

# TOLERANCE TO HISTOCOMPATIBILITY DETERMINANTS IN TETRAPARENTAL BONE MARROW CHIMERAS

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Chimeric mice have been produced by various procedures such as neonatal injection of histoincompatible hemopoietic cells (1), fusion of eight-cell-stage embryos (2, 3), or X irradiation and reconstitution with allogeneic bone marrow cells (4, 5). Unresponsiveness to histocompatibility determinants has been assessed by measuring various parameters, among them skin allograft survival, graft-vs.-host (GVH)<sup>1</sup> reactivity, responsiveness in the mixed leukocyte reaction (MLR), and capacity to destroy appropriate target cells. The results obtained are not easy to interpret: some authors reported the specific absence of GVH-reactive cells in neonatally induced chimeras (6-8) and in contrast others demonstrated specific cytotoxic cells in neonatally induced chimeras (9, 10), bone marrow chimeras (11), and tetraparental or allophenic mice produced by fusion of embryos (12). On the basis of these findings some authors maintain that in chimeras tolerance is due to the absence or reactive cell clones whereas others postulate that tolerance is a manifestation of an active immune response leading to the production of blocking serum factors (9-11) or suppressor cells (13). It is difficult to reconcile the different results or interpretations, partly because of the different test systems used and also because the degree of lymphoid cell chimerism is missing in some reports (10, 12).

In most studies on the induction of tolerance in lymphoid cell chimeras little attention has been given to the presence of immunocompetent T cells, either of host or donor origin. Thus, T cells of host origin would tend to reject the graft while donor T cells would induce a GVH reaction and thereby interfere nonspecifically with the immunocompetence of the host. To avoid this complication we have attempted to study tolerance in a situation where host and donor T cells are virtually absent during the induction of chimerism. For this purpose lethally X-irradiated F<sub>1</sub> hybrid mice were repopulated with equal proportions of T-cell-depleted bone marrow cells from both parental strains. This procedure allows stem cell differentiation in the absence of mature functional T cells in a histoincompatible environment, a situation possibly resembling that of physiological stem cell differentiation where during the generation of immunocompetent cells self-reactive cells arise and become tolerant. Lymphocyte reactivity in such mice for which we use the term "tetraparental bone marrow chimeras" (TBM) was studied in MLR as well as in T-cell-mediated lympholysis (CML).

<sup>1</sup> *Abbreviations used in this paper:* CML, cell-mediated lympholysis; GVH, graft-vs.-host; MLR, mixed leukocyte reaction; TBM, tetraparental bone marrow chimeras; TDL, thoracic duct lymphocytes.

## Material and Methods

### MICE

CBA/J ( $H-2^k$ ,  $Mls^d$ ) (CBA), DBA/2 ( $H-2^k$ ,  $Mls^d/H-2^k$ ,  $Mls^d$ ) (DBA),  $F_1$  (CBA  $\times$  DBA), C57BL ( $H-2^b$ ) (C57), and  $F_1$  (DBA  $\times$  C57) mice were used and kept under conventional conditions.

### CELLS

Thymus, lymph node, spleen, and bone marrow cells were obtained as described previously (14). Thoracic duct cannulation was carried out as described by Sprent (15).

### BONE MARROW CHIMERAS

$F_1$  (CBA  $\times$  DBA) mice of either sex were kept for 1 day without food and then X-irradiated with 900 R. Immediately after irradiation the mice received an intravenous injection of a mixture of  $7.5 \times 10^4$  CBA and  $7.5 \times 10^6$  DBA viable bone marrow cells. T cells were removed from the marrow cells before injection by pretreatment with AKR anti- $\theta$ C3H serum and guinea pig complement (C) as described elsewhere (16). Damaged cells were removed from the suspension by the method of von Boehmer and Shortman (17). After injection the mice were kept in a conventional mouse breeding room. 100 mg/liter Neomycin (Médial, Geneva, Switzerland) and 10 mg/liter Polymyxin B (Nova, Copenhagen, Denmark) were added to the drinking water for a period of 2 mo. The mice were checked for signs of sickness, e.g. hunched back, ruffling of fur, and loss of weight.

### ESTIMATION OF LYMPHOID CELL CHIMERISM

*Antisera.* Anti-CBA and anti-DBA sera were produced by injecting DBA or CBA mice at weekly intervals with  $5 \times 10^6$  CBA or DBA spleen cells intraperitoneally for 4 wk. The animals were bled at the end of the 5th week, and the serum was collected, and stored frozen.

*Cytotoxic Assay.* Lymph node cells freed of damaged cells (17) or thoracic duct lymphocytes (TDL) were suspended at a concentration of  $5 \times 10^6$  cells/ml in a balanced salt solution containing 10% fetal calf serum (18). 100  $\mu$ l of cell suspension were incubated with 100  $\mu$ l of diluted antiserum for 30 min at 4°C. The cells were washed twice and then incubated with 100  $\mu$ l 1:4 diluted guinea pig C for 30 min at 37°C. The proportion of viable cells was estimated after adding eosin to the suspension without further washing of the cells.

### MIXED LEUKOCYTE REACTION

$3 \times 10^6$  thymus cells or  $1 \times 10^6$  lymph node cells were cultured with  $5 \times 10^5$  mitomycin-C-treated (19) stimulator cells in 1 ml tissue culture medium as described in detail elsewhere (19). After 72 h 2  $\mu$ Ci [ $^3$ H]thymidine (The Radiochemical Center, Amersham, England; sp act 5 Ci/mmol) in 0.5 ml medium was added. Cells were collected after 96 h and DNA-incorporated [ $^3$ H]thymidine was determined as reported (20).

### CML

*Generation of Cytotoxic Lymphocytes.* The in vitro system used for generation of cytotoxic lymphocytes has been described by Nabholz et al. Briefly: 10 or  $20 \times 10^6$  lymph node or spleen cells (responders) were cultured from 5 days with  $8 \times 10^6$  mitomycin-C-treated (19) stimulator cells in 4.5 ml culture medium in a 30-ml plastic tissue culture flask (Falcon no. 3012, Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) in an humidified atmosphere of 5%  $\text{CO}_2$  in air at 37°C. On day 5 the cultured cells were collected, centrifuged, and resuspended to a volume corresponding to a predetermined concentration of responder cells as counted on day 0. Two three-fold serial dilutions were made of this suspension.

*Target Cells.*  $15 \times 10^6$  viable spleen cells in 3 ml medium were cultured in 30-ml Falcon flasks and stimulated with a 1:100 final dilution of PHA-M (Difco 0528-56, Difco Laboratories, Detroit, Mich.) for 72 h. The cells were then spun down, resuspended to 0.2 ml, and 500  $\mu$ Ci  $^{51}\text{Cr}$ , sp act 200-

400 mCi/mg, (Eidg. Inst. f. Reaktorforschung, Würenlingen, Switzerland) was added for 1.5 h at 37°C. After incubation the cells were washed twice. A suspension enriched for blast cells and devoid of damaged cells was obtained by centrifugation on a Ficoll (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) Urovision (Schering-Kahlbaum, Berlin, West Germany) step gradient, (density 1.077).

**CML Assay.** The killing assay was performed in round-bottomed microtiter plates (Greiner, Nürtingen, West Germany, no. M220-24AR). Varying numbers of cytotoxic cells were incubated with  $2 \times 10^4$  target cells in a total vol of 200  $\mu$ l. The plates were incubated for 4 h at 37°C. After incubation they were centrifuged at 1,000 *g* for 15 min. 100  $\mu$ l of the supernate containing the released  $^{51}\text{Cr}$  was collected and counted in a well-type gamma scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Total releasable  $^{51}\text{Cr}$  was determined by adding 100  $\mu$ l of Zaponin (Coulter Electronics, Inc., Fine Particle Group, Hialeah, Fla.) diluted 1:10 to each well and incubating the plate at 37°C over-night. For each well the specific release was determined as % specific release =  $100 \times [\% \text{ experimental release } (AB_M/C_T) - \% \text{ mean spontaneous release } (C_T)] / [100 - \% \text{ mean spontaneous release } (C_T)]$  where A is the responder,  $B_M$  the stimulator, and  $C_T$  the target. For each group of three replicas the mean specific release and standard deviation was computed. Since the standard deviation in all experiments was small (2-3%) only the mean value of specific release is given in the figures.

#### IN VIVO ASSAY FOR PROLIFERATION OF LYMPHOCYTES

The assay system used was that described by Sprent and Miller (22). Mice were irradiated with 800 R and injected with varying numbers of TDL. DNA synthesis in spleen was determined 4 days later by injecting 25  $\mu\text{Ci}$  [ $^3\text{H}$ ]thymidine (sp act 25 Ci/mmol) intravenously. After 1 h the spleens were removed and solubilized in 1 ml Soluene (Packard Instrument Co, Inc.). A 1:100 dilution of the solution was counted in a Packard tricarb scintillation counter. Four mice were injected for each cell dose tested. The results are expressed as arithmetic mean with standard deviation.

## Results

**Condition of TBM.** 10  $F_1$  (CBA  $\times$  DBA) hybrids 8 wk of age were X irradiated with 900 R and injected with T-cell-depleted bone marrow cells from both parental strains. All mice survived in good health after irradiation; and they were killed 2-7 mo after irradiation. After initial loss of weight the animals regained the weight of normal  $F_1$  hybrids of corresponding age. At autopsy no signs of GVH reaction were found, e.g. enlarged spleen, lymph node, or liver. Normal cell numbers were obtained from spleen ( $\sim 10^8$  viable cells) and thymus ( $7 \times 10^7$  viable cells). TDL outputs measured in two mice were close to normal levels, i.e.,  $6-8 \times 10^7$  cells over a 14-h collection period (15).

**Degree of Lymphoid Cell Chimerism.** 2-7 mo after irradiation the lymphoid cell chimerism in lymph node cells and TDL was checked in a cytotoxic assay using appropriate anti *H-2* sera. As shown in Table I, both anti-CBA and anti-DBA sera were highly specific and killed up to 99% of  $F_1$  (CBA  $\times$  DBA) lymph node cells. With lymphoid cells from the chimera, however, neither anti-CBA serum nor anti-DBA serum alone produced more than 71% lysis of lymph node cells or TDL. By contrast, incubation with both antisera simultaneously produced up to 100% lysis. Of the five chimeras tested the anti-CBA serum killed on the average 62% of the cells and the anti-DBA serum 43%. Summation of these values gives a figure of 105% (range 101-110%). It is clear that the vast majority of cells in the chimeras were of donor origin. It is to be noted that the proportions of cells derived from each of the two parental populations were quite

TABLE I  
Chimerism in Tetraparental Bone Marrow Chimeras

Cells	Incubation with:			
	Anti-CBA + C'	Anti-DBA/2 + C'	Saline + C'	Anti-CBA anti-DBA + C'
	%	%	%	%
CBA lymph node	94	14	19	95
DBA lymph node	15	99	10	99
F <sub>1</sub> (CBA × DBA) lymph node	99	98	11	—
Chimera I (CBA + DBA) lymph node	71	30	13	99
Chimera II (CBA + DBA) lymph node	67	35	10	100
Chimera III (CBA + DBA) lymph node	51	58	19	100
Chimera IV (CBA + DBA) lymph node	60	50	22	98
Chimera V (CBA + DBA) TDL	63	40	13	—

Incubation of cells with the alloantisera alone did not increase the percentage of dead cells above values observed after incubation with saline and complement (C'). TDL from chimera V contained 80% of lymphocytes killed by anti- $\theta$  serum plus C'.

similar, irrespective of the time after irradiation at which the mice were tested. Chimerism was thus comparatively stable and predictable.

**MLR.** The capacity of cells from the chimeras to respond in a "one-way" MLR was tested using extensively (six times) washed thymus (Table II) and lymph node cells (Table III). When these cell populations were cultured in the absence of stimulator cells [<sup>3</sup>H]thymidine uptake was low and no higher than that obtained with normal F<sub>1</sub> (CBA × DBA) cells. There was thus no evidence that the chimeric cells were being mutually stimulated *in vivo*. Similarly, [<sup>3</sup>H]thymidine incorporation was low when the chimeric cells (unlike normal parental cells) were cultured with CBA, DBA, or F<sub>1</sub> (CBA × DBA) spleen cells as stimulators. By contrast, a significant response was observed against "third-party" (C57) stimulator cells. Similar results were obtained with normal F<sub>1</sub> (CBA × DBA) responder cells. It is therefore evident that the cells from the chimeras behaved essentially as cells from "genetically tolerant" F<sub>1</sub> (CBA × DBA) mice.

Results similar to the above were obtained when the reactivity of the chimeric cells was tested in a one-way MLR *in vivo*, i.e. by intravenous transfer to heavily irradiated mice (see Materials and Methods). It is evident from Table IV that transfer of even high doses of chimeric cells failed to give more than minimum levels of [<sup>3</sup>H]thymidine incorporation in F<sub>1</sub> (CBA × DBA) mice. This finding made it unlikely that the unresponsiveness observed *in vitro* reflected the presence of specific blocking factors.

The possibility that blocking factors were synthesized by suppressor cells after transfer, however, was not excluded. To study this point, chimeric lymph node

TABLE II  
*Specific Unresponsiveness of Chimeric Thymus Cells to Host-Type MLR Determinants*

Responder cells	Stimulator cells				
	F <sub>1</sub> (CBA × DBA) spleen	C57 spleen	CBA spleen	DBA spleen	No stimulator cells
Chimera (CBA + DBA) thymus	2,380 ± 111	9,750 ± 1,403	2,278 ± 589	2,432 ± 132	2,252 ± 551
F <sub>1</sub> (CBA × DBA) thymus	3,822 ± 442	22,751 ± 1,806	3,838 ± 110	3,635 ± 427	3,418 ± 167
CBA thymus	17,048 ± 1,502	15,608 ± 3,086	3,079 ± 683	26,663 ± 1,739	2,654 ± 819

TABLE III  
*Specific Unresponsiveness of Chimeric Lymph Node Cells to Host-Type MLR Determinants*

Responder cells	Stimulator cells				
	F <sub>1</sub> (CBA × DBA) spleen	C57 spleen	CBA spleen	DBA spleen	No stimulator cells
Chimera (CBA + DBA) lymph node	11,654 ± 1,399	40,899 ± 1,205	12,737 ± 1,233	11,603 ± 1,893	12,630 ± 1,435
F <sub>1</sub> (CBA × DBA) lymph node	11,709 ± 494	64,084 ± 2,760	11,826 ± 2,262	12,058 ± 1,329	11,070 ± 1,467
CBA lymph node	77,810 ± 5,186	78,777 ± 4,209	12,728 ± 671	95,236 ± 4,869	13,470 ± 1,356

cells were cultured in vitro with normal parental cells to determine whether the proliferative response of the latter would be impaired, (neither population was treated with mitomycin C). It is evident from Table V that normal CBA lymph node cells responded as well when cultured with the chimeric cells as with normal F<sub>1</sub> (CBA × DBA) cells. It may be pointed out that in addition to providing evidence against the presence of specific blocking factors or suppressor cells this experiment also demonstrates that the capacity of the chimeric cells to stimulate other cell populations was unimpaired. Similar findings were reported by Meo et al. (23) with embryo fusion chimeras.

*CML.* To study reactivity of chimeric spleen cells in CML the cells were prepared and tested immediately, i.e. without in vitro stimulation, for their capacity to lyse F<sub>1</sub> (CBA × DBA) targets. As shown in Fig. 1, even high numbers of chimeric spleen cells failed to produce any significant lysis. By contrast, both CBA and DBA spleen cells when cultured in vitro for 5 days with F<sub>1</sub> (CBA ×

TABLE IV  
Specific Unresponsiveness of Chimeric TDL Injected into X-Irradiated  
 $F_1$  (CBA  $\times$  DBA) Hosts

Injected cells	X-irradiated recipient	
	$F_1$ (CBA $\times$ DBA)	$F_1$ (CBA $\times$ C57)
—	2,886 $\pm$ 581	2,991 $\pm$ 762
$5 \times 10^5$ chimera (CBA + DBA) TDL	2,790 $\pm$ 545	10,938 $\pm$ 1,548
$2.5 \times 10^6$ chimera (CBA + DBA) TDL	3,390 $\pm$ 604	—
$5 \times 10^5$ CBA TDL	7,959 $\pm$ 362	8,501 $\pm$ 795
$5 \times 10^5$ DBA TDL	15,041 $\pm$ 2,184	8,427 $\pm$ 308

TABLE V  
Stimulatory Capacity of Chimeric Lymphocytes for Normal Parental Lymph Node Cells

Strain + organ	Cell no. per culture	Calculated background	Observed [ $^3\text{H}$ ]thymidine uptake
Chimera (CBA + DBA) lymph node	$1 \times 10^6$	—	12,630 $\pm$ 1,435
CBA lymph node	$1 \times 10^6$	—	13,470 $\pm$ 1,356
Chimera lymph node + CBA lymph node 1:1	$1 \times 10^6$	13,050	75,253 $\pm$ 7,028
$F_1$ (CBA $\times$ DBA) lymph node	$1 \times 10^6$	—	11,070 $\pm$ 1,467
$F_1$ lymph node + CBA lymph node 1:1	$1 \times 10^6$	12,270	62,243 $\pm$ 822

In these experiments stimulating cells were not treated with mitomycin C.

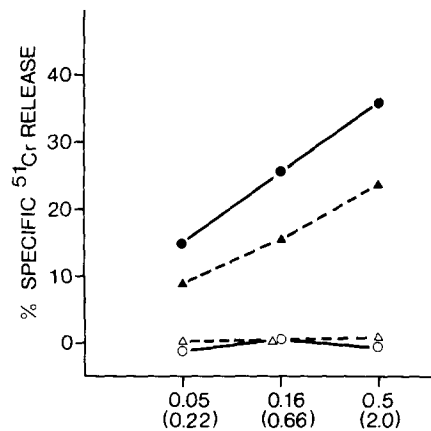


FIG. 1. Killing of  $F_1$  (CBA  $\times$  DBA) targets. (●—●), DBA spleen cells activated in vitro by  $F_1$  (CBA  $\times$  DBA) mitomycin-C-treated stimulators. (▲---▲), CBA spleen cells activated in vitro by  $F_1$  (CBA  $\times$  DBA). (○—○), spleen cells from chimera I. (Δ---Δ), spleen cells from chimera II. To each well are added an unknown number of killer cells which are the descendants of the known number ( $\times 10^6$ ) of responders indicated on the abscissa (see also Materials and Methods section). In parenthesis are given the numbers ( $\times 10^6$ ) of unstimulated chimera cells per well.

TABLE VI  
*Test System for Generation of Cytotoxic Lymphocytes*

Responder cells	Stimulator cells	Target cells
Chimera (CBA + DBA)	F <sub>1</sub> (CBA × C57)	CBA C57
Chimera (CBA + DBA)	F <sub>1</sub> (DBA × C57)	DBA C57

DBA) stimulators lysed the target cells effectively. No specific lysis of F<sub>1</sub> targets was found with unstimulated CBA or DBA responding cells.

To study whether the chimeras contained the precursors of cytotoxic lymphocytes, 10<sup>7</sup> chimeric lymph node cells or 10<sup>7</sup> CBA or DBA lymph node cells were cultured with either F<sub>1</sub> (CBA × C57) or F<sub>1</sub> (DBA × C57) stimulators for 5 days. The lytic activity of these cells on various <sup>51</sup>Cr-labeled target cells was then tested as shown in Table VI. The chimeric lymph node cells stimulated by F<sub>1</sub> (DBA × C57) or F<sub>1</sub> (CBA × C57) lymphocytes were able to lyse C57 targets (Fig. 2 a) but neither CBA nor DBA targets (Fig. 2 b and c). On the other hand, DBA cells stimulated by F<sub>1</sub> (CBA × C57) killed CBA targets while CBA cells stimulated by F<sub>1</sub> (DBA × C57) lysed DBA targets (Fig. 2 b and c). Thus, even during a 5-day culture period in the presence of appropriate stimulator cells the chimeric lymph node cells apparently failed to generate cytotoxic T lymphocytes against *H-2* antigens present in the chimera despite the fact that in the same culture a good cytotoxic response was generated against third-party C57 antigens. The use of F<sub>1</sub> stimulator cells (DBA × C57) or (CBA × C57) ruled out the possibility that cytotoxic cells were not produced because of the absence of a significant MLR (24).

*Failure to Demonstrate Specific Suppressor Cells.* In the above experiments it was conceivable that the unresponsiveness observed in CML reflected the

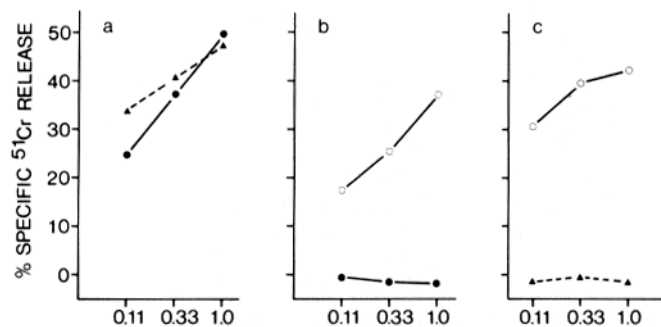


FIG. 2. (a) Killing of C57 targets by chimeric spleen cells activated in vitro by F<sub>1</sub> (DBA × C57) (▲---▲), or by F<sub>1</sub> (CBA × C57) (●---●). (b) Killing of CBA targets by DBA spleen cells (○---○), or chimeric spleen cells (●---●), activated in vitro by F<sub>1</sub> (CBA × C57) cells. (c) Killing of DBA targets by CBA spleen cells (○---○), or chimeric spleen cells (▲---▲), activated in vitro by F<sub>1</sub> (DBA × C57) cells. Numbers on abscissa indicate the number (×10<sup>6</sup>) of responders per well (see Fig. 1).

presence of specific suppressor cells which prevent activation of killer precursor cells. To test this possibility, chimeric cells were mixed with normal CBA or DBA spleen cells to see whether they were capable of specifically suppressing the generation of cytotoxic lymphocytes from precursors. In control cultures  $F_1$  (CBA  $\times$  DBA) spleen cells were cultured at the same ratio with both parental strain lymphocytes. The results illustrated in Fig. 3 *a* and *b* show that chimeric cells cultured at a 1:1 ratio with CBA or DBA cells did not suppress the response of the latter against DBA or CBA, respectively. As before no killing of CBA or DBA targets was observed when chimeric cells alone were cultured with the corresponding stimulator cells of the parental strains (Fig. 3 *a* and *b*)

Fig. 4 *a* shows that CBA cells plus chimeric lymphocytes stimulated with  $F_1$

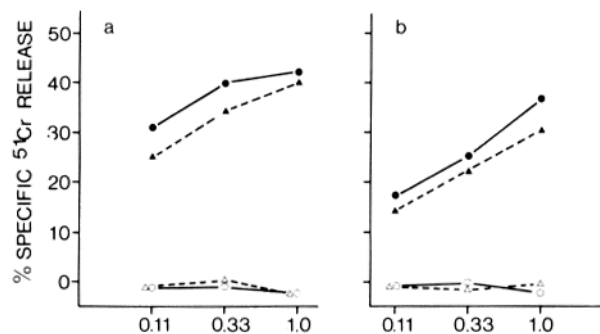


FIG. 3. (a) Killing of CBA targets by DBA spleen cells cultured at a 1:1 ratio with chimeric spleen cells (●—●) or  $F_1$  (CBA  $\times$  DBA) spleen cells (▲---▲) and stimulated by  $F_1$  (CBA  $\times$  C57) cells. (O—O), chimeric cells alone and, (Δ---Δ),  $F_1$  (CBA  $\times$  DBA) cells alone, both stimulated by  $F_1$  (CBA  $\times$  C57). (b) Killing of DBA targets by CBA spleen cells cultured at a 1:1 ratio with chimeric spleen cells (●—●) or with  $F_1$  (CBA  $\times$  DBA) spleen cells (▲---▲) and stimulated by  $F_1$  (DBA  $\times$  C57) cells. (O—O), chimeric spleen cells alone; and (Δ---Δ),  $F_1$  (CBA  $\times$  DBA) spleen cells alone, both stimulated by  $F_1$  (DBA  $\times$  C57) cells. Numbers on abscissa indicate responders ( $\times 10^6$ ) per well (see Fig. 1).

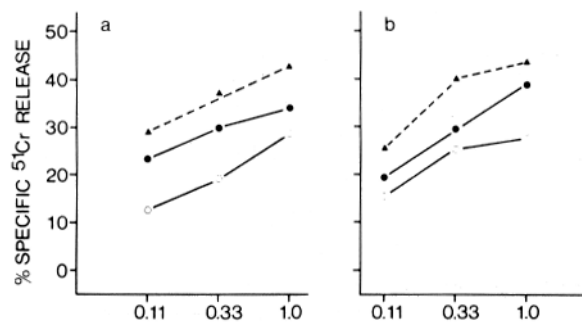


FIG. 4. (a) Killing of C57 targets by CBA spleen cells cultured at a 1:1 ratio with  $F_1$  (CBA  $\times$  DBA) spleen cells (▲---▲), or chimeric spleen cells (●—●), and stimulated by  $F_1$  (DBA  $\times$  C57) cells. (O—O), chimeric cells alone cultured with  $F_1$  (CBA  $\times$  C57) stimulating cells. (b) Killing of C57 targets by DBA spleen cells cultured at a 1:1 ratio with  $F_1$  (CBA  $\times$  DBA) (▲---▲), or chimeric spleen cells (●—●), and stimulated by  $F_1$  (CBA  $\times$  C57) cells. (O—O), chimeric cells alone cultured with  $F_1$  (CBA  $\times$  C57) stimulating cells. Numbers on abscissa indicate responders ( $\times 10^6$ ) per well (see Fig. 1).



(CBA  $\times$  C57) cells produced less lysis on C57 targets than CBA plus F<sub>1</sub> (CBA  $\times$  DBA) responders. The same results were found when DBA cells were mixed with chimeric cells or F<sub>1</sub> (CBA  $\times$  DBA) cells and stimulated with F<sub>1</sub> (DBA  $\times$  C57) (Fig. 4 b). We have no explanation for this finding but it seems possible that F<sub>1</sub> spleen cells produce relatively more cytotoxic lymphocytes against the C57 antigens than the chimeric cells. This was in fact observed in some experiments not shown here. Thus, whereas combinations of cells containing normal parental strain cells plus chimeric cells gave a lower response to third-party C57 antigens they responded to either CBA or DBA antigens as strongly as parental responding cells plus F<sub>1</sub> (CBA  $\times$  DBA) hybrid cells. These experiments clearly contradict the

TABLE VII  
*Effect of Serum on Lympholysis*

Responder cells	No. ( $\times 10^6$ )	Stimulator cells	Serum*	Dilution	Targets		
					CBA	DBA	C57
CBA	1.0	C57 $\times$ DBA	—		15 $\pm$ 2	56 $\pm$ 5	38 $\pm$ 1
	0.5		—		17 $\pm$ 2	59 $\pm$ 1	40 $\pm$ 3
	0.25		—		12 $\pm$ 0	23 $\pm$ 3	21 $\pm$ 2
	1.0	CBA $\times$ DBA	Chim	$\frac{1}{6}$	7 $\pm$ 1	14 $\pm$ 3	12 $\pm$ 2
	1.0		—	$\frac{1}{12}$	11 $\pm$ 1	50 $\pm$ 4	36 $\pm$ 1
	1.0		NMS	$\frac{1}{6}$	9 $\pm$ 2	15 $\pm$ 2	13 $\pm$ 1
	1.0		—	$\frac{1}{12}$	12 $\pm$ 0	60 $\pm$ 2	18 $\pm$ 1
DBA	1.0	C57 $\times$ CBA	—		33 $\pm$ 4	15 $\pm$ 0	30 $\pm$ 4
	0.5		—		30 $\pm$ 4	15 $\pm$ 0	26 $\pm$ 2
	0.25		—		13 $\pm$ 1	13 $\pm$ 1	16 $\pm$ 1
	1.0	DBA $\times$ CBA	Chim	$\frac{1}{6}$	17 $\pm$ 6	10 $\pm$ 1	13 $\pm$ 2
	1.0		—	$\frac{1}{12}$	49 $\pm$ 5	13 $\pm$ 0	34 $\pm$ 3
	1.0		NMS	$\frac{1}{6}$	27 $\pm$ 3	10 $\pm$ 0	19 $\pm$ 1
	1.0		—	$\frac{1}{12}$	43 $\pm$ 1	12 $\pm$ 0	27 $\pm$ 0
C57	1.0	CBA $\times$ DBA	—		58 $\pm$ 3	52 $\pm$ 1	18 $\pm$ 1
	0.5		—		62 $\pm$ 3	48 $\pm$ 3	18 $\pm$ 1
	0.25		—		41 $\pm$ 1	48 $\pm$ 7	18 $\pm$ 1
	1.0	CBA $\times$ DBA	Chim	$\frac{1}{6}$	36 $\pm$ 2	39 $\pm$ 4	12 $\pm$ 1
	1.0		—	$\frac{1}{12}$	59 $\pm$ 2	49 $\pm$ 3	15 $\pm$ 1
	1.0		NMS	$\frac{1}{6}$	41 $\pm$ 3	31 $\pm$ 4	11 $\pm$ 1
	1.0		—	$\frac{1}{12}$	49 $\pm$ 5	42 $\pm$ 4	12 $\pm$ 2
Spontaneous release					16 $\pm$ 1	23 $\pm$ 1	24 $\pm$ 1

Equal numbers of responders and irradiated stimulator cells from normal mice were cultured in 120  $\mu$ l medium containing, where indicated, mouse serum at various concentrations in wells of Falcon 3040 microculture plates. On day 3, 50  $\mu$ l of medium was added to each culture. On day 5, 50  $\mu$ l of PHA-stimulated target cells ( $10^4$ ) were added to each well and cytolytic activity determined as described in the Material and Methods. Results are given as  $^{51}\text{Cr}$  released as % of the total uptake determined by Zaponin cytotoxicity.

\* Sera: Chim, pooled serum from four (CBA + DBA) TBM. NMS, normal serum from F<sub>1</sub> (CBA  $\times$  DBA) mice. Sera were not heat inactivated.

hypothesis that chimeric cells have an antigen-specific suppressive effect on normal parental strain cells.

*Failure to Demonstrate Specific Serum Blocking Factors in CML.* The possibility that serum blocking factors were involved in maintaining a state of tolerance was investigated by testing the ability of serum from the chimera to suppress the generation of cytotoxic cells. Table VII demonstrates that at high concentration both normal mouse serum as well as the chimera serum blocked the production and/or action of killer cells but that this effect was nonspecific.

## Discussion

The present studies indicated that when equal proportions of T-cell-depleted bone marrow cells from two allogeneic parental strains were transferred to heavily irradiated  $F_1$  mice, the lymphoid organs became repopulated with cells derived equally from the two marrow populations; chimerism was stable and lasted at least 7 mo after irradiation. The possibility that the lymphoid cells in the chimeras were derived not from the two parental strains but from the host is excluded for two reasons: First, the antiparental strain alloantisera used to test chimerism lysed close to 100% of normal  $F_1$  cells but only a proportion of cells in the chimeras; incubation with both alloantisera, however, killed virtually all chimeric cells. Second, lymphoid cells obtained when the irradiated  $F_1$  mice were injected with marrow cells from only one of the parental strains were all lysed by alloantiserum directed against this strain but were entirely resistant to anti-serum against the other parent; the cells tested in this situation must thus have been entirely of donor and not host origin (J. Sprent and H. von Boehmer, unpublished data).

Thymus cells and peripheral lymphocytes from the chimeras showed specific unresponsiveness in MLR towards the host determinants. Since the mouse strains studied differed not only for the major histocompatibility complex but also for the M locus unresponsiveness to both types of determinants was apparently involved. The mechanism governing tolerance in this situation has yet to be elucidated. In contrast to the findings of Phillips and Wegmann (13) but in agreement with those of Meo et al. (23), in embryo fusion chimeras we could find no evidence that unresponsiveness resulted from specific blocking serum factors or suppressor cells. Thus, although suppressor cells might conceivably have been involved in the induction of tolerance they did not seem to be involved in maintaining the tolerant state. The results suggest rather that unresponsiveness resulted either from deletion or permanent inactivation of newly arising lymphocytes reactive to host MLR determinants. Possibly this was generated by a process of exhaustive proliferation of the responding cells (25).

No evidence could be found that the chimeras had either cytotoxic lymphocytes or precursors of cytotoxic lymphocytes reactive against the host *H-2* antigens; the unresponsiveness observed could not be accounted for by blocking factors present in serum produced by suppressor cells in culture. Thus, as with the MLR studies, these data suggest that tolerance to *H-2* antigens involved deletion or irreversible inactivation of the reactive cell clones rather than

reversible suppression by blocking factors. The CML data is not in accordance with the studies of Hellström and Hellström (11) on non-TBM and by Wegmann et al. (12) on embryo fusion chimeras. These workers reported that unresponsiveness observed in their respective chimeras and in their cytotoxic assay stemmed at least in part from the presence of specific blocking factors. It is difficult to account for the discrepancy between these findings and our own. It is not known whether the cytotoxic assay used by the authors reflects recognition of *H-2* antigens by T cells and T-cell killing. Furthermore, in the case of the studies of Hellström (11) it is conceivable that the results obtained were related to the failure of these workers to ensure that T cells were removed from the marrow cells used to prepare the chimeras; thus, T cells contaminating the marrow cells might have influenced the subsequent pathway of tolerisation. A strict comparison of the present studies with those of Wegmann et al. (12) is difficult because the latter did not provide precise data on the degree of lymphoid cell chimerism in the mice studied. On this point, however, it is perhaps worth stressing that although the present findings were obtained with mice in which chimerism was evenly balanced between the two parental strains it is not known whether this is a critical factor.

TBM chimeras are of potential value for studying a number of basic immunological problems. For example, they are particularly suitable for investigating the role of *H-2* determinants in T-B collaboration across allogeneic barriers and I-region-controlled responsiveness to defined antigens. Such studies are currently in progress. The ease with which TBM chimeras can be prepared, their predictable and stable chimerism, and apparent lack of blocking factors, all make these mice perhaps a more convenient tool for studies of this nature than embryo fusion chimeras.

### Summary

Tetraparental bone marrow chimeras were produced by injecting lethally X-irradiated  $F_1$  hybrids with relatively high numbers of T-cell-depleted bone marrow cells from both allogeneic parental strains. The mice survived in excellent health and showed a stable, approximately 50:50 (parent:parent), lymphoid cell chimerism lasting for at least 7 mo after irradiation; regeneration of host-type hemopoietic cells was very limited.

Thymus, lymph node, and thoracic duct lymphocytes showed specific unresponsiveness to host mixed leukocyte reaction (MLR) determinants. Similarly specific tolerance to *H-2* antigens of host type was demonstrated in spleen and lymph node. No suppressor cells could be demonstrated in either system and blocking serum factors could not be found. The results suggest specific deletion of functional T cells reactive to host-type MLR and cell-mediated lympholysis determinants.

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