restricted CTL-P were comparable in both MHC-compatible and MHC-incompatible thymus- and bone marrow-grafted (A × B) chimeras. These results were taken as evidence that in quantitative terms, the thymic MHC type imposes no measurable constraint on the maturation of either self-MHC-restricted or allo-MHC-restricted virus-specific CTL-P. Furthermore, because in the radiation chimeras tested here, the frequencies of self-MHC-restricted and allo-MHC-restricted virus-specific CTL-P were comparable to those present in lymphocytes of normal mice (20, 34), the previously reported lack of in vivo CTL responsiveness (21) must be

explained either by regulatory mechanisms counteracting an effective in vivo sensitization (37) or by a relative lack of T helper cell function (35).

It is noteworthy that both in lymphocytes of normal mice (20), as well as in H-2-compatible and in H-2-incompatible thymus- and bone marrow-grafted (A × B)F_1 chimeras (Table III and Fig. 4), the frequencies of self-MHC-restricted CTL-P were found to be in a three- to fivefold excess over allo-MHC-restricted, virus-specific CTL-P. This parallels the observation that lymphocytes from athymic nu/nu mice grafted with an allogeneic thymus preferentially generate self-MHC-restricted, virus-specific CTL (39). These data do not support the view that the observed self-MHC preference is dependent on the thymic MHC-haplotype. Because both Thy-1$^+$ (40) and Thy-1$^-$, Lyt-123 (41) lymphocytes of nu/nu mice contain self-MHC-restricted, TNP-specific CTL-P, at present we are determining the frequency of self-MHC-restricted and of allo-MHC-restricted CTL-P in nu/nu lymphocytes after removal of alloreactive CTL-P. If a similar ratio as in thymus-bearing normal mice could be observed, we then could conclude that prethymic events are responsible for the preferential generation of self-MHC-restricted CTL-P.

On the basis of the results reported here, the demonstration of high numbers of allo-MHC-restricted CTL-P in lymphocytes of normal mice (20) and the presence of self-MHC-restricted CTL-P in lymphocytes from athymic (nu/nu) mice (40, 42) it is difficult to defend the previously proposed view (1, 7) that thymic MHC-products, via selection, are responsible for the generation of the T cell repertoire. We rather propose that at least part of the T cell repertoire with specificity for exogenous antigens is expressed prethymically. In analogy to the clonal abortion model for B cells (43), prethymic CTL-P expressing receptors for endogenous antigens would be clonally deleted. We view the prime role of the thymus in the expansion and maturation of precommitted prothymocytes. Because of the increased intrathymically occurring proliferation rate, the probability of mutational events is high. Consequently a thymus-dependent diversification of the prethymic receptor repertoire of both self-MHC-restricted and allo-MHC-restricted CTL-P will take place. A third step of diversification of the T cell repertoire may take place postthymically via antigen-driven T cell proliferation. This prediction can be tested experimentally by comparing the CTL-P repertoire of lymphocytes from nu/nu mice with that of thymocytes and peripheral T cells from normal mice.

Summary

To study whether the thymic major histocompatibility complex (MHC) imposes a constraint on the receptor repertoire of maturating cytotoxic T lymphocyte (CTL) precursors, the restriction phenotypes of virus-specific CTL of MHC-compatible and of MHC-incompatible thymus- and bone marrow-grafted (A × B)F_1 chimeric mice were compared. Dependent on the mode of in vitro sensitization, thymocytes or splenocytes of both types of chimeric mice generated Sendai virus-specific, self-MHC- or allo-MHC-restricted CTL. By applying the limiting-dilution technique, the CTL-precursor (CTL-P) frequencies of self-MHC-restricted and allo-MHC-restricted virus-specific T cells as well as of alloreactive T cells were determined. The data obtained revealed that independent of MHC differences between thymus and bone marrow, the frequencies of self-MHC-restricted and allo-MHC-restricted CTL-P were comparable, and in the same order of magnitude as those previously determined in

conventionally reared mice. Self-MHC-restricted, virus-specific CTL-P were in a three- to fivefold excess over allo-MHC-restricted CTL-P. A segregation analysis revealed that clonally distinct CTL-P give rise to either self-MHC-restricted or allo-MHC-restricted, virus-specific CTL. Both sets were found not only in the spleen, but also in the thymus of chimeric mice, formally demonstrating the intrathymic differentiation pathway of self-MHC as well as of allo-MHC-restricted CTL-P. These data reveal no major constraint of the thymic MHC on the capacity of T cells to recognize viral antigens either in the context of self-MHC or of allogeneic MHC products.

References

1. 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.
2. Shearer, G. M., and A. M. Schmitt-Verhulst. 1977. Major histocompatibility complex restricted cell-mediated immunity. *Adv. Immunol.* **25:**55.
3. Bevan, M. J., and J. P. Fink. 1978. The influence of thymus H-2 antigens on the specificity of maturating killer and helper cells. *Immunol. Rev.* **42:**3.
4. von Boehmer, H., C. G. Fathman, and W. Haas. 1977. H-2 gene complementation in cytotoxic T cell responses of female against male cells. *Eur. J. Immunol.* **7:**443.
5. Levy, J. P., and J. C. Leclerc. 1977. The murine sarcoma virus-induced tumor: exception of general model in tumor immunology? *Adv. Cancer Res.* **24:**2.
6. Wagner, H., K. Pfizenmaier, and M. Röllinghoff. 1980. The role of the major histocompatibility complex in murine cytotoxic T cell responses. *Adv. Cancer Res.* **31:**77.
7. 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.
8. Pfizenmaier, K., G. Trinchieri, D. Solter, and B. B. Knowles. 1978. Mapping of H-2 genes associated with T cell-mediated cytotoxic responses to SV40-tumor-associated specific antigens. *Nature (Lond.).* **274:**691.
9. Matsunaga, T., and E. Simpson. 1978. H-2 complementation in anti H-Y cytotoxic T cell responses can occur in chimeric mice. *Proc. Natl. Acad. Sci. U. S. A.* **75:**6207.
10. 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 anti-viral cytotoxic T cells. Mapping to *K* or *D* and dominance of unresponsiveness. *J. Exp. Med.* **148:**592.
11. Doherty, P. C., Biddison, W. E., J. R. Bennink, and B. B. Knowles. 1978. Cytotoxic T-cell responses in mice infected with influenza and vaccinia viruses vary in magnitude with *H-2* genotype. *J. Exp. Med.* **148:**534.
12. Kurrle, R., M. Röllinghoff, and H. Wagner. 1978. H-2-linked murine cytotoxic T cell responses specific for Sendai virus-infected cells. *Eur. J. Immunol.* **8:**910.
13. Schendel, D. J., and F. H. Bach. 1974. Genetic control of cell-mediated lympholysis in mouse. *J. Exp. Med.* **140:**1534.
14. Nabholz, M., J. Vives, H. M. Young, T. Meo, V. Miggiano, A. Rijsbeek, and D. C. Shreffler. 1974. Cell-mediated cell lysis in vitro: genetic control of killer cell production and target specificities in the mouse. *Eur. J. Immunol.* **4:**378.
15. Sprent, J. 1978. Role of H-2 gene products in the function of T helper cells from normal and chimeric mice measured in vivo. *Immunol Rev.* **42:**108.

16. Waldmann, H. 1978. The influence of the major histocompatibility complex on the function of T-helper cells in antibody formation. *Immunol. Rev.* **42**:202.

17. Katz, D., and B. Benacerraf. 1976. Genetic control of lymphocyte interactions and differentiation. *In* The Role of Products of the Histocompatibility Gene Complex in Immune Responses. D. H. Katz and B. Benacerraf, editors. Academic Press, Inc., New York. 355.

18. Yamashita, U., and E. Shevach. 1978. The histocompatibility restrictions on macrophage T-helper cell interaction determine the histocompatibility restrictions on T helper cell B cell interactions. *J. Exp. Med.* **148**:1171.

19. Benacerraf, B. 1978. A hypothesis to relate the specificity of T lymphocytes and the activity of I region specific Ir genes in macrophages and B lymphocytes. *J. Immunol.* **120**:1809.

20. Stockinger, H., K. Pfizenmaier, C. Hardt, H. Rodt, M. Röllinghoff, and H. Wagner. 1980. H-2 restriction as a consequence of intentional priming: T cells of fully allogeneic chimeric mice as well as of normal mice respond to foreign antigens in the context of H-2 determinants not encountered on thymic epithelial cells. *Proc. Natl. Acad. Sci. U. S. A.* **77**: 7390.

21. Zinkernagel, R. M., G. N. Callahan, A. Althage, S. 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.

22. Bevan, M. J. 1977. In a radiation chimera, host H-2 antigens determine immune responsiveness of donor cytotoxic cells. *Nature (Lond.).* **269**:417.

23. Doherty, P. C., and J. R. Bennink. 1979. Vaccinia-specific cytotoxic T-cell responses in the context of H-2 antigens not encountered in thymus may reflect aberrant recognition of a virus-H-2 complex. *J. Exp. Med.* **150**:1187.

24. Wagner, H., M. Röllinghoff, H. Rodt, and S. Thierfelder. 1980. T cell mediated cytotoxic immune responsiveness of chimeric mice bearing a thymus graft fully allogeneic to the graft of lymphoid stem cells. *Eur. J. Immunol.* **10**:521.

25. Stockinger, H., R. Bartlett, K. Pfizenmaier, M. Röllinghoff, and H. Wagner. 1981. H-2 restriction as a consequence of intentional priming. Frequency analysis of alloantigen-restricted, trinitrophenyl-specific cytotoxic T lymphocyte precursors within thymocytes of normal mice. *J. Exp. Med.* **153**:1629.

26. Matzinger, P., and G. Mirkwood. 1978. In a fully H-2 incompatible chimera, T cells of donor origin can respond to minor histocompatibility antigens in association with either donor or host H-2 type. *J. Exp. Med.* **148**:84.

27. Pfizenmaier, K., R. Delzeit, M. Röllinghoff, and H. Wagner. 1980. T-T cell interactions during in vitro cytotoxic T lymphocyte responses. III. Antigen-specific T helper cells release nonspecific mediator(s) able to help induction of H-2 restricted cytotoxic T lymphocyte responses across cell impermeable membranes. *Eur. J. Immunol.* **10**:577.

28. Wagner, H., C. Hardt, K. Heeg, K. Pfizenmaier, W. Solbach, R. Bartlett, H. Stockinger, and M. Röllinghoff. 1980. T-T cell interactions during cytotoxic T lymphocyte (CTL) responses: T cell derived helper factor (interleukin-2) as a probe to analyse CTL responsiveness and thymic maturation of CTL progenitors. *Immunol. Rev.* **51**:215.

29. Miller, R. G., T. S. Teh, E. Harley, and R. A. Phillips. 1977. Quantitative studies of the activation of cytotoxic lymphocyte precursor cells. *Immunol. Rev.* **35**:38.

30. Wagner, H., M. Röllinghoff, K. Pfizenmaier, C. Hardt, and G. Johnscher. 1980. T-T cell interactions during in vitro cytotoxic T lymphocyte responses. II. Helper factor from activated Lyt 1$^+$ T cell is rate limiting (i) in T cell responses to non immunogenic alloantigen, (ii) in thymocyte responses to allogeneic stimulator cells, and (iii) recruits alloreactive or H-2 restricted CTL precursors from the Lyt 123$^+$ T cell subset. *J. Immunol.* **124**:1958.

31. Cohn, M., and R. Epstein. 1978. T cell inhibition of humoral responsiveness. II. The role of restrictive recognition in immune regulation. *Cell. Immunol.* **39:**125.

32. Taswell, C., H. R. MacDonald, and J. C. Cerottini. 1979. Limiting dilution analysis of alloantigen-reactive T lymphocytes. II. Effect of cortisone and cyclo-phosphamide on cytolytic T lymphocyte precursor frequencies in the thymus. *Thymus.* **1:**119.

33. MacDonald, H. R., J. C. Cerottini, J. E. Ryser, J. L. Maryanski, C. Taswell, M. B. Widmer, and T. Brunner. 1980. Quantitation and cloning of cytolytic T lymphocytes and their precursors. *Immunol. Rev.* **51:**93.

34. Bartlett, R., C. Hardt, K. Heeg, M. Röllinghoff, and H. Wagner. Studies of cytotoxic immune responsiveness of thymocytes. II. CTL-precursor frequencies of thymocyte subpopulations. *J. Immunol.* In press.

35. Zinkernagel, R. M., G. N. Callahan, A. Althage, J. 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.

36. Zinkernagel, R. M., and A. Althage. 1979. Search for suppression of T-cells specificity for the second non-host haplotype in $F_1 \rightarrow P$ irradiation bone marrow chimeras. *J. Immunol.* **122:** 1742.

37. Smith, F. I., and J. F. A. P. Miller. 1980. Suppression of T cells specific for the nonthymic parental H-2 haplotype in thymus-grafted chimeras. *J. Exp. Med.* **151:**246.

38. Katz, D. 1980. Adaptive differentiation of lymphocytes: theoretical implications for mechanisms of cell-cell recognition and regulation of immune responses. *Adv. Immunol.* **29:**139.

39. Zinkernagel, R. M., A. Althage, E. Waterfield, B. Kindred, R. M. Welsh, G. Callahan, and P. Pincetl. 1980. Restriction specificities, alloreactivity, and allotolerance expressed by T cells from nude mice reconstituted with H-2-compatible or -incompatible thymus grafts. *J. Exp. Med.* **151:**376.

40. Hünig, T., and M. J. Bevan. 1980. Specificity of cytotoxic T cells from athymic mice. *J. Exp. Med.* **152:**688.

41. Wagner, H., C. Hardt, H. Stockinger, K. Pfizenmaier, R. Bartlett, and M. Röllinghoff. The impact of the thymus on the generation of immunocompetence and diversity of antigen-specific, MHC-restricted cytotoxic T-lymphocyte precursors. *J. Immunol.* In press.

42. Wagner, H., C. Hardt, R. Bartlett, K. Pfizenmaier, M. Röllinghoff, and K. Heeg. 1980. T-lymphocyte progenitors from thymus deficient (nu/nu) mice differentiate into H-2 restricted, hapten specific cytotoxic effector cells. *Behring Institut Mitteilungen.* **67:**105.

43. Nossal, G. J. V., and B. L. Pike. 1975. Evidence for the clonal abortion theory of B lymphocyte tolerance. *J. Exp. Med.* **141:**904.