

A NATURALLY OCCURRING  
BONE MARROW-CHIMERIC PRIMATE  
II. Environment Dictates Restriction on  
Cytolytic T Lymphocyte-Target Cell Interactions

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Cytolytic T lymphocytes (CTL)<sup>1</sup> directed against virus, hapten, minor histocompatibility, or tumor antigens demonstrate specificity for self major histocompatibility complex (MHC) antigens as well as the immunizing foreign antigen (1-5). Studies (6) using constructed murine bone marrow chimeras have demonstrated that CTL can be generated that recognize targets expressing MHC-encoded determinants that differ from those expressed by the CTL. For example, genetically homozygous H-2<sup>d</sup> CTL that have matured in an irradiated H-2<sup>d</sup>/H-2<sup>k</sup> mouse can recognize antigen in the context of H-2<sup>d</sup> or H-2<sup>k</sup>.

Similarly performed experiments have shown (7) that the helper T lymphocyte's environment imposes a restriction on its collaborative potential. T lymphocytes from parent A that mature in x-irradiated (A × B)F<sub>1</sub> mice have an expanded potential for collaboration with B cells, being capable of collaborating with parent A or parent B cells. T lymphocytes of F<sub>1</sub> origin, if they mature in a parental environment, will have a contracted potential for collaboration with B lymphocytes. They will only interact productively with B lymphocytes of that parental strain. Using radiation chimeric mice, it has been shown (8), both for the CTL and the helper T cell, that the thymus controls differentiation of self-recognition in maturing T cells. It has been suggested (9) that postthymic events may also play a role in dictating interactional restrictions on these lymphocytes.

However, a number of artifacts exist in constructed chimeric mice, which might significantly bias the results of these studies (10-13). Insufficient irradiation of such mice before they receive allogeneic bone marrow can result in allogeneic effects in vivo that obscure real biologic interactions that might otherwise occur. Similarly, allogeneic effects may be initiated in these animals by

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<sup>1</sup> *Abbreviations used in this paper:* APC, antigen-presenting cell; CM, conditioned medium; CTL, cytolytic T lymphocyte; EBV, Epstein-Barr virus; HBSS, Hanks' balanced salt solution; MHC, major histocompatibility complex; PBL, peripheral blood lymphocyte; PHA, phytohemagglutinin; TCGF, T cell growth factor; TNP, trinitrophenyl.

a radiation-resistant cell population or a newly emerging population that arises from radiation-resistant progenitors. It has also been shown (14) that the inter-actonal restrictions on the T cells of chimeric mice can change during the months following the construction of these experimental animals. In fact, the effect of the environment on T cell restrictions has been studied in detail only in these constructed murine systems.

The primate species *Saguinus oedipus*, the cotton-top tamarin, provides a unique system for studying the role of the T cell's environment in the lymphocyte's development of self-recognition. This primate has a most unusual bone marrow construction, resulting from rare developmental events. Tamarins regularly give birth to twins. While each member of a twin pair arises from a separate ovum, the pair share a circulation in utero. This leads to their sharing one another's genetically distinct bone marrow elements. Thus, cotton-top tamarins have naturally occurring chimeric bone marrow.

We have assessed the restriction on CTL-target interactions in the naturally occurring bone marrow-chimeric primate species *S. oedipus* to determine the possible role of the T cell environment in dictating that restriction. In these experiments, we have (a) shown that both the lymphocyte and monocyte/macrophage populations of these animals are chimeric, (b) developed an in vitro system for assessing the target cell restrictions on trinitrophenyl (TNP)-specific CTL clones generated from their peripheral blood lymphocytes, (c) selected chimeric animals populated by fully MHC-disparate cell populations for study, and (d) demonstrated that the environment dictates the target cell preference of the tamarin's TNP-specific CTL.

### Materials and Methods

**Animals.** *S. oedipi* were maintained in the New England Regional Primate Research Center breeding colonies. All twin pairs were colony born, enabling us to document that the animals studied were truly members of twin pairs. The animals used in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. A maximum of 2 ml of blood per week was taken from each animal.

**Peripheral Blood Lymphocyte (PBL) Isolation.** 2 ml of heparinized peripheral blood were mixed with 2 ml of Hanks' balanced salt solution (HBSS) (Gibco, Grand Island, NY) and carefully layered over 2 ml of a mixture of 9% Ficoll (Sigma Chemical Co., St. Louis, MO) and 34% sodium diatrizoate (Sterling Drug, Inc., NY) with a specific gravity of 1.0755 gm/ml. The gradient was spun at 3,000 rpm at room temperature for 20 min. The mononuclear cell layer was removed and washed twice with HBSS.

**Establishment of Genetically Homogeneous Cell Populations.** B cells from *S. oedipus* were transformed by cocultivating PBL with supernatants from the Epstein-Barr virus (EBV)-producing tamarin cell line B95-8 (provided by Dr. J. L. Strominger, Dana-Farber Cancer Institute, Boston, MA). The cells were expanded, and then cloned in soft agar. Briefly, culture media containing 0.8% Bacto agar (Difco Laboratories, Detroit, MI) was plated, and the cells, mixed into 0.4% agar, were layered above. Colonies derived from single cells were picked off within 10 d and expanded.

**Karyotype Determinations.** Karyotype determinations were carried out on three types of cell populations: lectin-stimulated peripheral blood cells, EBV-transformed B cell clones, and bone marrow myeloid precursors. All of these cell populations were derived from animals with sex-opposite siblings. The lectin-stimulated cells were prepared by adding 0.4 ml of heparinized blood to 5 ml of RPMI 1640 (Gibco) containing 20% fetal calf

serum (Sterile Systems, Inc., Logan, UT) and 5  $\mu\text{g}/\text{ml}$  phytohemagglutinin (PHA) (Wellcome Laboratories, Beckenham, Great Britain). These cells were incubated for 3 d at 37°C. EBV-transformed B cell clones were prepared as described previously (18). Myeloid precursors were prepared from bone marrow harvested from *S. oedipus* that died from natural causes. At necropsy, marrow cells were flushed from femurs of the animals under sterile conditions, purified by centrifugation over a Ficoll/diatrizoate gradient, treated with the B lymphocyte-specific monoclonal antibody anti-B1 plus complement to eliminate B lymphocytes (15), and placed in culture at  $10^6$  cells/ml in Iscove's medium, supplemented with 20% human AB serum and 10% conditioned medium (CM) from the GCT cell line (Gibco). GCT-CM contains granulocyte/monocyte colony stimulating factors and stimulates growth of granulocyte/monocyte colony forming cells from human and most nonhuman primates (16 and J. Griffin, unpublished observation). GCT-CM does not support growth of T cells or B cells. After 3 d in culture, the cells were treated with  $\text{Tris-NH}_4\text{Cl}$  to eliminate mature erythroid cells, then placed in culture for a final 24 h. The purity of this population was confirmed by preparing a Wright-Giemsa-stained spin preparation of these cells and determining the percentage of granulated cells. >95% of such cells were of myeloid origin in all experiments.

Karyotype determinations were carried out on each of these cell populations. Five drops of colchicine (Gibco) at 40  $\mu\text{g}$  in 100 ml were added to the cultures. The cells were washed 2 h later, and 5 ml of 0.075 M KCl were added for a 30-min incubation. They were then washed three times with a fixative of 3:1 methanol/acetic acid, dropped on slides, air-dried, and stained with Giemsa (Scientific Products, Boston, MA). Metaphase spreads containing exactly 46 chromosomes were examined so that only normal diploid cells were assessed. The presence or absence of a Y chromosome was noted, allowing each cell to be assigned a genetic origin.

*TNP Coupling.* The method followed is similar to that described by Shearer (17). A 10 mM solution of 2,4,6-trinitrobenzenesulfonic acid (Fisher Scientific, Fairlawn, NJ) was prepared with the pH adjusted to 7.4.  $10^7$  cells were incubated in 0.1 ml of this solution at 37°C for 10 min. The cells were then washed three times in HBSS. In some experiments, these TNP-conjugated cells were used immediately as stimulator cells; in others, they were incubated overnight in complete medium and then extensively washed before use as stimulator cells. In preparing stimulator cell populations in the latter fashion, all TNP is eliminated from the system, except for TNP tightly bound to the surface of the B cells. Cells were treated with mitomycin C if used as stimulators, or labeled with  $^{51}\text{Cr}$  when used as targets in cytotoxicity assays.

*Preparation of T Cell Growth Factor (TCGF).* TCGF was prepared as previously described (18). Human PBL at a concentration of  $2.5 \times 10^6$  cells/ml were stimulated in HBSS with PHA (5  $\mu\text{g}/\text{ml}$ ), phorbol myristate acetate (0.5 ng/ml) (Sigma Chemical Co.) and  $5 \times 10^5$  cells/ml mitomycin C-treated allogeneic cells. After 2 h, the cells were washed and resuspended in culture media. After 40 h of incubation, the supernatants were harvested. TCGF activity was quantitated by a proliferation assay according to the method of Gillis et al. (19). The TCGF was used at a concentration of 25% of final volume in these experiments.

*Limiting-dilution CTL Assays.* In vitro stimulations were performed in 96-well, round-bottom plates with a limiting number of responder cells.  $4.0 \times 10^4$  TNP-conjugated mitomycin C-treated cloned B cells were placed in each well as the stimulator population. PBL prepared as previously described were aliquoted into the wells at cell numbers varying from 50–15,000 cells/well. The wells were brought up to a 0.2 ml final volume with culture medium and 25% TCGF. Control wells consisted of stimulator cells only. At least 24 wells were set up at each responder cell concentration.

After an 8-d incubation at 37°C in a 5%  $\text{CO}_2$  humidified atmosphere, three equal aliquots of cells were removed from each well. Each aliquot was added to a well containing a different  $^{51}\text{Cr}$ -labeled target cell population. Target cells were TNP-conjugated cloned B cell populations. After a 6-h incubation, aliquots of supernatants from each well were counted in a  $\gamma$  counter (Tracor Analytic, Elk Grove Village, IL). Wells were determined to be positive when the supernatant contained counts >3 SD above background. Data are

expressed as the percentage of wells plated at each responding cell concentration that exhibit each possible pattern of lytic specificity.

## Results

*Chimeric Immune Cell Populations of S. oedipus.* The interpretation of studies of the interactional restrictions of the CTL of cotton-top tamarins are critically dependent on knowing which cell populations in the immune system of these animals are chimeric. We have chosen to study individual tamarins that are members of male-female twin pairs. The presence or absence of a Y chromosome in any cell serves as a marker to indicate the twin member in which it genetically originated. Karyotypic analysis of PHA-stimulated *S. oedipus* PBL indicated a clear-cut chimerism of the T cell populations of this species (Table I). Similarly, cloned EBV-transformed B cell lines from such individual tamarins consisted of both male and female cell populations (Table I). Since mature monocytes do not undergo mitosis, our approach to assessing the chimerism of the monocyte/macrophage population in the tamarin is based upon the observation that the granulocyte and monocyte diverge from a common progenitor cell late in ontogeny. Thus, the demonstration of chimerism in the myeloid bone marrow

TABLE I  
*Degree of Bone Marrow Chimerism of Tamarins and Establishment of Genetically Homogeneous B Cell Populations*

Animal	Sex	Peripheral blood karyotype %*		Karyotype of cloned EBV-transformed lines <sup>‡</sup>	
		XX	XY	XX	XY
76-81	Male	66	34	51-A6 3-F1	5-H5 71-D2
77-81	Female	70	30	72-C6	72-C9
33-79	Male	44	56	73-C1	73-A7
34-79	Female	18	82	74-E7	74-G5 74-H8
91-82	Male <sup>§</sup>			25-D4	
92-82	Female	50	50		116-E7 116-E3
254-79	Female	72	28	254-G1	254-D6
255-79	Male	46	54	255-F1 255-A6	
159-80	Female	54	46		
160-80	Male	38	62	160-B2	OB4-E7 OA4-B3

\* PHA-stimulated peripheral blood cells were placed in culture for 3 d, then incubated with colchicine for 4 h. The cells were treated with hypotonic KCl for 30 min and fixed in a methanol/acetic acid solution. These cells were dropped onto slides, air dried, and stained with Giemsa; 50 metaphase spreads containing 46 chromosomes were analyzed.

<sup>‡</sup> PBL were infected with EBV, then cloned and subcloned using soft agar techniques. The resulting clones were analyzed for karyotype and homogeneity by incubating with colchicine and following the above protocol to Giemsa-stain the chromosomes. At least 10 metaphase spreads containing 46 chromosomes were analyzed, and all cells were shown to be of the same sex.

<sup>§</sup> Animal 91-82 died before its karyotype was analyzed.

populations of *S. oedipus* is tantamount to documenting monocyte/macrophage chimerism. Karyotype analysis of B cell-depleted bone marrow, harvested from *S. oedipus* with sex-opposite siblings, and grown for 4 d in the presence of granulocyte/monocyte colony stimulating factors, showed that such populations included both male and female cells (data not shown). Therefore, the monocyte/macrophage populations of the cotton-top tamarin are chimeric.

*Analysis of Genetic Restriction of CTL-Target Interaction.* We then developed an in vitro system for analyzing at the clonal level the genetic restrictions on CTL-target interactions in this species. We have previously shown (18) that the EBV-transformed B cell clones, when TNP conjugated, can serve to stimulate the generation of TNP-specific CTL, and act as targets in the assay of that function. We have also established that primary TNP-specific CTL can be generated under conditions of limiting dilution (18). The CTL activity generated in one well containing a limited number of responder cells under these conditions reflects the activity of a single clone of CTL. The target cell preference of that clone might be determined by assessing the lytic function of aliquots of that CTL clone on a panel of genetically distinct target cell populations. Control experiments established the validity of such an approach. When CTL generated in a single well under these limiting-dilution conditions are assayed on two identical target populations, there is an extremely high concordance for the presence or absence of killing. That is, if an aliquot of a CTL clone kills a given target cell, a second aliquot of cells from the same clone will kill an identical target cell population (data not shown). This finding indicates that aliquots of a single clone of CTL generated in this fashion can be assayed for lytic function on a panel of genetically distinct target cell populations to determine the target cell preference of that clone.

*Functional MHC Typing of Members of Each Twin Pair.* We must know the extent of MHC compatibility between members of each twin pair to interpret experiments performed to assess the relative roles of environment and genetic program on the restrictions manifested in their CTL-target cell interactions. That is, we must know whether the members of a twin pair are MHC identical, have one haplotype in common, or are fully MHC incompatible.

The experiments shown in Tables II, III, and IV document, through indirect means, the extent of MHC compatibility in some of these tamarin twin pairs. The rationale for the approach used in these experiments is as follows. T lymphocytes, upon interaction with alloantigen and antigen-presenting cells (APC), will generate CTL with specificity for those alloantigenic determinants. The degree to which such CTL are able to kill a target cell population that originated genetically in an animal other than that from which the stimulating-cell population arose will reflect the degree of MHC sharing between the stimulating-cell population and that second cell population. Thus, an EBV-transformed B cell clone from a twin pair is used to stimulate the generation of alloantigen-specific CTL under limiting-dilution conditions. Each generated clone is then split and assayed for killing function on three target populations: the B cell clone used to stimulate the CTL generation, a clone that arose genetically in the co-twin of the animal from which the original stimulating cell arose, and a B cell clone from an unrelated tamarin. If the members of the

TABLE II  
*CTL Generated Against Self Alloantigens Do Not Kill Twin Target  
 Cells in Twin Pair 76-81 and 77-81*

Responder cells/well*	Specificity <sup>‡</sup>			
	Targets: <sup>§</sup>			
	Self	+	+	-
	Twin	-	+	+
	Allogeneic	-	-	-
500		0 <sup>¶</sup>	0	0
1,000		0	0	0
1,500		0	0	0
2,000		58	0	0
2,500		79	0	0
3,500		92	0	0
6,000		100	0	0
Total		41	0	0

\* Limiting numbers of responding PBL of a *S. oedipus* were placed in culture with  $4 \times 10^4$  mitomycin C-treated, EBV-transformed *S. oedipus* cells from a genetically homogeneous clone in the presence of TCGF for 8 d. 12-36 wells were plated at each responding cell concentration.

<sup>‡</sup> CTL activity was determined in a 6-h <sup>51</sup>Cr-release assay. Aliquots of responder cells were transferred into V-bottom wells with  $10^4$  <sup>51</sup>Cr-labeled genetically homogeneous EBV-transformed target cells. Lytic activity was scored as positive when <sup>51</sup>Cr released into a supernatant was >3 SD above the average of the activity in the supernatants of background wells. Only 6% of the wells demonstrated allogeneic target cell lysis.

<sup>§</sup> The genetic homogeneity of each target population of EBV-transformed B cells was confirmed by karyotype analysis. These targets included self (the clone from animal 254-79 used to stimulate the CTL [254-G6]), twin (a clone that originated genetically in the twin of 254-79 [254-D6]), and allogeneic (a clone from an animal unrelated to this twin pair).

<sup>¶</sup> The numerical value represents the percentage of wells, plated at that responding cell concentration, that were scored as positive.

tamarin twin pair are genetically identical, any clone that kills B cells of the stimulating cell population will also kill B cells derived from the twin of the animal from which that stimulating cell arose. If the twin members are fully MHC incompatible, an insignificant number of these alloantigen-specific clones will kill the co-twin targets. Finally, if the twin members share a single haplotype in common, approximately half of the clones that kill the stimulating cell population will kill the twin cells.

In the experiment shown in Table II, very few of these alloantigen-specific clones kill cells from the twin of the stimulating population. Reciprocal experiments using self and twin stimulating clones and the same responding PBL were simultaneously performed to demonstrate that the twin target populations were indeed lysable. Members of this twin pair thus can be assumed to be fully MHC incompatible. In the experiment shown in Table III, a large number of alloantigen-stimulated clones kill B cells from both self and twin, but not target cells of allogeneic origin. These data suggest that members of this twin pair have at least one haplotype in common. A tabulation of the results of 15 such experiments

TABLE III  
*CTL Generated Against Self Alloantigen Kill Twin Target Cells in  
 Twin Pair 91-82 and 92-82*

Responder cells/well	Specificity			
	Targets:	+	+	-
	Self	-	+	+
	Twin	-	-	-
	Allogeneic	-	-	-
1,000		6	0	3
2,000		36	17	8
3,000		22	49	0
3,500		8	83	0
5,000		8	71	3
7,500		0	100	0
Total		16	47	2

See also Table II. The targets in this experiment included self (the clone from animal 91-82 used to stimulate the CTL [116E7]), twin (a clone which originated genetically in the twin of 91-82 [25D4]), and allogeneic (a clone from an animal unrelated to this twin pair). 24-72 wells were plated at each responding cell concentration. Only 6% of the wells demonstrated allogeneic target cell lysis.

is shown in Table IV. This approach was used to select fully MHC-mismatched twin pairs for further study.

*Chimeric PBL Population Generates Self-restricted CTL after Stimulation with Hapten-conjugated Self APC.* CTL were generated under limiting-dilution conditions using TNP-conjugated EBV-transformed B cells of genetic self origin as stimulator cells. B cells from a male member of a male-female twin pair are referred to as self if they are male and twin if they are female by karyotype analysis. These CTL were assayed for target cell lysis on self, twin, and allogeneic target populations. An example of such an experiment, in which a large number of lytic clones were generated, is shown in Table V. When  $>2 \times 10^3$  responder T cells were plated per well in the stimulating culture, nonspecific killing was generated in most of those wells in this particular experiment. When fewer responder cells were plated per well, the killing seen appeared to be specific. Such specific killing from a single well generated at very low responder cell dilutions can be assumed to represent the activity of an individual CTL clone. Most clones, following stimulation with TNP-conjugated self cells, killed self targets but not twin targets. This result was seen in repeated experiments.

The responder cells of this species are made up of two genetically distinct T cell populations. Thus, if most or all of the generated clones show the same pattern of target cell preference, a preference for killing the target that is identical to the stimulating cell population, we might predict that this reflects the process of clonal selection. That is, the clonal response of A + B T lymphocytes to TNP presented on A cells should be A-restricted. The following experiments were done to determine if clonal selection explains the target cell preference seen in these experiments.

*Chimeric PBL Population Generates Self-restricted CTL after Stimulation with*

TABLE IV  
*Summary of Functional MHC Typing Data of Four S. oedipus  
 Twin Pairs*

Twin pair	Specificity			
	Targets: Self Twin Allogeneic	+	+	-
254-79 and 255-79		12	0	0
		17	1	1
		12	0	1
		8	0	0
76-81 and 77-81		41	0	0
		36	0	0
91-82 and 92-82		25	11	10
		19	11	8
		43	7	8
		10	11	4
		11	5	7
		5	1	3
159-80 and 160-80		13	11	5
		22	43	5

Tabulation of the results of 15 separate experiments, each done as described in Tables II and III. Each line of data represents the summary of one such experiment, with each number representing the percentage of wells that were scored as positive.

*Hapten-conjugated Twin APC.* If clonal selection is the only factor dictating which CTL clones will be generated, and therefore what the restriction of these clones will be under these culture conditions, TNP-coupled B cells of twin origin should select for CTL clones that kill hapten-coupled twin but not self targets. That predicted finding was not observed. Two examples of such experiments, in which a large number of lytic clones were generated, are shown in Table VI. CTL generated after stimulation with TNP-coupled B cells of twin origin did not show a preference for killing twin targets. Rather, the CTL showed an overwhelming preference for killing targets that arose genetically in the animal in which the T cells matured. Moreover, these same results were seen when reciprocal experiments were performed to document that the twin target cells were indeed lysable.

A summary of an unselected series of such experiments, using fully MHC-mismatched twin pairs, is shown in Table VII. These studies demonstrate that TNP-specific CTL show a marked preference, even when pushed in the opposite direction during their stimulation, for killing targets whose genotype is the same as that of the animal in which the T cells matured.

*Do MHC-mismatched T Cells and APC Interact In Vitro?* We might have predicted that very few CTL should be generated by the interaction in vitro

TABLE V  
Chimeric PBL Population Generates Self-restricted CTL after  
Stimulation with Hapten-conjugated Self Cells

Responder cell/well*	Specificity <sup>‡</sup>								
	Targets: <sup>§</sup>	Self-TNP		Twin-TNP		Allo-TNP			
		+	-	+	-	+	-	+	-
		-	+	+	-	-	+	+	-
		-	-	-	+	+	+	+	-
1,000		8 <sup>¶</sup>	0	0	0	0	0	0	92
1,500		29	0	0	0	0	0	0	71
2,000		50	0	0	0	0	0	0	50
Total		29	0	0	0	0	0	0	71

\* Limiting numbers of responding PBL of a *S. oedipus* were placed in culture with  $5 \times 10^4$  mitomycin C-treated, TNP-conjugated, EBV-transformed *S. oedipus* cells from a genetically homogeneous clone of self cells in the presence of TCGF for 8 d. Control wells contained stimulator cells alone.

<sup>‡</sup> CTL activity was determined in a 6-h  $^{51}\text{Cr}$ -release assay. Aliquots of responder cells were transferred into V-bottom wells with  $10^4$   $^{51}\text{Cr}$ -labeled genetically homogeneous EBV-transformed target cells. Lytic activity was scored as positive when  $^{51}\text{Cr}$  released into a supernatant was  $>3$  SD above the average of the activity in the supernatants of background wells.

<sup>§</sup> The genetic homogeneity of each target population of EBV-transformed B cells was confirmed by karyotype analysis. These TNP-conjugated clones were designated self if their sex was identical to the host animal of the responding cell population, twin if the sex was that of the twin animal, and allogeneic if the cells were from an unrelated animal.

<sup>¶</sup> The numerical value represents the percentage of wells plated at that responding cell concentration which were scored as positive.

between the twin APC and any T cell clones that might, on expansion, be capable of lysing self targets. Genetically mismatched, albeit tolerant, immune cells may be incapable of productive interactions. Furthermore, the precursor frequency in a PBL population of A lymphocytes that can lyse TNP-conjugated B cells was expected to be quite low. We may therefore have expected that a chimeric PBL population might generate self-restricted CTL after stimulation with hapten-conjugated self APC, but very little CTL activity after stimulation with hapten-conjugated twin APC. Yet this was not observed. One in vitro phenomenon that might explain the unpredicted observation that TNP-conjugated twin APC appear to stimulate self-restricted TNP-specific killing is antigen representation. TNP shed by the twin APC may be picked up and presented to self T cells by self monocytes in the responder cell population.

A number of experimental approaches were used to determine whether antigen representation occurs to a significant extent in this in vitro system. TNP was introduced into the stimulating cultures on autologous T cell lines rather than on EBV-transformed B cell clones. The presumption underlying this approach was that such T cell lines should not be capable of presenting antigen; any antigen presentation that might occur under these conditions should, of necessity, be occurring via monocytes in the responder cell population. In fact, in these experiments, a large number of self-restricted CTL clones were gener-

TABLE VI  
*Chimeric PBL Population Generates Self-restricted CTL after  
 Stimulation with Hapten-conjugated Twin Cells*

Responder cell/well	Specificity									
	Targets:									
	Self	+	-	+	+	-	-	+	-	
	Twin	-	+	+	-	+	-	+	-	
	Allogeneic	-	-	-	+	+	+	+	-	
Exp. 1:										
2,500		38	8	4	0	0	0	0	50	
3,000		29	16	13	0	0	0	0	42	
3,500		34	8	12	0	0	2	0	45	
Total		34	9	9	0	0	0	0	46	
Exp. 2:										
1,500		6	0	0	2	0	3	0	91	
2,000		13	0	0	0	0	0	0	87	
2,500		22	0	0	0	0	0	0	78	
3,500		27	0	0	0	0	0	0	73	
5,000		71	0		0	0	0	0	29	
Total		19	0	0	0	0	1	0	79	

See legend for Table V. The hapten-conjugated stimulator cell population was of twin genetic origin.

ated, but at responder cell concentrations markedly lower than that seen when TNP was introduced into the system on B cell clones (data not shown). The new variables introduced into the system using this approach, as well as the recent demonstration (20) that at least activated T cells may be capable of presenting antigen to T cells in vitro, made these experiments difficult to interpret.

Attempts were also made to deplete the responding population of cells capable of presenting antigen by nylon wool passage of those cells followed by treatment with anti-Ia plus complement. *S. oedipus* PBL treated in such a fashion remained 2% Ia<sup>+</sup> by cell staining and analysis by fluorescence-activated cell sorter, and maintained approximately one-third of their capacity to proliferate after an Ia-dependent antigen stimulation (data not shown). Thus, we were incapable of fully depleting the responder cell populations of Ia<sup>+</sup> cells. Nevertheless, such Ia<sup>+</sup> cell-depleted responder populations did not generate TNP-specific CTL after stimulation with hapten-conjugated twin B cell clones (data not shown).

Experiments of the type shown in Table VIII provide evidence that antigen re-presentation may be occurring to some extent in these cultures. Previous studies (21) have shown that considerable free TNP is introduced into cell culture systems when hapten-conjugated cells are added into such cultures immediately following haptentation. Much less free TNP is introduced into these systems if the hapten-conjugated cells are maintained in culture overnight and washed extensively prior to their addition to cell cultures. Therefore, the generation of TNP-specific CTL was assessed after the stimulation of *S. oedipus* PBL with TNP-conjugated self or twin B cell clones introduced into the cultures

TABLE VII  
*Genetic Restriction of CTL Clones Generated from Chimeric PBL  
 Populations after In Vitro Stimulation with TNP-conjugated  
 Self or Twin Cells*

Animal	Stimulating population	Lytic specificities		
		Self	Twin	Both
76-81	Self	18	4	1
		3	0	0
		37	0	0
	Twin	8	6	0
		2	1	0
		19	0	10
77-81	Self	8	4	6
		17	3	6
		6	3	0
		15	4	3
	Twin	7	2	0
		6	2	0
254-79	Self	11	1	0
	Twin	10	5	3
255-79	Twin	8	0	0
		3	0	0

Tabulation of the results of 15 separate experiments, each done as described in Tables V and VI. Every line of data represents the summary of one such experiment. Previous studies have shown that the members of the twin pairs used for these experiments are fully MHC mismatched. The numerical values represent the percentage of wells in the entire experiment that were scored as positive. These results indicate that CTL generated under these in vitro conditions, whether stimulated with hapten-conjugated self or twin cells, show a preference for lysing self targets.

after an overnight incubation and extensive washing. TNP-conjugated twin APC prepared without such an incubation and washing stimulated self-restricted CTL, as had been previously observed. The same responding cell population, when stimulated with self APC after incubation and washing, generated self-restricted, antigen-specific CTL, but at a level quantitatively less than that observed after stimulation under the usual conditions. However, very little CTL activity was generated when the stimulation was done with hapten-conjugated twin APC. While experiments of this type do not rule out the possibility that MHC-mismatched immune cells are interacting in this system, or that the maturation of the T lymphocytes in this chimeric environment alters the expected precursor frequencies of MHC-restricted antigen-specific CTL clones, these findings suggest that at least a portion of the self-restricted CTL activity generated in this system after education with TNP-conjugated twin APC may result from antigen reprocessing by self APC in the responder cell population.

TABLE VIII  
*Chimeric PBL Population Generates CTL after Stimulation with Hapten-conjugated Self But Not Twin Cells if APC Are Incubated Overnight before Use*

Responder cell/well	Specificity								
	Targets:								
	Self	+	-	+	+	-	-	+	-
	Twin	-	+	+	-	+	-	+	-
Allogeneic	-	-	-	+	+	+	+	-	
A. TNP-coupled twin stimulating cells prepared using routine conditions:									
1,500	25	4	2	0	0	0	0	0	69
2,500	30	5	10	0	0	0	0	0	51
3,500	42	11	0	0	2	0	0	0	45
5,000	61	3	25	0	0	0	0	3	11
Total	37	6	8	0	1	0	1	1	47
B. TNP-coupled twin stimulating cells used after overnight incubation:									
2,500	0	6	0	0	0	0	0	0	94
3,000	2	0	0	0	0	0	0	0	98
3,500	4	1	0	0	0	0	0	0	95
5,000	0	0	0	0	0	0	0	0	100
Total	1	1	0	0	0	0	0	0	98
C. TNP-coupled self stimulating cells used after overnight incubation:									
2,500	0	8	0	0	8	0	0	0	83
3,000	6	0	8	0	0	0	0	0	86
3,500	21	0	0	0	0	0	0	0	79
5,000	27	0	9	0	14	5	0	0	45
Total	16	0	4	0	3	0	0	0	76

### Discussion

These experiments have been carried out in naturally occurring allogeneic chimeric primates. Some earlier studies (8, 22, 23) suggested that CTL are not generated in A → B allogeneic chimeric mice, even when lymphocytes from such animals are acutely transferred into irradiated F<sub>1</sub> recipients to provide appropriate accessory cells for those lymphocytes. Such CTL may not have been seen by those investigators because of interference generated by an allogeneic effect in the interaction of the chimeric T cells with the unique F<sub>1</sub> determinant expressed on cells of the F<sub>1</sub> host (24). Other reports (25–28) have, in fact, demonstrated the immunocompetence of T cells from allogeneic murine chimeras. Our previous studies (18) of the immune system of *S. oedipus* clearly indicate the functional competence of the lymphocytes from this bone marrow chimeric species.

No consensus among various investigators is readily apparent as to the nature of the restrictions on CTL-target cell interactions when CTL have been generated in allogeneic radiation chimeric mice. For example, Matzinger and Mirkwood (27), using fully allogeneic chimeric mice, showed that CTL generated against minor histocompatibility antigens show a preference but not an absolute

restriction for targets bearing the MHC of the host. Lattime et al. (28), using  $(A \times B)F_1 \rightarrow (A \times C)F_1$  semiallogeneic radiation chimeric mice, found that tolerance to MHC antigens of the APC was sufficient to allow a cytotoxic antihapten response. Such mice could generate CTL to TNP-modified A, B, or C, but not to nontolerated TNP-coupled allogeneic cells. Kruisbeek et al. (24) assessed the CTL generated by splenocytes of allogeneic chimeric mice following in vitro sensitization with TNP-modified stimulator cells of either host or donor haplotype. They found that TNP-specific CTL could only be generated from such a cell population when stimulated by TNP-modified cells of the host haplotype.

The present studies have been done in a unique, naturally occurring  $A + B \rightarrow A$  primate species, where A and B have been carefully shown to be fully MHC disparate. It is critical that the experiments to assess the role of the environment on T lymphocytes be performed using cells from members of tamarin twin pairs that are fully MHC disparate. If two unrelated tamarins share even one MHC haplotype in common, one would expect to see the generation of a significant number of CTL clones from PBL of one animal capable of killing target cells from the other. Since the degree of conservation of membrane antigens on bone marrow-derived cells of various primate species appears to be correlated to the phylogenetic distance between any two such species (29–32), one would expect the sharing of near-identical structures between man and *S. oedipus* to be quite limited. Thus, one could not expect the serologic reagents developed for human transplantation tissue typing to prove useful for determining the MHC relationship between members of tamarin twin pairs. We have therefore used an indirect, functional approach for assessing this relationship. This particular approach for MHC typing is valid if one can be certain that the responding PBL population and the stimulating B cell clone are derived from tamarins that share no MHC-encoded determinants in common. Such sharing is unlikely in view of the fact that the parents of the twin pairs in these studies were all wild-caught rather than inbred. Furthermore, MHC typing experiments on each twin pair have been repeated using responding PBL from a number of different unrelated tamarins. This approach has proven to be quite reproducible.

We have found that tolerance to foreign MHC determinants in this  $A + B \rightarrow A$  primate species is not, of itself, sufficient to facilitate the generation of CTL specific for target cells expressing those MHC determinants. Rather, a marked preference for the expansion of CTL clones with a restriction for target cells bearing the host animals' MHC determinants was seen. TNP-specific CTL generated from PBL of *S. oedipus*, even when pushed in the opposite direction during their stimulation, showed a marked preference for killing target cells whose genotype was the same as that of the animal in which the T cells existed. This is the first demonstration of the phenomenon of an environment dictating interactional restrictions on CTL in a naturally occurring bone marrow chimera. It is also the first demonstration of the profound influence of environment on the repertoire of the T lymphocyte in a primate species.

Longo and Schwartz (14) showed, in constructed chimeric mice, a correlation between the genotype of bone marrow-derived APC present in the thymus and the restriction of proliferating T cells which emerge from that thymus. These findings suggested that cells of bone marrow rather than thymic origin dictate

the MHC restriction of developing T lymphocytes. In the  $A + B \rightarrow A$  chimeric *S. oedipus*, a species that we have shown to be populated by both A- and B-type cells of myelomonocytic origin, CTL show a consistent preference for killing A target cells. These findings differ from those of Longo and Schwartz (14), but are consistent with those of Zinkernagel (33), who also was unable to demonstrate a role for bone marrow-derived cellular elements in determining CTL-target restrictions.

Thus, if the target cell restriction of CTL in the tamarin reflects the influence of an education process during the T cell's maturation, these data suggest that hematopoietically derived cells do not effect the repertoire of the T lymphocytes. Rather, nonhematopoietic cells, perhaps thymic epithelial cells, must be dictating the MHC restriction of the T lymphocytes. This interpretation of these experiments is valid if one assumes that the majority of T lymphocytes are formed after birth. If, however, most T cells are formed before birth, these experiments suggest that the restriction on T lymphocytes may result, at least in part, from an ongoing regulatory process in the tamarin. Because *S. oedipus* twin pairs share an anastomosing placental circulation through the complete period of gestation, we expect that T cells that have matured in the thymus of one tamarin during gestation will be present at birth in the other animal in substantial numbers. If thymic education were the sole determinant of the restrictions on T cells in the tamarin, two T cell populations should exist in each animal: one population restricted to killing self targets, and another restricted to killing twin targets. Yet a sizeable CTL population capable of killing twin targets could not be detected in these animals. This finding therefore argues, given the assumption that most T cells are formed before birth, for the existence in these chimeric tamarins of a postthymic ongoing regulatory process that suppresses this twin-restricted CTL population.

While this set of experiments clearly indicates the role of the environment of the T cell in dictating eventual restrictions on the interactions of the mature effector T lymphocyte, the system used in these studies does not allow us to characterize with any precision the detailed cellular interactions leading to these apparent restrictions. For example, we cannot determine with certainty how CTL are generated from a PBL population after stimulation by twin APC. The experiment shown in Table VIII suggests that antigen reprocessing by self APC in the responder population may play an important role in the generation of such CTL. The number of monocytes in the wells of the responding cell populations that might potentially present antigen, however, is extremely small when compared with the number of hapten-conjugated B cells present in these cultures. This fact alone argues against the notion that antigen representation by self APC is fully accounting for the generation of these CTL. Indeed, Nagy et al. (34) have shown that, in the presence of interleukin 2, MHC-mismatched T cells and APC can interact productively. Furthermore, the precursor frequency of pre-CTL that will expand and mature after an interaction with an APC expressing a given foreign MHC determinant may be significantly higher in a bone marrow-chimeric animal than in one with a single genetic bone marrow population. Any number of these mechanisms may be contributing to the generation of CTL after in vitro stimulation with hapten-coupled twin APC.

We are currently expanding antigen-specific, MHC-restricted CTL clones derived from PBL of these *S. oedipus* in order, among other things, to perform karyotype analyses of these populations. The data provided by these studies will allow us to determine the extent to which cells of twin genetic origin play a role in antigen-specific CTL responses in these chimeric animals.

The existence of this restriction in CTL–target cell interactions may have enormous implications for immune surveillance in *S. oedipus*. Virus-infected cells of twin genetic origin may not be killed by CTL in this unusual species. Such infected cells may therefore escape from normal host immune surveillance. These circumstances could explain how viral infections of some cells might go unchecked by the usual mechanisms of defense against infections in this species, perhaps accounting for the extraordinary incidence of chronic colitis seen in *S. oedipus* (35), as well as the striking evolution of lymphoproliferative disorders that occur in these animals after infection with a number of herpesviruses, including EBV (36, 37). Abnormal immune surveillance may also account for the large number of deaths from adenocarcinoma of the colon in this primate species (35).

### Summary

Restrictions on cytolytic T lymphocyte (CTL)–target cell interactions are studied in the primate *S. oedipus*, a naturally occurring A + B → A bone marrow–chimeric species. We show that the T cell, B cell, and myelomonocytic progenitor cell populations are chimeric in this species. We selected animals for study that are populated by fully major histocompatibility complex (MHC)-disparate hematopoietic cell populations, using a functional assay system. We then developed an in vitro system for analyzing at the clonal level the genetic restrictions on the trinitrophenyl-specific CTL–target cell interactions of this species. In this system, we have shown that tolerance to foreign MHC determinants was not, of itself, sufficient to facilitate the generation of CTL specific for target cells expressing those foreign MHC determinants. Rather, a marked preference for the expansion of CTL clones with a restriction for target cells bearing the host animals' MHC determinants was seen. Hematopoietically derived cells did not affect the repertoire of these T lymphocytes.

These studies represent the first demonstration of the phenomenon of an environment dictating interactional restrictions on CTL in a naturally occurring bone marrow–chimeric animal. This is also the first demonstration of the profound influence of the environment on the repertoire of the T lymphocyte in a primate species.

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