Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 3045–3049, May 1983
Immunology

Functional clonal deletion of cytotoxic T-lymphocyte precursors in chimeric thymus produced *in vitro* from embryonic *Anlagen*

(immunologic tolerance/third pharyngeal pouch/fetal liver/H-2 antigens)

MICHAEL F. GOOD*, KENNETH W. PYKE†, AND G. J. V. NOSSAL*

*The Walter and Eliza Hall Institute of Medical Research and †The Ludwig Institute for Cancer Research, Post Office Royal Melbourne Hospital, Victoria 3050, Australia

Contributed by G. J. V. Nossal, February 16, 1983

ABSTRACT Chimeric thymus, formed by fusing the prelymphoid third pharyngeal pouches of fetal mice with fetal liver, have been allowed to develop entirely *in vitro*. Syngeneic and allogeneic chimeras were prepared and both types of thymus were shown to contain substantial numbers of functional cytotoxic T lymphocyte precursors reactive against "third party" alloantigens. However, alloreactivity specific for H-2 antigens present on either the third pharyngeal pouch or the fetal liver was minimal. In three different allogeneic chimeric thymuses, the frequencies of cytotoxic T lymphocyte precursors reactive to H-2 antigens present on the third pharyngeal pouches were reduced to 1%, 4%, and 0% of control values, whereas, in the one allogeneic chimera tested for alloreactivity to H-2 antigens present on the fetal liver, the cytotoxic T lymphocyte precursor frequency was reduced to less than 1% of control values. The phenotype of the H-2 tolerance is shown to be one of functional clonal deletion of the cytotoxic T lymphocyte precursor.

The classical experiments of Billingham, Brent, and Medawar provided evidence that immunologic tolerance was a somatically acquired characteristic of lymphocyte populations (1). Since the demonstration that tetraparental mice show tolerance to all four parental H-2 haplotypes (2), the idea that T-cell populations become tolerant of "self" H-2 antigens during ontogeny has become widely accepted, but the inductive mechanisms concerned remain controversial. The two dominant and not mutually exclusive mechanisms that have been proposed are: (*i*) the development of suppressor T cells preventing self-reactivity (3–7); and (*ii*) a purging of self-reactive cells from the T-cell repertoire, through either destruction or functional inactivation of self-reactive cells (1, 8–11). Studies in our laboratory have recently revealed evidence in favor of functional clonal deletion of cytotoxic T lymphocyte precursors (CTL-P) as one mechanism operative in a model of tolerance dependent on the injection of semi-allogeneic spleen cells into newborn mice (12). This study had the advantage that the frequencies of functional CTL-P were quantitated by limiting-dilution cloning techniques. However, its disadvantages are that the newborn mouse already possesses readily detectable numbers of functional T cells in its thymus, and thus the sudden introduction of foreign cells does not mimic the physiologic situation in which, presumably, each potential self-reactive T cell must be dealt with in some way as it arises, thus ensuring that self-tolerance is acquired coincidentally with functional maturation of the immune system.

The pioneering studies of Le Douarin and colleagues (13, 14) have suggested a different approach to this problem. These techniques, as further modified in our laboratory (15–17), allow the development of a thymus entirely *in vitro*, beginning with embryonic rudiments. The third pharyngeal pouch (TPP), which includes the *Anlage* of the thymic epithelial-reticular framework, is removed at 10 days' gestation. At this early stage, it is entirely devoid of infiltrating hemocytoblasts. When it is fused *in vitro* with 11-day fetal liver (FL) and then placed into organ culture for 3 weeks, a functional thymus with typical cortico-medullary demarcation develops and can be shown to have Thy-1-positive lymphocytes and Ia-positive cells of FL origin. Accordingly, we have constructed such chimeric thymuses by using both syngeneic and allogeneic FL–TPP combinations.

In this paper, we show that syngeneic and allogeneic preparations both contain substantial numbers of functional CTL-P reactive against irrelevant ("third party") H-2 antigens. Moreover, a specific state of tolerance develops to the H-2 antigen of both FL and TPP. The phenotype is one of functional clonal deletion of CTL-P.

MATERIALS AND METHODS

Mice. Fetuses from the following strains of mice were used: BALB/c AnBradley (H-2^d); CBA/CaH (H-2^k); and C57BL/6J (H-2^b).

Preparation of Tissues and Organ Fusion. This has been described (16). Briefly, the TPP were obtained from 10-day fetuses by sterile microdissection. Three or four TPP were fused together by overnight incubation (37°C, 10% CO_2/90% humidified air) on the surface of a gel composed of 1% agar and 10% fetal calf serum in Dulbecco's modified Eagle medium. They were then transferred to 45-μm Millipore filters floating on rafts of Gelfoam (Upjohn) in modified Eagle medium supplemented with 15% fetal calf serum. These organ cultures were accumulated over a period of 2 weeks; then, all were colonized by placement of one lobe of 11-day FL beside or on top of each TPP in culture. Medium was changed at 3- to 4-day intervals for 3 weeks. Then the cultures were harvested by trypsinization to obtain a single-cell suspension. Average yield was 10^5 lymphocytes per organ.

Limit-Dilution Microcultures. The method has been described (11, 12). Limiting numbers of responder cells were cultured (37°C, 7 days, 10% CO_2/90% humidified air) with 4×10^5 x-irradiated "stimulator" spleen cells in 0.2 ml of 10 mM Hepes-buffered Eagle minimal essential medium supplemented with 10% fetal calf serum, 50 μM 2-mercaptoethanol, and 10% concanavalin A spleen cell-conditioned medium (see ref. 11) in 96-well, V-bottomed Linbro microtiter trays (no. 76-023-05, Flow Laboratories, McLean, VA). Each tray included

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: TPP, third pharyngeal pouch; FL, fetal liver; CTL, cytotoxic T lymphocyte; F_0, fraction of nonresponding cultures; CTL-P, cytotoxic T lymphocyte precursor.

two rows (24 control wells) of microcultures that lacked responder cells.

Analysis of Lytic Potential. Each culture was individually assayed for its capacity to lyse 5×10^3 ^{111}In oxine-labeled P815 (*H-2^d*), C1.18 (*H-2^k*), or El-4 (*H-2^b*) target cells over a 4-hr incubation (37°C, 10% CO_2) according to the radioautographic method of Shortman and Wilson (18). Positive wells were then determined (19).

CTL-P Frequency Estimation. Best-fit lines of the number of responder cells per well (*x* axis) vs. natural logarithm of the fraction of nonresponding cultures (ln F_0; *y* axis) have been plotted by using the maximal likelihood estimator (19). The precursor frequency can be shown to be equivalent to the slope of this line.

RESULTS

Various *in vitro* chimeric thymuses of the type A→B, in which A refers to the strain of the FL and B refers to the strain of the TPP, were constructed. Cells from these thymuses were then tested for their ability to respond to different allogeneic stimulator cells *in vitro* and so generate cytotoxic effector cells. Fig. 1 shows the case in which cells from three different types of chimeric thymus were challenged with CBA (*H-2^k*) stimulator cells and assayed against C1.18 (*H-2^k*) target cells. Whereas cells from both C57→C57 and C57→BALB/c (*H-2^b*→*H-2^d*) were able to generate a significant and essentially equivalent response to CBA stimulation (CTL-P frequencies, 62.7×10^{-6} and 93.7×10^{-6}, respectively), cells from C57→CBA chimeric thymus were virtually unable to respond to CBA stimulation (CTL-P frequency, 0.7×10^{-6}). To check that this low response after CBA stimulation was truly the result of immunologic tolerance and not merely from failed chimerization, cells from the same pools (C57→C57, C57→BALB/c, and C57→CBA) were challenged *in vitro* with BALB/c stimulation and assayed against P815 (*H-2^d*) targets. Cells from the C57→CBA chimeras could mount a good response against BALB/c alloantigens, as could cells from C57→C57 chimeras

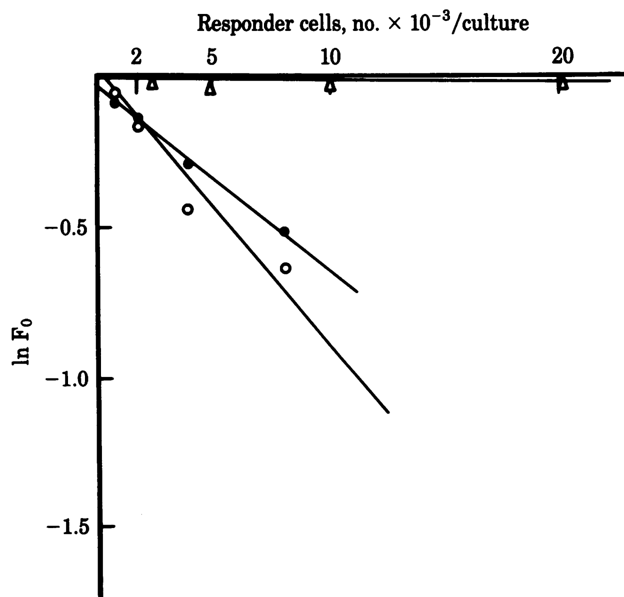

FIG. 1. Limit-dilution analysis of C57→C57 (●), C57→BALB/c (○), and C57→CBA (△) chimeric cells responding to CBA stimulation. CTL-P frequencies were: C57→C57, 62.7×10^{-6}; C57→BALB/c, 93.7×10^{-6}; and C57→CBA, 0.7×10^{-6}. Cells from the same pools were used for the experiment represented by Fig. 2. Each point represents 72 microcultures.

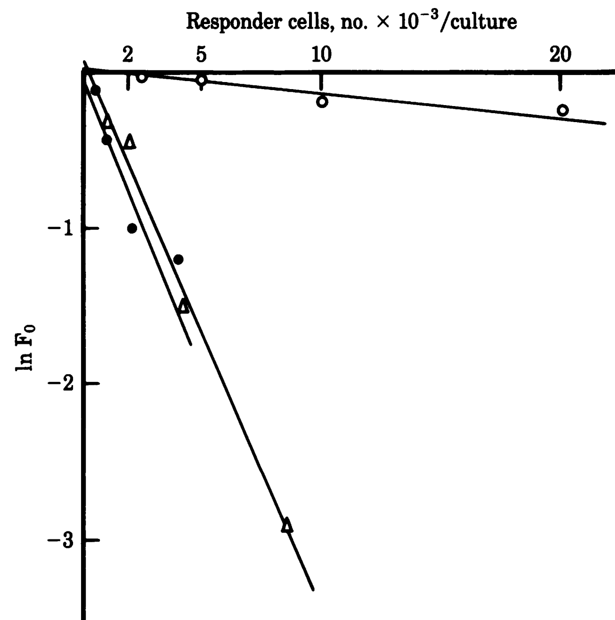

FIG. 2. Limit-dilution analysis of C57→C57 (●), C57→BALB/c (○), and C57→CBA (△) chimeric cells responding to BALB/c stimulation. CTL-P frequencies were: C57→C57, 391×10^{-6}; C57→BALB/c, 16.7×10^{-6}; and C57→CBA, 350×10^{-6}. For this experiment, cells from the same pools were used for the experiment represented by Fig. 1. Each point represents 72 microcultures.

(CTL-P frequencies, 350×10^{-6} and 391×10^{-6}, respectively) (Fig. 2). Furthermore, cells from C57→BALB/c chimeras could mount only a poor response to BALB/c alloantigens (CTL-P frequency, 16.7×10^{-6}) compared to cells from C57→C57 and C57→CBA chimeras, even though cells from the same pool were functional against CBA alloantigens (Fig. 1).

Figs. 1 and 2 also demonstrate that, when cells from the chimeras show tolerance, the best-fit lines exhibit zero-order kinetics [i.e., straight lines passing through a point near the origin (0, 0)]. This can be shown to indicate that only one cell type (the CTL-P) is limiting the response (19, 20). Consequently, suppressor mechanisms can be excluded as having contributed to this tolerance, unless the frequency of suppressors is so high that they were not limiting even with the lowest cell number used.

We were concerned at the overall discrepancy in CTL-P frequencies between H-2^d and H-2^k stimulation. That is, the frequency of H-2^d-reactive CTL-P in the C57→C57 chimera was 391×10^{-6}, whereas the frequency of H-2^k reactive CTL-P in the C57→C57 chimera was only 62.7×10^{-6}. To test that this was not a chimeric artifact, thymus cells from 2-week-old C57 mice were tested for their H-2^d and H-2^k responses. CTL-P reactive against H-2^d alloantigens were also more frequent than those reactive against H-2^k alloantigens (frequencies, 189×10^{-6} and 70.8×10^{-6}, respectively) (Fig. 3). Possibly, P815 functions better as a target than C1.18 does.

We have also examined BALB/c→C57 chimeras. Fig. 4 shows the results of an experiment in which cells from BALB/c→C57 and BALB/c→BALB/c chimeras were challenged *in vitro* with C57 stimulation and assayed on El-4 targets. Cells from the BALB/c→C57 chimeras were totally unable to mount a response against C57 alloantigens (CTL-P frequency, 0), whereas cells from BALB/c→BALB/c chimeras were able to mount a response against C57 alloantigens (CTL-P frequency, 27.5×10^{-6}). Again, to show that this unresponsiveness reflected true immunologic tolerance and not failed chimerization, cells from the same pool were assessed for their ability to

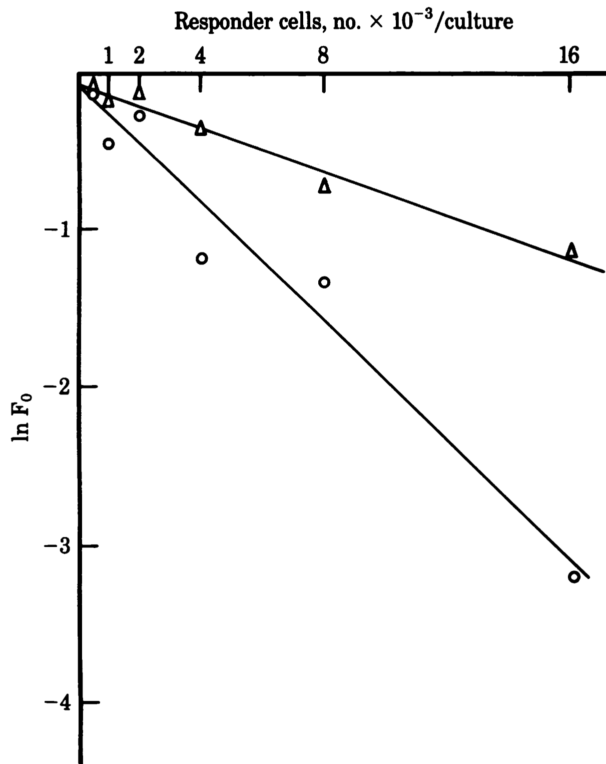

FIG. 3. Limit-dilution analysis of 2-week-old C57 thymus cells responding to BALB/c stimulation (○) and to CBA stimulation (△). CTL-P frequencies were 189×10^{-6} and 71×10^{-6}, respectively.

generate a response against H-2^k stimulation, as assayed on C1.18 targets. Cells from the same BALB/c → C57 chimeras were able to mount a good response to CBA, in fact higher than that of cells from syngeneic BALB/c → BALB/c chimeras (CTL-P frequencies, 232×10^{-6} and 77.3×10^{-6}) (Fig. 5). Again, tolerance had the phenotype of clonal deletion, with no evi-

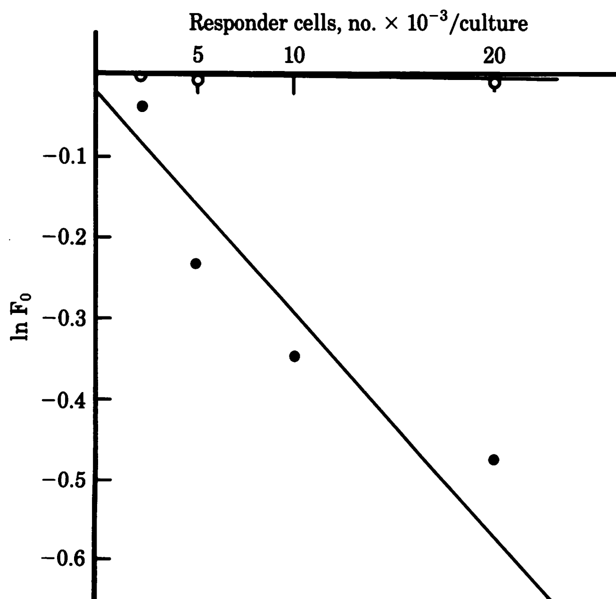

FIG. 4. Limit-dilution analysis of BALB/c → BALB/c (●) and BALB/c → C57 (○) chimeric cells responding to C57 stimulation. CTL-P frequencies were: BALB/c → BALB/c, 27.5×10^{-6}; BALB/c → C57, 0.0×10^{-6}. Cells from the same pools were used for the experiment represented by Fig. 5. Each point represents 24 microcultures for the BALB/c → BALB/c line and 12 for the BALB/c → C57 line.

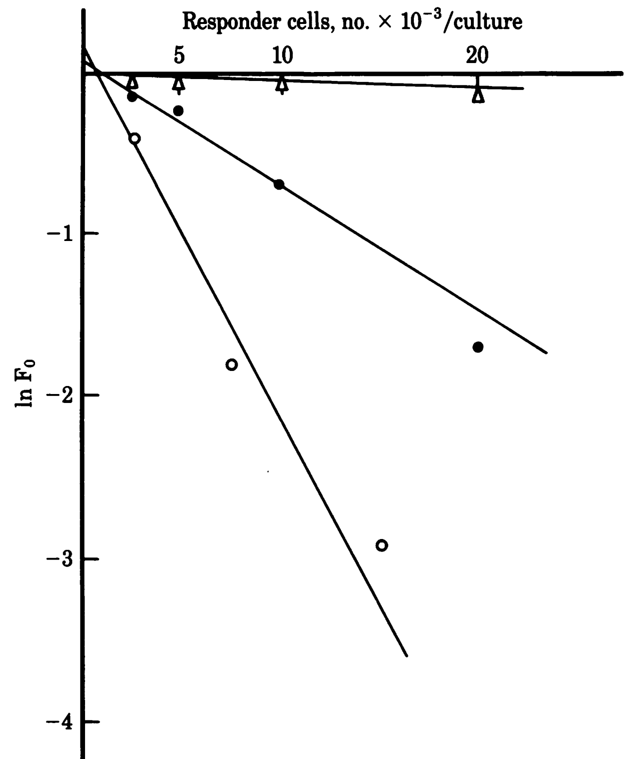

FIG. 5. Limit-dilution analysis of BALB/c → BALB/c (●), BALB/c → C57 (○), and BALB/c → CBA (△) chimeric cells responding to CBA stimulation. CTL-P frequencies were: BALB/c → BALB/c, 77.3×10^{-6}; BALB/c → C57, 232×10^{-6}; BALB/c → CBA, 6.2×10^{-6}. Each point represents 24 microcultures.

dence of suppression. Fig. 5 also shows that cells from BALB/c → CBA chimeric thymus could not mount a significant anti-CBA response (CTL-P frequency, 6.2×10^{-6}).

Fig. 6 represents a different experiment showing that, although cells from BALB/c → BALB/c chimeras can mount a strong response against CBA alloantigens (CTL-P frequency, 118×10^{-6}), cells from the same chimera and cells from a BALB/c → CBA chimera are unable to mount a response against BALB/c alloantigens (CTL-P frequencies, 1.2×10^{-6} and 0.9×10^{-6}, respectively). Taken alone, the tolerance to BALB/c antigens demonstrated by the BALB/c → BALB/c chimera could mean (from the previous results) that it was the TPP (of BALB/c origin) that was creating this tolerance. However, cells from BALB/c → CBA chimeras also were unable to mount a response against BALB/c alloantigens. This shows that tolerance is also engendered to the genotypic antigens of the developing lymphocytes themselves.

DISCUSSION

In this study, we created a situation in which prethymic stem cells can mature into T lymphocytes *in vitro* within either a syngeneic or an allogeneic epithelioreticular framework. This involved allowing hemocytoblasts from 11-day fetal liver to invade and colonize early hemocytoblast-free thymic *Anlagen*, in a culture system that allows the development of a functional thymus. Previous work has shown that the T cells and Ia-positive cells in allogeneic combinations all come from the FL source (16).

The first point of interest is that allogeneic as well as syngeneic fusions permitted the development of CTL-P. This strongly suggests that, whatever factors are involved in promoting extensive cell division and differentiation within the thymus, they are not major histocompatibility complex re-

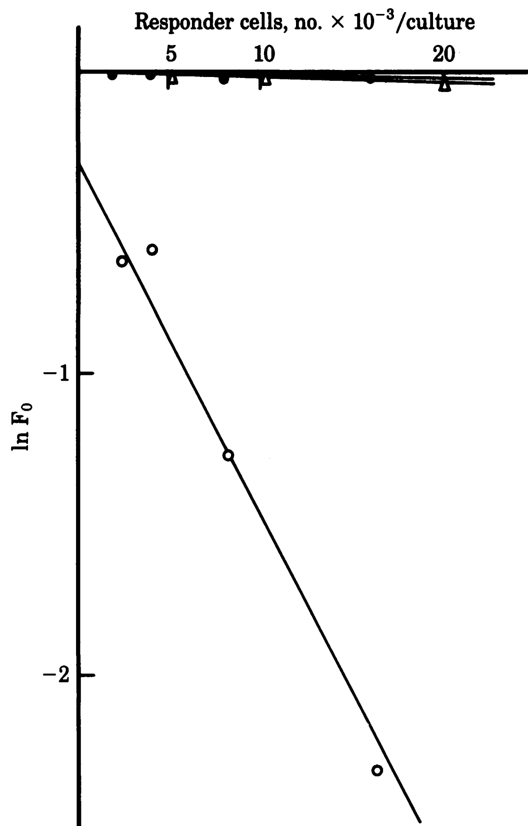

FIG. 6. Limit-dilution analysis of BALB/c → BALB/c chimeric cells responding to BALB/c stimulation (△) (CTL-P frequency, 1.2×10^{-6}) and to CBA stimulation (○) (CTL-P frequency, 118×10^{-6}) and of BALB/c → CBA chimeric cells responding to BALB/c stimulation (●) (CTL-P frequency, 0.9×10^{-6}).

stricted. It was also noted that the proportions of CTL-P generally were somewhat higher in the cultured thymuses than in thymuses allowed to mature normally *in vivo*. This could be due to the *in vitro* conditions favoring the development of a disproportionately high number of medullary-type thymocytes (21), which in turn could be associated with an inability of cells to migrate from the thymus into the peripheral pool.

The quantitative cloning technique for CTL-P permitted determination of the degree and nature of tolerance that might have developed within T cells maturing in an allogeneic thymus *Anlage*. Clear evidence of tolerance toward the H-2 antigens of the thymus framework and of the antigens of the thymocytes themselves was obtained within lymphocyte populations retaining adequate third-party reactivity. Furthermore, the limiting-dilution analysis strongly suggested a functional clonal deletion in both cases. If suppressor cells were at work, they failed to allow clonal expression even when small numbers of total thymus responder cells were present in a well, and so the suppressors must have been present at an extraordinarily high frequency. If a functional clonal deletion was indeed achieved, it appears that antigens from the FL-derived T cells or accessory cells and from the thymic epithelioreticular framework were equally effective. Of course, the results do not exclude the possibility that clonal deletion was induced by a population of suppressor/cytotoxic lymphocytes active within the original thymus organ culture—e.g., an anti-idiotypic cell as has been proposed (7, 12).

Recent elegant experiments in the rat (22, 23) bear on this point. Neonatal rats were rendered tolerant by injection with semi-allogeneic adult lymphocytes, and suppressor T cells were generated which prevented graft rejection in suitable irradiated recipients. Adoptive transfer experiments showed an active role for chimeric donor lymphocytes, best understood as an immune response to the idiotypes of alloreceptors on host T cells. The target of the suppressor cells so generated was therefore the alloreactive T cell bearing receptors for tolerated antigen. If such cells were frequent, they could mask the activity of competent anti-allo CTL-P even with few responders present in a well.

Theoretically, the two-cell nature of the functional incompetence should show up in a departure from linearity in the limiting-dilution graphs, but in practice it may not be possible to dilute out suppressors if they are very frequent because then the number of positive wells would be too low to score. It must be stressed that the transplantation tolerance induced by semi-allogeneic lymphocytes is occurring in an animal that has a substantial number of foreign but mature lymphocytes. Such a system may favor the development of suppressor networks. In contrast, our system allows the synchronous development of a genotypically homogeneous population. Nevertheless, we have preliminary evidence in another system (antihapten cytotoxic tolerance) suggesting that suppressor influences in cytotoxic cultures can be overcome by suitable amounts of stimulatory lymphokines, under which circumstance clonal deletion can still be shown. Thus, it may be that suppression and deletion coexist in transplantation tolerance.

We have no evidence bearing on the question of whether thymic epithelial cells bearing H-2D or H-2K antigens directly interact with emerging, potentially self-reactive T cells, or whether a processing step—e.g., via an Ia-positive FL-derived cell—is necessary. If the latter, the results stand in contrast to experiments suggesting that allogeneic differences within the *I* region facilitate tolerance induction to class I major histocompatibility complex antigens (24). As regards the tolerance of the thymocytes themselves toward the H-2 antigens, it is possible that CTL-P for self H-2 would be destroyed by some clonal abortion mechanism on contact with a neighboring lymphoid cell (25). Because one cannot detect CTL-P of a given specificity other than through their function, it is impossible to determine whether the self-reactive cells are actually killed or simply rendered anergic, as appears to be the case for B cells under some circumstances (26).

Our interpretation differs from that of Morrissey *et al.* (27) who suggested that self-tolerance was a prethymic event. In their studies $(A \times B)F_1$ mice were thymectomized and then grafted with a type A thymus. They were then x-irradiated and given an injection of type A bone marrow cells. Although no type B H-2K antigens could be detected within their new thymus, which had been repopulated with type A lymphocytes, the thymus cells nevertheless showed tolerance to both A and B alloantigens. These authors argued that prothymocytes present in marrow must express antigen receptors and that tolerance must already have been present prethymically.

Our model differs in two important ways. First, the TPP in our system have been populated by very immature cells—from 11-day FL and not from adult bone marrow—and the existence of a clonally committed repertoire within this population seems unlikely. Second, as Morrissey *et al.* pointed out, the presence of some $(A \times B)F_1$ cells within the implanted A thymus or of small amounts of host H-2 antigens undetectable by immunofluorescence cannot be ruled out. Whatever the reason for these results, an intrathymic event appears to cause tolerance in our system. However, our results must be reconciled with those of Wagner *et al.* (28) who have shown that CTL generated from athymic (*nu/nu*) mice are self-tolerant but able to react to third party antigens. Tolerance in their system also has the phenotype of clonal deletion. They argue that an encounter with

self-antigens, whether in the thymus or prethymically, can render maturing CTL-P self-tolerant. Whereas an athymic mouse has very few CTL-P, a normal mouse has many and thus needs an efficient means for engendering self-tolerance coincidentally with functional maturation. The thymus would appear to be a logical place for this to occur, and the results shown here clearly demonstrate its capability.

The thymic epithelium has already been implicated in the development of various fundamental immunologic properties. For example, it has been claimed to influence immune responsiveness to synthetic antigens (29). In those experiments it was shown that low-responder lymphocytes maturing in a high-responder thymus acquired the high-responder phenotype even though high/low response is genetically based. Similarly, the *H-2* restriction preference for lymphocytes has been claimed to occur in the thymus (30). In this case, however, the key cell that educates the developing lymphocytes may be of bone marrow origin, even though it is not a lymphocyte (31). It seems probable that the thymus serves more functions than simply allowing lymphocytes to divide and mature. It may be the site of clonal diversification, selection for low-affinity self-reactivity, and elimination of potentially destructive, high-affinity anti-self cells (32). If so, it is not surprising that the thymic framework can influence toleragenesis. It will be of interest to determine the nature of tolerance to class II major histocompatibility complex antigens and the major histocompatibility complex restriction specificities of cells from such chimeric thymuses.

We thank Helen Bathard and Richard Gogerly for their technical assistance. This work was supported by the National Health and Medical Research Council, Canberra, Australia; by Grant AI-03958 from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service; and by the generosity of a number of private donors to The Walter and Eliza Hall Institute. M.F.G. is the holder of a National Health and Medical Research Council Fellowship (Australia).

1. Billingham, R. E., Brent, L. & Medawar, P. B. (1953) *Nature (London)* **172,** 603–606.
2. Matsunaga, T., Simpson, E. & Meo, T. (1980) *Transplantation* **30,** 34–39.
3. Gorczynski, R. M. & MacRae, S. (1979) *J. Immunol.* **122,** 747–752.
4. Hasek, M. & Chutná, J. (1979) *Immunol. Rev.* **46,** 3–26.
5. Hilgert, I. (1979) *Immunol. Rev.* **46,** 27–53.
6. Roser, B. & Dorsch, S. (1979) *Immunol. Rev.* **46,** 55–86.
7. Gorczynski, R. M., Khomasurya, B., MacRae, S. & Short, L. (1981) *Cell. Immunol.* **57,** 183–200.
8. Miller, S. D., Sy, M.-S. & Claman, H. N. (1977) *Eur. J. Immunol.* **7,** 165–170.
9. Bunce, J. V. & Mason, D. W. (1981) *Eur. J. Immunol.* **11,** 889–896.
10. Nash, A. A., Phelan, J., Gell, P. G. H. & Wildy, P. (1981) *Immunology* **43,** 363–369.
11. Good, M. F. & Nossal, G. J. V. (1983) *J. Immunol.* **130,** 78–86.
12. Nossal, G. J. V. & Pike, B. L. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 3844–3847.
13. Le Douarin, N. M. & Jotereau, F. V. (1975) *J. Exp. Med.* **142,** 17–40.
14. Fontaine-Perus, J. C., Calman, F. M., Kaplan, C. & Le Douarin, N. M. (1981) *J. Immunol.* **126,** 2310–2316.
15. Bartlett, P. F. & Pyke, K. W. (1981) in *In Vivo Immunology: Histophysiology of the Lymphoid System,* ed. Hanna, M. G., Jr. (Plenum, New York), p. 375.
16. Pyke, K., Bartlett, P. F. & Mandel, T. E. (1983) *J. Immunol. Methods,* in press.
17. Pyke, K., Bartlett, P. F. & Mandel, T. E. (1983) *Thymus* **5,** 95–104.
18. Shortman, K. & Wilson, A. (1981) *J. Immunol. Methods* **43,** 135–152.
19. Good, M. F., Boyd, A. W. & Nossal, G. J. V. (1983) *J. Immunol.,* in press.
20. Lefkovits, I. & Waldman, H. (1979) *Limiting Dilution Analysis of Cells in the Immune System* (Cambridge Univ. Press, London).
21. Mandel, T. E. & Kennedy, M. M. (1978) *Immunology* **35,** 317–331.
22. Dorsch, S. & Roser, B. (1982) *Transplantation* **33,** 518–524.
23. Dorsch, S. & Roser, B. (1982) *Transplantation* **33,** 525–529.
24. Streilein, J. W. (1979) *Immunol. Rev.* **46,** 125–146.
25. Nossal, G. J. V. (1983) *Annu. Rev. Immunol.,* in press.
26. Nossal, G. J. V. & Pike, B. L. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 1602–1606.
27. Morrissey, P. J., Kruisbeek, A. M., Sharrow, S. O. & Singer, A. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 2003–2007.
28. Wagner, H., Hardt, C., Stockinger, H., Pfizenmaier, K., Bartlett, R. & Röllinghoff, M. (1981) *Immunol. Rev.* **58,** 95–129.
29. Longo, D. L. & Schwartz, R. H. (1980) *J. Exp. Med.* **151,** 1452–1467.
30. Zinkernagel, R. M., Callahan, G. N., Althage, A., Cooper, S., Klein, P. A. & Klein, J. (1978) *J. Exp. Med.* **147,** 882–896.
31. Longo, D. L. & Schwartz, R. H. (1980) *Nature (London)* **287,** 44–46.
32. Dröge, W. (1981) *Cell. Immunol.* **57,** 251–264.