keratin 5 has also been implicated as the genetic defect in another family affected with EBS (*24*).

The mutations described in these patients suggest that defects in keratins not only cause cytolysis, as observed in EBS and EHK, but also acanthosis and hyperkeratosis, as observed in EHK. Thus, the integrity of the keratin intermediate filament network contributes to mechanical stability of the keratinocyte and to the maintenance of a functional epidermis. EHK may be heterogenous with respect to the underlying defect, as is EBS, and it is likely that mutations will be discovered in other conserved regions of these keratins (*26*).

REFERENCES AND NOTES

1. E. H. Epstein, Jr., *Science* **256**, 799 (1992).
2. M. L. Williams, in *Pediatric Dermatology*, L. A. Schachner and R. C. Williams, Eds. (Churchill Livingstone, New York, 1988), vol. 1, pp. 400–406.
3. L. Brocq, *Ann. Dermatol. Syphiligr.* **4**, 1 (1902); S. Lapiere, *ibid.* **3**, 401 (1932); P. Frost and E. Van Scott, *Arch. Dermatol.* **94**, 113 (1966).
4. A. B. Ackerman, *Arch. Dermatol.* **102**, 253 (1970); P. Frost, G. D. Weinstein, E. Van Scott, *J. Invest. Dermatol.* **47**, 561 (1966).
5. P. M. Steinert and D. R. Roop, *Ann. Rev. Biochem.* **57**, 593 (1988); D. A. D. Parry and P. M. Steinert, *Curr. Opin. Cell Biol.* **4**, 94 (1992).
6. B. L. Bader, L. Jahn, W. W. Franke, *Eur. J. Cell Biol.* **47**, 300 (1988); S. R. Lessin, K. Huebner, M. Isobe, C. M. Croce, P. M. Steinert, *J. Invest. Dermatol.* **91**, 572 (1988); V. Romano *et al.*, *Cytogenet. Cell Genet.* **48**, 148 (1988); N. C. Popescu, P. E. Bowden, J. A. DiPaolo, *Hum. Genet.* **82**, 109 (1989); P. Heath *et al.*, *ibid.* **85**, 669 (1990); M. Rosenberg, E. Fuchs, M. M. LeBeau, R. L. Eddy, T. B. Shows, *Cytogenet. Cell Genet.* **57**, 33 (1991).
7. P. A. Coulombe and E. Fuchs, *J. Cell Biol.* 111, 153 (1990); M. Hatzfeld and K. Weber, *ibid.* 110, 1199 (1990); P. M. Steinert, *J. Biol. Chem.* **265**, 8766 (1990).
8. E. Fuchs and H. Green, *Cell* **19**, 1033 (1980); J. Woodcock-Mitchell, R. Eichner, W. G. Nelson, T.-T. Sun, *J. Cell Biol.* **95**, 580 (1982); D. R. Roop, P. Hawley-Nelson, C. K. Cheng, S. H. Yuspa, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 716 (1983); J. Schweizer, M. Kinjo, G. Furstenberger, H. Winter, *Cell* **37**, 159 (1984).
9. G. F. Wilgram and J. B. Caulfield, *Arch. Dermatol.* **94**, 127 (1966); T. Hirone, *J. Electron Microsc.* **18**, 63 (1969); I. Anton-Lamprecht and U. W. Schnyder, *Arch. Dermatol. Forsch.* **250**, 207 (1974).
10. K. A. Holbrook, B. A. Dale, V. P. Sybert, R. W. Sagebiel, *J. Invest. Dermatol.* **80**, 222 (1983).
11. H. Ogawa, M. Hattori, Y. Ishibushi, *Arch. Dermatol. Res.* **266**, 109 (1979); A. Ishida-Yamamoto *et al.*, *J. Invest. Dermatol.* **99**, 19 (1992).
12. J. Kanitakis *et al.*, *Br. J. Dermatol.* 117, 479 (1987).
13. J. W. H. Mali *et al.*, *ibid.* **95**, 627 (1976).
14. J. M. Bonifas *et al.*, *J. Invest. Dermatol.* **98**, 573 (1992); J. G. Compton *et al.*, *Nature Genetics* 1, 301 (1992).
15. DNA was extracted from blood samples [J. Sambrook, E. G. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, ed. 2, 1989)] or (EHK-PL) tissue embedded in paraffin [D. P. Jackson, F. A. Lewis, G. R. Taylor, A. W. Boylston, P. Quirke, *J. Clin. Pathol. (Lond.)* **43**, 499 (1990)]. Primers for PCR and sequencing were based on sequences for the human keratin 1 and 10 genes [L. D. Johnson, W. W. Idler, X-M. Zhou, D. R. Roop, P. M. Steinert, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 1896 (1985); M. Rieger and W. W. Franke, *J. Mol. Biol.* **204**, 841 (1988)]. The sequences are shown for their respective DNA: HK10, 5′-GAGGAGATGGTGGCCTTCTCTCTGG-3′ and 5′-GCATAGTGAACAGCCACATTGTGC-3′ (for PCR) 5′-TAAGATTCATCTGTCTGG-3′ (for sequencing), HK1, 5′-TGGACTCATTATTGGCCTCACTGG-3′ and 5′-TCACAGCTGCAAGAGGAAGCTCAG-3′ (for PCR), and 5′-GCGAGAATGCCCTCAAGG-3′ (for sequencing). PCR amplification was modified from R. A. Gibbs, P-N. Nguyen, A. Edwards, A. B. Civitello, and C. T. Caskey [*Genomics* **7**, 235 (1990)]. Reactions were preheated (95°C, 5 min), 2.5 units of Taq DNA polymerase added (AmpliTaq, Perkin-Elmer Cetus), cycled 30 times (68°C, 3 min; 94°C, 30 s; 60°C, 50 s), then extended at 72°C for 15 min. PCR products were purified (Magic PCR Preps, Promega), biotinylated DNA strands were captured on streptavidin-coated magnetic beads (Dynal), and the resultant single-stranded DNA was sequenced (Sequenase, U.S. Biochemicals).
16. J. A. Rothnagel *et al.*, data not shown.
17. D. Hohl, U. Lichti, D. Breitkreutz, P. M. Steinert, D. R. Roop, *J. Invest. Dermatol.* **96**, 414 (1991).
18. N. Geisler and K. Weber, *Nature* **296**, 448 (1982); T. M. Magin, M. Hatzfeld, W. W. Franke, *EMBO J.* **6**, 2607 (1987); J. F. Conway and D. A. D. Parry, *Int. J. Biol. Macromol.* **10**, 79 (1988).
19. M. Hatzfeld and K. Weber, *J. Cell Science* **99**, 351 (1991).
20. D. A. D. Parry *et al.*, *Int. J. Biol. Macromol.* **9**, 137 (1987).
21. A. Letai, P. A. Coulombe, E. Fuchs, *J. Cell Biol.* **116**, 1181 (1992).
22. P. A. Colombe *et al.*, *Cell* **66**, 1301 (1991).
23. R. Heald and F. McKeon, *ibid.* **61**, 579 (1990).
24. E. B. Lane *et al.*, *Nature* **356**, 244 (1992).
25. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
26. J. M. Bonifas, A. L. Rothman, E. H. Epstein, Jr., *Science* **254**, 1202 (1991).
27. R. D. B. Fraser, T. P. MacRae, D. A. D. Parry, E. Suzuki, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 1179 (1986).
28. B. P. Korge, S-Q. Gan, O. W. McBride, D. Mischke, P. M. Steinert, *ibid.* **89**, 910 (1992); J. A. Rothnagel and D. R. Roop, unpublished observations.

29. We thank R. A. Gibbs for the PCR sequencing protocol; D. A. D. Parry for calculations and discussions; C. Scaletta and M. Benathan for keratinocyte cultures; L. A. Applegate for RNA extraction; T. Gedde-Dahl, T. Schwarz, and B. Held for patient referrals and samples; the EHK families for their generous support; and N. J. Laminack for preparation of the manuscript. Supported by funds from NIH grant HD25479 to D.R.R. A.M.D. was a recipient of a Dermatology Foundation Research Fellowship.

25 June 1992; accepted 29 July 1992

Maintenance of in Vivo Tolerance by Persistence of Antigen

Fred Ramsdell* and B. J. Fowlkes

T cells of the immune system respond only to foreign antigens because those cells with reactivity for self proteins are either deleted during their development or rendered nonresponsive (anergic). The maintenance of the nonresponsive state was found to require the continual exposure of the anergic T cells to antigen. When anergic T cells were removed from the self antigen by adoptive transfer to a mouse strain lacking the antigen or by in vitro culture, nonresponsiveness was reversed and the anergic cells returned to normal functional status.

Although the thymus is probably the major site for inducing T cell tolerance (*1*, *2*), peripheral mechanisms of tolerance also exist (*3*, *4*). Data from several of these systems indicate that peripheral tolerance may result in the generation of clonal anergy rather than clonal deletion. A nondeletional form of developmental tolerance is induced in radiated bone marrow chimeras (*5*, *6*). This model system takes advantage of the fact that T cells bearing particular T cell receptor (TCR) V_β chains react with minor lymphocyte stimulatory (Mls) or major histocompatibility complex (MHC) antigens. In certain strains that possess specific self antigens, this reactivity results in the deletion of entire subsets of T cells that bear a particular TCR V_β chain. For example, T cells bearing $V_\beta6$ and $V_\beta17$ are specific for Mls-1^a and I-E, respectively (*1*, *7*). However, when chimeras are constructed with *Mls-1*^{b}, I-E$^-$ bone marrow cells that are injected into mice that are heavily irradiated (1000 R) and are *Mls-1*^{a}, I-E$^+$, the majority of T cells bearing these receptors are not clonally deleted.

Despite the lack of deletion in such chimeras, no T cell reactivity toward host antigens in mixed lymphocyte reactions in vitro or in graft-versus-host reactions in vivo can be detected (*5*, *6*). The proliferative response to stimulation by monoclonal antibodies to $V_\beta6$ and $V_\beta17$ is decreased by 80 to 99% (*5*). This effect can be overcome in the CD8 subset, but not in the CD4 subset, by the addition of exogenous interleukin-2 (IL-2) (*8*). In order to determine whether the nonresponsive state in these chimeric animals was reversible, we adoptively transferred lymphocytes from chimeric and control mice into irradiated host animals that lacked the Mls-1^a and I-E antigens and then assessed the phenotypic and functional status of the transferred cells.

For the purposes of this study, two types of

Laboratory of Cellular and Molecular Immunology, National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, MD 20892.

*To whom correspondence should be addressed at Department of Immunology, Immunex Corp., Seattle, WA 98101.

chimeras were constructed. Control chimeras consisted of B10.S mice (*Mls-1*^{b}, I-E$^-$) injected with SJL (*Mls-1*^{b}, I-E$^-$) bone marrow [SJL → B10.S mice]. In such chimeras, approximately 10% of mature T cells express either $V_\beta6$ or $V_\beta17$, and these cells respond normally to stimulation with V_β-specific antibodies (Table 1 and Fig. 1). These results are comparable to those seen with a normal SJL mouse. We generated the second chimera by injecting (B10.S × AKR)F_1 mice (*Mls-1*^{a}, I-E$^+$) with SJL bone marrow [SJL → (B10.S × AKR)F_1 mice]. Consistent with other data (*5*, *6*), mice of this type delete only 10 to 30% of their total $V_\beta6$- and $V_\beta17$-bearing T cells (Table 1). These same T cells are deleted efficiently (83 to 95%) when the Mls-1^a and I-E antigens are expressed on bone marrow–derived cells, as in (SJL × AKR)F_1 mice. However, the remaining $V_\beta6$ and $V_\beta17$ T cells show a decreased responsiveness to direct stimulation by antibodies to the TCR (anti-TCR) and thus appear to be in a state of clonal anergy (Fig. 1, B and C).

To determine if there is a requirement for antigen in maintaining the nonresponsive state, we adoptively transferred lymph node cells from control or nonresponsive chimeras into irradiated (750 R) B10.S hosts. We predicted both sets of chimeras to be tolerant of B10.S antigens, and, as expected, no graft-versus-host reactivity was noted. At 1 to 3 weeks after transfer, lymph node cells from the B10.S hosts were removed and assayed for the presence of donor cells. The relative percentage of $V_\beta6$- and $V_\beta17$-bearing T cells did not change significantly after adoptive transfer (Table 1). Because the original SJL bone marrow cells express the CD45.1 allele, we were able to accurately determine the percentage of $V_\beta6$ and $V_\beta17$ cells of donor origin in the B10.S hosts. No significant differences were noted at any time between the relative percentages of $V_\beta6$ or $V_\beta17$ cells as compared to the original starting populations.

We assessed the functional capabilities of these adoptively transferred cells by determining the ability of T cells "parked" in B10.S lymph nodes to proliferate in response to antibodies to $V_\beta17$ (anti-$V_\beta17$) or $V_\beta6$ (anti-$V_\beta6$). In these proliferation assays, cells from B10.S recipients that were not given chimeric lymph node cells made only minimal proliferative responses to anti-TCR stimulation because the mice were previously irradiated. In contrast, donor cell proliferation was easily detected although the maximal proliferative responses decreased with time after transfer (*9*). At 10 days after transfer, the difference in responsiveness to anti-$V_\beta17$ or anti-$V_\beta6$ between control and anergized cells became less pronounced than in the analysis before transfer (Fig. 1). By 20 days after transfer, there was no difference noted between the control and anergized groups. Because the B10.S host lacks the relevant self antigens, this result indicates that if cells are maintained in the absence of antigen (Mls-1^a or I-E), the nonresponsive state induced in the original SJL → (B10.S × AKR)F_1 chimeras could be reversed.

In order to address the possibility that adoptive transfer into the irradiated B10.S mouse resulted in a nonspecific effect that led to the reversal of anergy (such as the spontaneous production of IL-2 or other lymphokines in the irradiated host), we transferred chimeric lymph node cells into (B10.S × AKR)F_1 mice, which do bear the relevant self antigens. Phenotypic analyses of these cells also demonstrated no significant differences in the proportions of $V_\beta6$- and $V_\beta17$-bearing T cells, relative to the original populations (Table 1). When transferred into antigen-bearing (B10.S × AKR)F_1 hosts, anergic $V_\beta6$- and $V_\beta17$-bearing T cells remained in the nonresponsive state (Fig. 2). As before, an aliquot from the same pool of cells parked in antigen-free (B10.S) mice reverted to a state of normal responsiveness. Although we cannot exclude the possibility that the adoptive transfer into B10.S mice results in the selection of a minor population of nonanergic cells, we find this unlikely for several reasons. As noted, B10.S mice lack the self superantigens recognized by $V_\beta6$ and $V_\beta17$ cells, and thus a minor subset of such cells should not expand preferentially. Additionally, the selection of a minor subpopulation of reactive cells would be expected to alter the relative V_β frequencies or CD4:CD8 ratios from those of the original starting populations. Because neither effect was noted for either $V_\beta6$ or $V_\beta17$ at any time point examined, we conclude that anergic cells require repeated contact with antigen to retain the nonresponsive state.

To further support the contention that the reversal of anergy is due to an antigen-free environment and not to the outgrowth of a minority population of cells, we used a short term in vitro culture system. Lymph node T cells from control and nonresponsive chimeras were cultured individually or mixed at a 1:20 ratio and incubated in vitro in the presence of IL-7. We used IL-7 as a trophic factor to maintain the viability of freshly isolated T cells (*10*). The cultures were harvested 7 days later, and the relative percentages of $V_\beta17$ and $V_\beta6$ cells in each population were assessed by flow cytometry

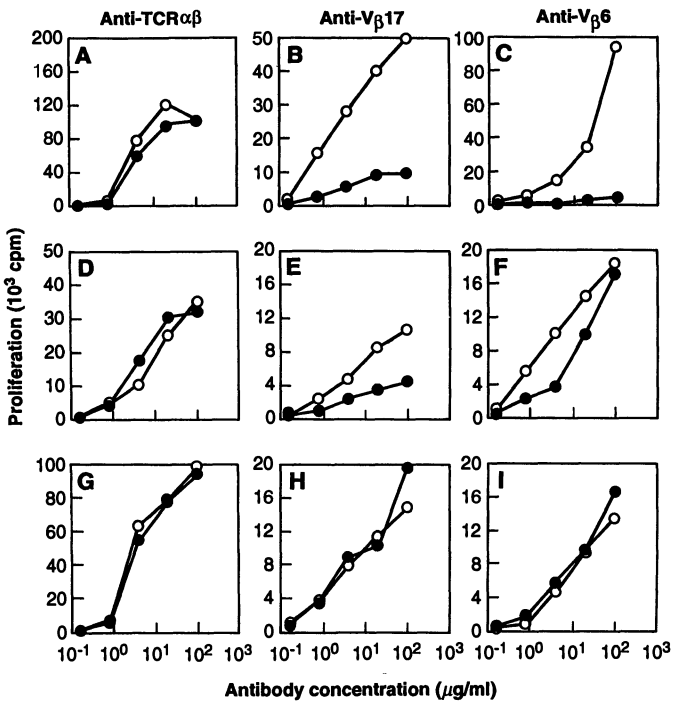

Fig. 1. Nonresponsiveness can be reversed after adoptive transfer into a mouse lacking the relevant self antigens. Lymph node cells (10^5 cells/well) from SJL → B10.S chimeras (open circles) or SJL → (B10.S × AKR)F_1 chimeras (closed circles) were stimulated with various concentrations of immobilized monoclonal antibodies to TCRαβ (*20*) (**A**, **D**, and **G**), $V_\beta17$ (*1*) (**B**, **E**, and **H**), or $V_\beta6$ (*21*) (**C**, **F**, and **I**) as described (*5*). In (A) through (C), lymph node cells were analyzed before adoptive transfer. In (D) through (F), lymph node cells were from irradiated B10.S mice that had received chimeric lymph node cells 10 days earlier. Cells in (G) through (I) were from irradiated B10.S mice that had received chimeric lymph node cells 20 days earlier. Chimeras were generated as described (*5*). Twelve weeks after reconstitution, chimeric lymph node cells were examined for TCR V_β expression by flow cytometric analysis and for functional responses to anti–TCR V_β stimulation. For anti-TCR stimulation, both antibody concentration and responding cell number were varied with comparable results. Chimeric lymph node cells (5×10^7) were transferred intravenously into B10.S mice that were irradiated (750 R). At 10 or 20 days after adoptive transfer, donor-derived TCR V_β expression (Table 1) and functional responses were determined. Irradiated B10.S mice that received no lymph node cells were unable to proliferate in response to antibody stimulation (<1000 cpm). Data are representative of five independent adoptive transfer experiments; proliferative responses were measured by [^{3}H]thymidine incorporation.

(Table 2). When normal (nonanergic) cells were added as a minor component (5%), they did not overgrow cells from the nonresponsive chimera. Additionally, the percent of $V_\beta 17$ and $V_\beta 6$ cells from both sources of T cells remained relatively constant throughout the culture period. When the functional responses were compared (Table 3), we found that the originally nonresponsive chimeric cells proliferated in response to anti-$V_\beta 17$ and anti-$V_\beta 6$ in a manner identical to that of the control cells. The mixed population also proliferated. $CD4^+8^-$ and $CD4^-8^+$ lymph node cells were examined individually, and both subsets from anergic mice were nonresponsive to anti-$V_\beta 6$ and anti-$V_\beta 17$ stimulation. After being cultured in IL-7, $CD4^+8^-$ and $CD4^-8^+$ cells from the anergic chimera proliferated to the same extent as control cells (Table 4). Thus, using a short-term in vitro assay system, we have shown that it is possible to reverse clonal anergy under conditions in which the outgrowth of minor subsets of responsive cells does not occur.

Our results indicate that antigen persistence is required for the maintenance of T cell anergy in vivo. When anergic T cells are removed from their specific antigen, they can revert to a state of normal responsiveness. The kinetics of this reversion and the ability to detect it in vitro indicate that it is independent of the production of new T cells by the thymus. This result appears to differ from tolerance to human gamma globulin (HGG), which is maintained in the apparent absence of antigen in adult thymectomized, but not in euthymic, animals (*11*). Whether there exists a cryptic source of antigen in HGG-primed mice is not clear. One potential source for antigen would be the follicular dendritic cell, which is thought to act as an antigen depot for the maintenance of memory cells (*12*) and could thus act as a long term depot for maintaining tolerance. A previous study suggested that the nonresponsive state for cytotoxic T lymphocyte activity was not reversed by in vivo parking, although it was not possible to examine specific T cells or

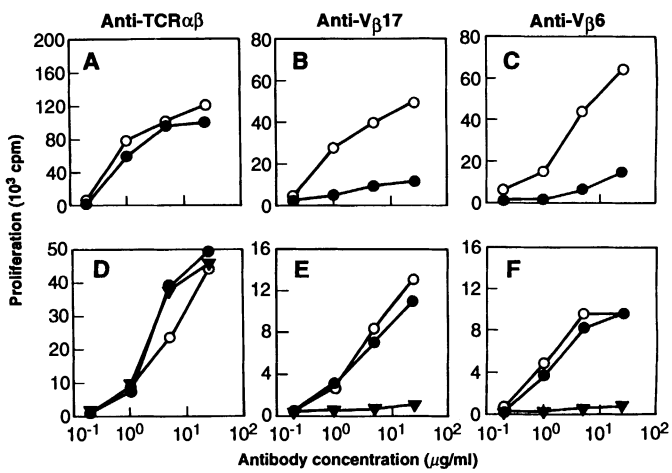

Fig. 2. Adoptive transfer of anergic cells into an antigen-bearing mouse maintains the nonresponsive state. Lymph node cells were stimulated as in Fig. 1. In (**A**) through (**C**), lymph node cells from SJL → B10.S (open symbols) or SJL → (B10.S × AKR)F_1 (closed symbols) chimeras were tested before adoptive transfer. In (**D**) through (**F**), lymph node cells were removed from B10.S (circles) or (B10.S × AKR)F_1 (triangles) mice 17 days after adoptive transfer. For adoptive transfers, lymph node cells (4×10^7) from SJL → B10.S (open symbols) or SJL → (B10.S × AKR)F_1 (filled symbols) chimeras were injected intravenously. (B10.S × AKR)F_1 recipients given lymph node cells from SJL → B10.S mice died of acute graft-versus-host disease by 12 days after transfer and are not included in the analysis.

Table 2. Reversal of clonal anergy in vitro is not due to the outgrowth of a minor subset of responsive cells. Lymph node T cells from SJL → (B10.S × AKR)F_1 chimeras were labeled in vitro with FITC (5 μg/ml), washed, and mixed 20:1 with T cells from SJL → B10.S chimeras. Cells were cultured in RPMI 1640 (10^6 cells per milliliter) with 10% fetal bovine serum and 100 units of recombinant IL-7) (rIL-7) per milliliter (Genzyme) for 7 days. Flow cytometry and TCR V_β normalization were performed essentially as in Table 1, except that cells were analyzed based on FITC expression to distinguish the original source of the responding cells. Values, from one representative experiment of three performed, indicate the percent positive.

Chimeric source	Total T	$V_\beta 17$	$V_\beta 6$
Before culture			
SJL → B10.S	4.4	11.6	10.3
SJL → (B10.S × AKR)F_1	95.6	8.1	8.5
After culture			
SJL → B10.S	5.5	11.1	10.2
SJL → (B10.S × AKR)F_1	94.5	7.4	8.0

Table 1. Clonal deletion in P → F_1 chimeras before and after adoptive transfer. Bone marrow chimeras were constructed as in Fig. 1. The before-transfer analysis was performed 12 weeks after bone marrow reconstitution, at which time the chimeras were >90% donor type. The after transfer analysis of irradiated hosts given chimeric lymph node cells was performed between 10 and 21 days after adoptive transfer. For flow cytometric analysis, lymph node cells were incubated with monoclonal antibodies to $V_\beta 6$ (RR4-7) or $V_\beta 17$ (KJ23), followed by fluorescein isothiocyanate (FITC)– or phycoerythrin (PE)–labeled goat antibody to rat immunoglublin G (IgG) or goat antibody to mouse IgG2a, respectively (Caltag, South San Francisco, California). Cells were simultaneously stained for CD4 (PE-coupled GK-1.5, Becton-Dickinson, Mountain View, California) and CD8 (biotin-conjugated 53-6.7, followed by streptavidin-APC, Caltag). Viable cells (10^5) were analyzed for each group with data collected on a FACS 440 (Becton-Dickinson). All percentages are normalized to include only cells bearing TCRαβ and CD45.1 (donor derived). Donor cells constituted between 23 and 67.5% (mean of 52.8% for control and 55.7% for nonresponsive chimera donors) of the total lymph cells in the population; most were T cells. There was no significant difference in the ratio of CD4 to CD8 cells bearing any of the V_β chains examined. Staining values are the mean of 12 chimeric animals and 5 independent adoptive transfer experiments. Numbers in parenthesis indicate standard error of the mean.

Before transfer			After transfer				
				B10.S Hosts		(B10.S × AKR)F_1 hosts	
Mice	$V_\beta 17$	$V_\beta 6$	Lymph node donor	$V_\beta 17$	$V_\beta 6$	$V_\beta 17$	$V_\beta 6$
SJL → B10.S	10.3 (1.5)	10.8 (1.7)	SJL → B10.S	10.2 (2.0)	11.2 (0.8)	NA*	NA
SJL → (B10.S × AKR)F_1	7.1 (0.8)	9.7 (2.3)	SLJ → (B10.S × AKR)F_1	7.3 (1.9)	10.7 (2.7)	7.1 (1.3)	10.8 (2.7)
SJL	10.5 (0.7)	11.1 (0.8)					
B10.S	0.2 (0.2)	10.3 (0.2)					
(B10.S × AKR)F_1	0.3 (0.2)	0.3 (0.4)					
(SJL × AKR)F_1	1.8 (0.4)	0.4 (0.6)					

*NA, not applicable. (B10.S × AKR)F_1 mice injected with SJL → B10.S lymph node cells died of acute graft-versus-host disease within 12 days of transfer.

Table 3. Reversal of anergy following in vitro culture. Lymph node T cells from control and anergic chimeras were analyzed for their proliferative response to anti-TCRαβ (2 μg/ml), anti-V_β17 (10 μg/ml), or anti-V_β6 (10 μg/ml) stimulation as in Fig. 1. Individual chimeras and a 20:1 mixture of anergic:control T cells were tested both before and after culture in rIL-7. Addition of rIL-7 to proliferation assays before culture does not reverse the anergic phenotype (*8*). Responder cell numbers were 8 × 10^4 cells per well in analyses before culture and 4 × 10^4 cells per well in analyses after culture. Numbers in parentheses indicate standard error of the mean of triplicate samples.

Responder cells	Proliferation (10^3 cpm)		
	Anti-TCRαβ	Anti-V_β17	Anti-V_β6
	Before culture		
SJL → B10.S	86.2 (1.1)	13.2 (1.3)	28.5 (3.1)
SJL → (B10.S × AKR)F_1	85.5 (7.7)	1.7 (0.2)	2.4 (0.4)
Mixture	79.7 (8.1)	1.6 (0.3)	2.7 (0.5)
	After culture		
SJL → B10.S	69.9 (5.6)	14.1 (1.4)	22.2 (2.7)
SJL → (B10.S × AKR)F_1	59.9 (8.4)	12.8 (1.0)	20.0 (2.1)
Mixture	60.0 (6.5)	11.0 (1.2)	20.0 (1.9)

Table 4. Both anergized $CD4^+$ and $CD8^+$ cells revert to normal functional status after in vitro culture. Lymph node cells from control and anergic chimeras were depleted of CD4 or CD8 cells by treatment with antibody plus complement. Remaining cells were analyzed for their proliferative response to anti-TCRαβ (2 μg/ml), anti-V_β17 (10 μg/ml), or anti-V_β6 (10 μg/ml) stimulation both before and after culture in rIL-7 as in Table 2. Responder cell numbers were 1 × 10^5 cells per well, and numbers in parentheses indicate standard error of the mean of triplicate samples.

Responder cells	Proliferation (10^3 cpm)		
	Anti-TCRαβ	Anti-V_β17	Anti-V_β6
	Before culture		
$CD4^+$ cells			
SJL → B10.S	54.3 (2.4)	5.9 (0.3)	8.4 (1.0)
SJL → (B10.S × AKR)F_1	45.1 (3.5)	0.5 (0.1)	0.8 (0.2)
$CD8^+$ cells			
SJL → B10.S	12.4 (0.8)	3.7 (0.4)	6.5 (0.2)
SJL → (B10.S × AKR)F_1	21.9 (3.4)	1.2 (0.5)	0.5 (0.1)
	After culture		
$CD4^+$ cells			
SJL → B10.S	197.7 (14.9)	36.4 (5.4)	43.9 (6.3)
SJL → (B10.S × AKR)F_1	175.2 (16.4)	58.5 (11.2)	69.8 (6.1)
$CD8^+$ cells			
SJL → B10.S	184.8 (20.2)	26.5 (3.5)	13.3 (2.3)
SJL → (B10.S × AKR)F_1	128.9 (18.9)	39.9 (5.2)	16.9 (1.5)

the amount of donor cell recovery in that system (*13*). Because the mechanism of inducing tolerance may not be the same, there may be distinct requirements for maintaining the nonresponsive state in these various systems.

The source of antigen in the irradiated chimera described here is as yet undetermined. Generally, only bone marrow–derived cells and thymic epithelial cells express class II MHC molecules; however, expression of these molecules can be induced in a variety of other cell types (*14*). In addition, Mls antigens are derived from endogenous retroviruses (*15*), and the identity of all the cells capable of producing such products is not established. The apparent ability of Mls antigens to be transferred between cell types for presentation makes identification of the Mls-bearing cell in vivo difficult.

T cell receptor occupancy in the absence of a second signal leads to clonal anergy in vitro (*16*). In this model of anergy, cells in the anergized state are still capable of generating a rise in intracellular Ca^{2+} in response to TCR signaling but fail to produce IL-2 (*17*). Whether the induction of nonresponsiveness seen in these bone marrow chimeras is due to a lack of second signals, perhaps because of antigen presentation on defective antigen-presenting cells (*18*), and whether the biochemical basis is identical to that of the clones remain to be determined. A distinct mechanism may be operating in the chimeras in which chronic stimulation of T cells prevents the cells from entering a resting state; such cells are therefore nonresponsive when assayed in vitro. Support for this idea comes from an in vivo transgenic model of T cell anergy in which V_β8.1 T cells in an Mls-1^a–expressing mouse are decreased in their response to stimulation by antibodies to V_β8 (*4*). Unlike the in vitro system, T cells in this transgenic mouse show a decreased Ca^{2+} mobilization after TCR cross-linking (*19*). Thus, prolonged TCR engagement may lead to a state of TCR desensitization and poor capacity to mobilize Ca^{2+} that prevents subsequent activation. If this is the mechanism of anergy in the chimeras, the data presented here suggest that allowing such nonresponsive cells to rest in the absence of antigen would permit the reappearance of functional responsiveness. Because nondeletional forms of tolerance are potentially involved in autoimmune disorders and allograft acceptance, it is important to determine not only the mechanisms that can induce this state but also the mechanisms that control its maintenance and reversal.

REFERENCES AND NOTES

1. J. W. Kappler *et al.*, *Cell* **49**, 273 (1987).
2. P. Kisielow, H. Blüthmann, U. D. Staerz, M. Steinmetz, H. von Boehmer, *Nature* **333**, 742 (1988); W. C. Sha *et al.*, *ibid.* **336**, 73 (1988).
3. D. Lo, L. C. Burkly, R. A. Flavell, R. D. Palmiter, R. L. Brinster, *J. Exp. Med.* **170**, 87 (1989); H.-G. Rammensee, R. Kroschewski, B. Frangoulis, *Nature* **339**, 541 (1989); S. Qin, S. Cobbold, R. Benjamin, H. Waldmann, *J. Exp. Med.* **169**, 779 (1989); S. Webb, C. Morris, J. Sprent, *Cell* **63**, 1249 (1990); S. E. Alters *et al.*, *J. Exp. Med.* **173**, 491 (1991); Y. Kawabe and A. Ochi, *ibid.* **172**, 1065 (1990); B. L. Rellahan, L. A. Jones, A. M. Kruisbeek, A. M. Fry, L. A. Matis, *ibid.*, p. 1071.
4. M. R. Blackman, J. Kappler, P. Marrack, *Science* **248**, 1335 (1990).
5. F. Ramsdell, T. Lantz, B. J. Fowlkes, *ibid.* **246**, 1038 (1989).
6. J. Roberts, S. O. Sharrow, A. Singer, *J. Exp. Med.* **171**, 935 (1990).
7. H. R. MacDonald *et al.*, *Nature* **332**, 40 (1988).
8. F. J. Ramsdell and B. J. Fowlkes, unpublished observations.
9. In most experiments, there was an overall drop in responsiveness between the before and after parking analyses. This is due at least in part to the fact that many (32 to 77%) lymph node cells from the parked animals were residual host T cells. Although these cells did not proliferate because of irradiation before transfer, their presence was able to dilute out the adoptively transferred, functional T cells. This decrease is not seen in the in vitro cultures described in Table 2.
10. We included IL-7 during in vitro cultures at 100 units/ml as a means of maintaining cell viability. Purified T cells did not proliferate in response to IL-7 at any time during the culture, as demonstrated by ^{3}H-labeled thymidine incorporation (<1000 cpm). Cell recoveries for IL-7–supplemented cultures ranged from 80 to 105% of the original input number. In addition, IL-7 did not reverse the clonal anergy seen when added directly to proliferation assays.
11. S. Qin *et al.*, *Eur. J. Immunol.* **20**, 2737 (1990).
12. J. G. Tew, R. P. Phipps, T. E: Mandel, *Immunol. Rev.* **53**, 29 (1980); D. Gray, M. Kosco, B. Stockinger, *Int. Immunol.* **3**, 141 (1991).
13. K. Wieties, R. E. Hammer, S. Jones-Youngblood, J. Forman, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 6604 (1990).
14. L. H. Glimcher and C. J. Kara, *Annu. Rev. Immunol.* **10**, 13 (1992).
15. W. N. Frankel, C. Rudy, J. M. Coffin, B. T. Huber, *Nature* **349**, 526 (1991); P. Marrack, E. Kushnir, J. Kappler, *ibid.*, p. 524; D. L. Woodland, M. P. Happ, K. J. Gollob, E. Palmer, *ibid.*, p. 529; P. J. Dyson, A. M. Knight, S. Fairchild, E. Simpson, K. Tomonari, *ibid.*, p. 531.

16. D. L. Mueller, M. K. Jenkins, R. H. Schwartz, *Annu. Rev. Immunol.* **7**, 445 (1989).
17. M. K. Jenkins *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5409 (1987).
18. J. Markmann *et al.*, *Nature* **336**, 476 (1988).
19. M. A. Blackman *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 6682 (1991).
20. R. T. Kubo, W. Born, J. W. Kappler, P. Marrack, *J. Immunol.* **142**, 2736 (1989).
21. O. Kanagawa, E. Palmer, J. Bill, *Cell. Immunol.* **119**, 412 (1989).
22. We thank R. Germain and L. Matis for critical reading of the manuscript and F. Hausman for flow cytometric analyses.

11 March 1992; accepted 29 June 1992

Transactivation by AP-1 Is a Molecular Target of T Cell Clonal Anergy

Sang-Mo Kang,* Bart Beverly, Annie-Chen Tran, Kurt Brorson, Ronald H. Schwartz, Michael J. Lenardo†

Anergy is a mechanism of T lymphocyte tolerance induced by antigen receptor stimulation in the absence of co-stimulation. Anergic T cells were shown to have a defect in antigen-induced transcription of the interleukin-2 gene. Analysis of the promoter indicated that the transcription factor AP-1 and its corresponding cis element were specifically down-regulated. Exposure of anergic T cells to interleukin-2 restored both antigen responsiveness and activity of the AP-1 element.

In pursuing the observations of Billingham and colleagues (*1*) on acquired immunological tolerance, Dresser showed that immunization with deaggregated foreign proteins rendered adult mice unresponsive to subsequent antigenic challenge (*2*). In contrast, the same antigen preparation initially administered with adjuvant was immunogenic. Models that use two signals for lymphocyte activation can potentially explain these early observations (*3*, *4*). Experimental evidence shows that stimulation of the T cell receptor (TCR) together with a second, nonantigen-specific signal, termed co-stimulation (*3–7*), causes T lymphocyte activation. Antigen receptor stimulation in the absence of co-stimulation, however, causes functional inactivation or anergy of the T cells (*4–7*). Anergic T cells have a greatly reduced capacity to produce interleukin-2 (IL-2) and to proliferate when re-stimulated with antigen–major histocompatibility complex (MHC) and co-stimulation. They also produce less of other lymphokines such as IL-3 and γ-interferon (IFN-γ). Studies with transgenic mice have provided compelling evidence that T cell tolerance in vivo can be due to the induction of anergy (*8*).

The $CD4^+$ mouse T cell clone A.E7 can be stimulated to produce IL-2 by a pigeon cytochrome c peptide (amino acids 81 to 104) presented by the MHC class II molecule E^k in the presence of co-stimulation (*7*, *9*). A.E7 cells can be rendered anergic by depleting them of residual antigen-presenting cells (APCs) and activating them with agents that trigger the TCR or raise intracellular Ca^{2+} concentrations (*4*, *10*). We induced anergy with concanavalin A (Con A), a lectin that binds the TCR and does not activate co-stimulatory pathways (*10*, *11*). A.E7 cells were treated with Con A (5 μg/ml) for 24 hours, after which the blocking sugar α-methyl mannoside (10 mg/ml) was added (*12*). The cells were subsequently incubated for 4 to 6 days before use. The Con A–treated group (termed anergic) had nearly identical surface expression of CD3, CD4, CD45, and IL-2 receptor α chain, as compared to cells treated in parallel without Con A (termed normal) (*13*). Normal and anergic T cells were then restimulated with antigen and splenic APCs, which provide full co-stimulation. Anergic A.E7 cells typically showed a 70 to 90% reduction in maximal incorporation of ^3H-labeled thymidine and a requirement for 25- to 50-fold more antigen to obtain half-maximal proliferation, although anergic cells were capable of proliferating in response to exogenous IL-2 (Fig. 1A). In anergic cells, maximal production of IL-2 was significantly decreased (to between one-tenth and one-fiftieth of the normal value), whereas maximal IFN-γ production was only diminished from one-third to one-half at 10 μM antigen (Fig. 1, B and C).

Northern (RNA) blot analysis of a time course of induction revealed that steady-state mRNA for IL-2 peaked sharply at 4 hours after antigen stimulation, whereas steady-state mRNA for IFN-γ appeared to plateau by 6 hours (Fig. 2A, lanes 1 through 7) (*14*). Thus, critical regulatory events for IL-2 mRNA production occur at times earlier than 4 hours. Induction of both mRNAs depended on peptide antigen (Fig. 2A, lanes 8 and 9). As observed for lymphokine production, IL-2 mRNA amounts were dramatically decreased in anergic A.E7 cells, whereas IFN-γ mRNA amounts were only modestly attenuated (lanes 11 and 13).

Studies have shown that IL-2 mRNA

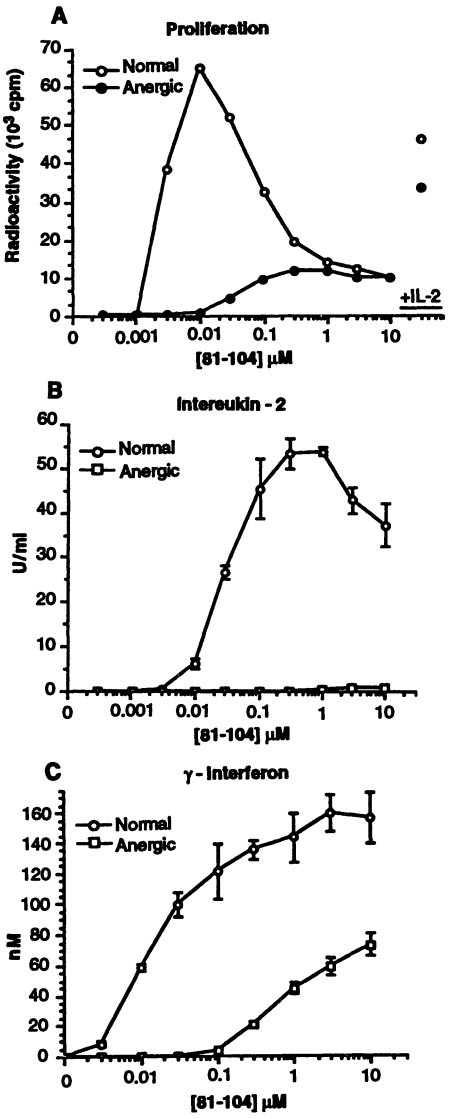

Fig. 1. Proliferation and lymphokine production assays of normal and anergic A.E7 cells. A.E7 cells induced into the anergic state or normal controls were rechallenged with B10.A spleen cells and increasing amounts of pigeon cytochrome c peptide (amino acids 81 to 104) (*12*). (**A**) Proliferation assay. [^3H]thymidine was added 24 hours after stimulation, and incorporation was measured after an additional 16 hours (*10*). Unconnected points above the "+IL-2" bar denote cells treated with IL-2 (30 units per milliliter) in the absence of antigen. (**B**) IL-2 bioassay on 24-hour culture supernatants. (**C**) IFN-γ assay on 24-hour culture supernatants. Error bars are for triplicate samples.

S.-M. Kang, A.-C. Tran, M. J. Lenardo, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892.
B. Beverly, K. Brorson, R. H. Schwartz, Laboratory of Cellular and Molecular Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892.
*Present address: Department of Surgery, UCSF School of Medicine, San Francisco, CA 94143.
†To whom correspondence should be addressed.