Pierce, *J. Exp. Med.* **170**, 1799 (1989).
22. Single letter 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.
23. J. Kyte and R. F. Doolittle, *J. Mol. Biol.* **157**, 105 (1982).
24. D. M. Engelman, T. A. Steitz, A. Goldman, *Annu. Rev. Biophys. Biophys. Chem.* **15**, 321 (1986).
25. The sequences shown were from the following publications: *CFTR*, J. R. Riordan *et al.*, *Science* **245**, 1066 (1989); *white*, K. O'Hare, C. Murphy, R. Levis, G. M. Rubin, *J. Mol. Biol.* **180**, 437 (1984); *araG*, J. B. Scripture *et al.*, *ibid.* **197**, 37 (1987); *ftsE*, D. R. Gill, G. F. Hatfull, G. P. C. Salmond, *Mol. Gen. Genet.* **205**, 134 (1986); *fecE*, H. Staudenmaier, B. Van Hove, Z. Yaraghi, V. Braun, *J. Bacteriol.* **171**, 2626 (1989); *oppD* and *oppF* (*18*); *malK*, E. Gilson, H. Nikaido, M. Hofnung, *Nucleic Acids Res.* **10**, 7449 (1982); *glnQ*, T. Nohno, T. Saito, J. Hong, *Mol. Gen. Genet.* **205**, 260 (1986); *ugpC*, P. Overduin, W. Boos, J. Tommassen, *Mol. Microbiol.* **2**, 767 (1988); *hisP*, C. F. Higgins *et al.*, *Nature* **298**, 723 (1982); *pstB*, B. P. Surin, H. Rosenberg, G. B. Cox, *J. Bacteriol.* **161**, 189 (1985); *STE6*, J. P. McGrath and A. Varshavsky, *Nature* **340**, 400 (1989); *cyaB* (*17*); *hlyB* (*16*); *mmdr1* (*14*).
26. These sequences were from the following publications: *brown*, T. D. Dreesen, D. H. Johnson, S. Henikoff, *Mol. Cell. Biol.* **8**, 5206 (1988); *btuD*, M. J. Friedrich, L. C. DeVeaux, R. J. Kadner, *J. Bacteriol.* **167**, 928 (1986); *chlD*, S. Johann and S. M. Hinton, *ibid.* **169**, 1911 (1987); *chvA*, G. A. Cangelosi *et al.*, *ibid.* **171**, 1609 (1989); *ndvA*, S. W. Stanfield, L. Ielpi, D. O'Brochta, D. R. Helinski, G. S. Ditta, *ibid.* **170**, 3523 (1988); *nodI*, I. J. Evans and J. A. Downie, *Gene* **43**, 95 (1986); *proV*, J. Gowrishankar, *J. Bacteriol.* **171**, 1923 (1989); *rbsA*, A. W. Bell, *J. Biol. Chem.* **261**, 7652 (1986).
27. G. Widera and R. A. Flavell, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 5500 (1985).
28. M. Steinmetz, D. Stephan, K. Fischer-Lindahl, *Cell* **44**, 895 (1986).
29. These bands do not correspond to any of the ethidium-stained Eco RI fragments. The 4.24, 5.22, 5.10, and 5.9 cosmids rearrange at relatively high frequency, probably due to the presence of a bacterial insertion sequence in the cosmid cloning vector (pNNL) [C. Fernandez, D. Larhammar, B. Servenius, L. Rask, P. A. Peterson, *Gene* **42**, 215 (1986)].
30. The authors thank M. Steinmetz and G. Widera for cosmid clones, T. Reynolds and the Massey Cancer Center Nucleic Acid Core Facility for synthesis of oligonucleotide primers and for technical assistance in obtaining portions of the nucleotide sequence, J. Howard for communicating unpublished data, and M. G. Brown, H. O. McDevitt, D. Conrad, and K. McCoy for reviewing the manuscript. Supported by USPHS grant GM38774 to J. M.

23 October 1990; accepted 28 November 1990

Peripheral Clonal Elimination of Functional T Cells

LORI A. JONES, L. THOMAS CHIN, DAN L. LONGO, ADA M. KRUISBEEK

A major mechanism for generating tolerance in developing T cells is the intrathymic clonal deletion of T cells that have receptors for those self antigens that are presented on hematopoietic cells. The mechanisms of tolerance induction to antigens not expressed in the thymus remain unclear. Tolerance to self antigens can be generated extrathymically through the induction of clonal nonresponsiveness in T cells with self-reactive receptors. A second mechanism of extrathymic tolerance was identified: clonal elimination of mature T cells with self-reactive receptors that had previously displayed functional reactivity.

CLONAL DELETION IS A MAJOR mechanism to achieve T cell tolerance to self antigens (*1–4*) and is thought to occur only in the thymus (*5, 6*). When this mechanism fails, tolerance may also be acquired by intra- and extrathymically induced clonal nonresponsiveness (anergy) (*7, 8*). The mechanism involved in generating T cell tolerance is probably determined by the manner in which self antigens are presented (*7–9*), and perhaps also by the developmental stage at which T cells encounter these antigens (*10*). Self antigens for which these rules have been established include H-Y antigens (*1*), products of the minor lymphocyte stimulatory (*Mls*) loci and staphylococcal enterotoxins (*2*), and largely unknown gene products that are recognized in association with $E_\alpha E_\beta$–class II molecules of the major histocompatibility complex (MHC) (*3*). In the present study, we established an environment in which both the intrathymic clonal deletion and the clonal anergy pathways were blocked and revealed yet another mechanism of self tolerance. As a model system, we used the Mls-1^a superantigen–$E_\alpha E_\beta$-MHC molecule combination, because potentially Mls-1^a–reactive cells that use $V_\beta 6$ as one of their T cell receptor (TCR) chains can be followed by analyzing $V_\beta 6$ expression among T cells (*2*).

Although some of the requirements for tolerance induction in Mls-1^a reactive cells have been addressed (*7, 11, 12*), several issues remain unresolved. Because some Mls-responsive cells are $CD8^+$, it is not clear how these cells depend on the MHC (*13*). Also, the presumed dependence of clonal deletion on $E_\alpha E_\beta$ in Mls responsive cells was shown only indirectly (*12*), in cell-transfer or chimera experiments. It is unclear whether induction of nonresponsiveness to Mls-1^a (*7, 14, 15*) is dependent on $E_\alpha E_\beta$, or $A_\alpha A_\beta$. Induction of in vitro responsiveness to Mls-1^a can be blocked by both $A_\alpha A_\beta$- and $E_\alpha E_\beta$-specific monoclonal antibodies (MAbs) (*16*).

To address whether Mls-1^a tolerance is dependent on $E_\alpha E_\beta$, Mls-1^a expressing mice [DBA/2 or (DBA/2 × C57Bl/6)F_1] were treated from birth with saturating doses of MAb to E_α, and the presence of potentially Mls-1^a reactive cells was analyzed by following the fate of $V_\beta 6^+$ T cells. MAb-mediated blocking of either class I or class II MHC molecules from birth can abrogate development of CD8 or CD4 expressing T lymphocytes, respectively (*17, 18*). Analysis of E_α and A_β expression in the spleen, lymph nodes, and thymus of treated mice confirmed the complete blocking of recognition of expression of E_α, whereas recognition of A_β was unaffected (*17, 19*). The effects of this treatment on development of the T cell repertoire were analyzed with MAbs specific for the variable (V) region of the T cell receptor β chain and flow cytometry. T cells that expressed $V_\beta 6$ were detected in both the thymus and the periphery of anti-E_α treated mice (Fig. 1) albeit at lower percentages than in $E_\alpha E_\beta^-$ mice, probably due to partial deletion through recognition of Mls-1^a in association with $A_\alpha A_\beta$ (*15*), but not in untreated controls. Additionally, this treatment prevented the deletion of other T cells that do not mature in the presence of $E_\alpha E_\beta$ [that is, $V_\beta 11$ and $V_\beta 5$; (*3, 20*)]. As a control, mice were also analyzed for expression of $V_\beta 14$, which identifies a TCR not dependent upon $E_\alpha E_\beta$ for selection (*21*). The percentage of T cells expressing $V_\beta 14$ was either unaffected or decreased (Fig. 1), most likely reflecting the concomitant increase in T cells with other receptors (V_βs 3, 5, 9, 11, 12, and 16) (*22*) normally deleted in $E_\alpha E_\beta$-expressing mice of the strains used here. The total number of αβ TCR expressing T cells in either the $CD4^+$ or $CD8^+$ subset of T cells was also not affected by the anti-E_α treatment (Fig. 1). Treatment with MAb for the other major class II molecule in DBA/2 mice, $A_\alpha A_\beta$, had no effect (*19*). Thus, $E_\alpha E_\beta$ expression is necessary and sufficient for clonal deletion of $V_\beta 6^+$ T cells.

Subsequent experiments focused on the functional reactivity of the $V_\beta 6^+$ T cells found in anti-E_α–treated mice. The $V_\beta 6^+$ T cells were analyzed both for their response to stimulation through direct crosslinking of their TCRs with MAbs and their response to syngeneic stimulator cells, to test for possible reactivity with self antigens. If, as for deletion, induction of nonresponsiveness

Biological Response Modifiers Program, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892.

requires $E_\alpha E_\beta$, the T cells might be functional since tolerance induction would be precluded by the blocking of $E_\alpha E_\beta$. Alternatively, tolerance induction may not require $E_\alpha E_\beta$ expression, but may utilize $A_\alpha A_\beta$ or may be entirely class II-independent. Both thymocytes and spleen cells from anti-E_α–treated mice proliferated in a dose dependent fashion to immobilized MAb to $V_\beta 6$ (Fig. 2). Whereas the responsiveness of these $V_\beta 6^+$ cells is lower than that of control Mls-1^b mice (*23*), the dose response curves are qualitatively similar. Because these cells were cultured in the absence of any exogenous lymphokines, the $V_\beta 6^+$ cells from treated mice apparently have no defects in either lymphokine production, or TCR and lymphokine receptor signalling. This was confirmed in a lymphokine production assay for interleukin-2 (IL-2) and IL-4 (*24*). In contrast, untreated littermates that do not express $V_\beta 6$ do not respond to anti-$V_\beta 6$ cross-linking over a range of MAb doses (Fig. 2, A and B). Yet T cells from control mice responded vigorously to MAbs to $V_\beta 14$ and to the αβ TCR (*25*), in a qualitatively similar fashion as T cells from anti-E_α–treated mice. Thus the effects induced by anti-E_α treatment are specific for $V_\beta 6$-expressing T cells.

We determined whether peripheral T cells from these mice were functionally tolerant of self antigens such as Mls-1^a and largely unidentified cotolerogens (*2, 3, 12*) in association with $E_\alpha E_\beta$. The number of T cells that escape deletion in anti-E_α–treated mice is potentially large, because it includes not only the populations that express V_βs 5, 6, and 11 detected here, but additional $E_\alpha E_\beta$-dependent T cell subsets that express V_βs 3, 9, 12, and 16 (*22*). However, responses to self antigens in vivo would not be elicited because the continued treatment with anti-E_α-MAbs blocks recognition of potential antigenic sites. Consistent with this prediction, anti-E_α–treated mice appear healthy, and no treatment related deaths are observed within the time frame of the experiments (4 to 5 weeks). We therefore compared the capacity of T cells from treated and control mice to respond in vitro to syngeneic and allogeneic stimulator cells in a mixed lymphocyte culture (MLC) assay. Whereas spleen cells (Fig. 2C) from control mice only responded to allogeneic stimulator cells, peripheral T cells from anti-E_α–treated (DBA/2 × C57B1/6)F_1 mice responded to a variety of syngeneic stimulator cells (Fig. 2C) (*26*). The target antigens in such an assay are undefined, but T cells from anti-E_α–treated mice were not functionally tolerant of self antigens, at least not those expressed on spleen cells. In an attempt to define at least some of the self antigens to which T cells from anti-E_α–treated mice are responding in MLCs, we determined the $V_\beta 6$-expression among peripheral T cells from anti-E_α–treated DBA/2 mice after in vitro activation with Mls-1^a–expressing (DBA/2) stimulator cells. As expected, peripheral T cells from anti-E_α–treated mice, after 5 days of in vitro culture with syngeneic DBA/2 stimulator cells, had increased the percentage of $V_\beta 6^+$

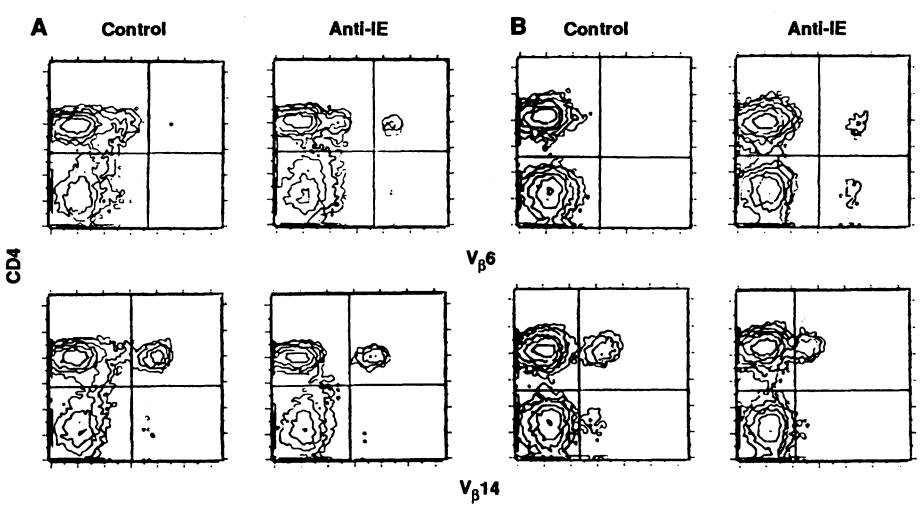

Fig. 1. Anti-E_α MAb treatment of Mls-1^a mice blocked deletion of $V_\beta 6^+$ T lymphocytes in (**A**) thymus and (**B**) lymph nodes. DBA/2 (A) or (DBA/2 × C57B1/6)F_1 (B) mice were daily injected intraperitoneally from birth with 500 μg of purified MAb to E_α (14-4-4) (*33*). Controls received saline injections, after previous studies demonstrated that irrelevant IgG injections had no effect (*17, 18*). Thymocytes were enriched for single positive mature T cells by treating mice with 2.5 mg hydrocortisone acetate 48 hours before analysis. T cells were purified from lymph nodes by treatment with a cocktail of MAbs to class II (anti-A^d_β plus anti-E_α, that is, MKD6 and 14-4-4) (*33*) and complement. Viable T cells were isolated after centrifugation over Lympholyte-M, and stained with either fluorescein isothiocyanate (FITC)-labeled $V_\beta 6$ [RR4-7 MAb (*33*)] or anti-$V_\beta 14$[14-2 MAb (*21*)]. Separate samples were stained with FITC-conjugated anti-αβ TCR [H57-597 MAb (*33*)]. After washing, cells were stained with biotinylated MAb specific for CD4 (GK1.5) (*32*) and then allophycocyanin labeled avidin (Caltag). Flow cytometry analysis was done on a Becton-Dickenson Dual Laser 440 interfaced to a PDP 11/24 computer. Data were collected on 50,000 cells. V_βs normalized to the percentage of αβ TCR^+ cells were as follows: (A) Thymus: Control, $V_\beta 6$: 0.5%, $V_\beta 14$: 8.2%; Anti-E_α–treated, $V_\beta 6$: 2.1%, $V_\beta 14$: 7.0%. (B) Lymph Nodes: Control, $V_\beta 6$: 0.2%, $V_\beta 14$: 8.3%; Anti-E_α–treated, $V_\beta 6$: 3.2%, $V_\beta 14$: 5.3%. Results are representative of 10 separate experiments.

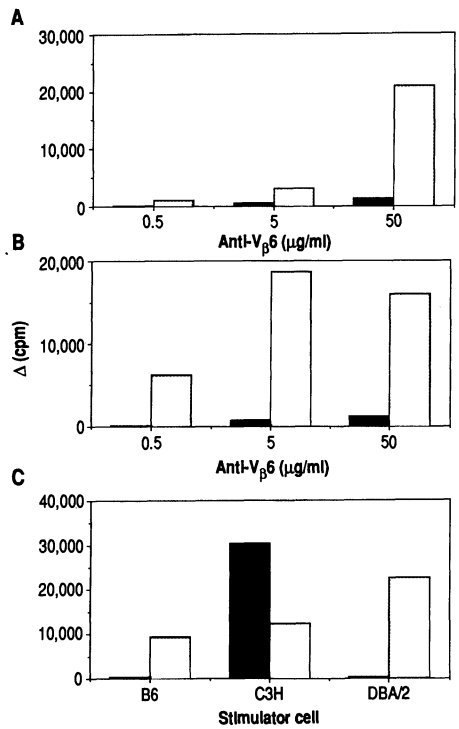

Fig. 2. $V_\beta 6^+$ thymocytes (**A**) and splenic T cells (**B**) from anti-E_α–treated Mls-1^a mice respond to specific MAb cross-linking, and spleen cells respond to syngeneic stimulator cells (**C**). Black bars, control; white bars, anti-Eα–treated (DBA/2 × C57B1/6)F_1 mice were daily injected intraperitoneally from birth with 500 μg of purified MAb to E_α. Thymocytes were enriched for mature single-positive T cells by treatment with J11D (*33*) and complement. Spleen cells were enriched for T cells by treatment with a cocktail of MAbs to class II (anti-A^d_β plus anti-E_α) and complement. For MAb cross-linking assays, purified MAb to $V_\beta 6$ was diluted to the indicated concentration in PBS, plates were incubated for 2 to 3 hours at 37°C, and were washed three times before use. Responder cells were added at a concentration of 2×10^5 cells per well. Assay was for 3 days with a label of 1 μCi of [^{3}H]thymidine added 18 hours before harvest. For mixed lymphocyte cultures (MLCs), stimulator spleen cells were added at 2, 4, and 8×10^5 cells/well, and responder cells at 2×10^5 cells/well; culture was for 4 days, including 18 hours of label with 1 μCi of [^{3}H]thymidine. Values shown are those obtained at the stimulator cell numbers giving optimal responsiveness (4×10^5). All determinations were performed in triplicate, and data are expressed as delta cpm (cpm values of experimental groups minus cpm values of responder cells alone); standard errors were generally less than 10%. Results are representative of 10 separate experiments.

T cells that expanded to 25% of all αβ TCR–expressing T cells by day 8 of culture. Stimulation with C3H/HeJ stimulator cells (Mls-1^b) had no effect on $V_\beta6$ expression (*27*). In summary, $V_\beta6$-expressing cells rescued from intrathymic clonal deletion were not functionally tolerant; they proliferated and produced lymphokines in response to TCR stimulation. Thus, not only clonal deletion, but also induction of clonal nonresponsiveness is dependent on $E_\alpha E_\beta$: developing T cells cannot become unresponsive to Mls because all $E_\alpha E_\beta$ molecules are blocked, and the unaltered expression of $A_\alpha A_\beta$ in anti-E_α–treated mice is insufficient for tolerance induction. Our model system, therefore, creates an environment in which the two known pathways of tolerance induction, clonal nonresponsiveness and intrathymic clonal deletion, are blocked.

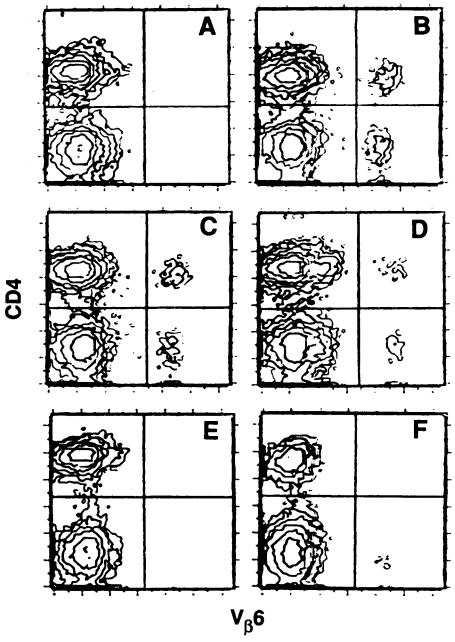

Fig. 3. Reexpression of self $E_\alpha E_\beta$ results in clonal elimination. Mice were treated with anti-E_α as described in Fig. 1, and tested at the age of 6 to 7 weeks. Control mice are shown in (**A**). Mice were treated either continuously (**B**) or until 12 days (**C**), 23 days (**D**), 31 days (**E**), or 34 days (**F**) before analysis. Mice in (F) were also thymectomized at the time the anti-E_α treatment was terminated, and were free of any thymic remnants on the day of analysis. Thymectomy alone (that is, with continued anti-E_α treatment) does not result in changes in the percentage of $V_\beta6$ expressing T cells. Data are representative of three independent experiments, and reflect $V_\beta6$ expression and CD4 expression in T cell–enriched lymph nodes (obtained as described in Fig. 1). Identical findings were obtained in the spleens of these mice. In the experiments shown, the percent of $V_\beta6$ cells normalized to the number of αβ TCR expressing cells is (A), 0.2; (B), 3.4; (C), 2.4; (D), 1.4; (E), 0.9; and (F), 3.1. The apparent increase in percentage of $V_\beta6$ in (F) reflects the fact that the $V_\beta6^+$CD8-cells did not change in absolute numbers, whereas a 75% reduction in absolute numbers of αβ TCR-expressing cells in the lymph nodes of thymectomized mice occurred.

We next asked how the $V_\beta6$ T cells that escaped deletion and anergy in anti-E_α–treated mice would respond to reexpression of the self antigens responsible for tolerance induction. Mice were first treated with anti-E_α MAb to allow $V_\beta6^+$ T cells to mature in the thymus and escape to the periphery. To achieve reexpression, MAb treatment was then terminated, such that mice could reexpress $E_\alpha E_\beta$; expression of $E_\alpha E_\beta$ and $V_\beta6$ were monitored by flow cytometry analysis during the subsequent weeks. The number of detectable $V_\beta6^+$ T cells in the periphery decreased with time after cessation of anti-E_α treatment (Fig. 3). There was no indication of activation of $V_\beta6$ expressing T cells as defined by IL-2 receptor expression or blast formation (*28*). T cells that expressed $V_\beta6$ disappeared from both the spleen and the lymph nodes, suggesting that elimination applies to the whole recirculating lymphocyte pool. Additional contributions to this elimination may be made by sequestration to nonlymphoid sites, such as mucosal tissues (*29*), but would still reflect a clonal event, since T cells with β chains that have no specificity for $E_\alpha E_\beta$ do not disappear after reexpression of $E_\alpha E_\beta$. However, the disappearance of $V_\beta6^+$ T cells was gradual. Termination of anti-E_α MAb treatment resulted in detection of $E_\alpha E_\beta$ by flow cytometry in the periphery by day 7. There were no significant changes in the number of $V_\beta6$-positive T cells until day 8 (Fig. 4), and even at 12 and 23 days after anti-E_α treatment, some $V_\beta6$-expressing T cells were detected (Fig. 3, C to E). Yet by 31 days after treatment, all $V_\beta6^+$ T cells disappeared from both the CD4 and the CD8 subset of peripheral T cells (Fig. 3E). Thus, clonal elimination can occur not only in immature thymocytes (*1–4, 30*) but also in mature single positive T cells that previously displayed functional reactivity; deletion, sequestration to nonlymphoid sites, or both, could be responsible.

Since previous studies demonstrated that clonal deletion during early T cell development occurs in the thymus (*1–6*), we addressed to what extent the clonal deletion of functional T cells was a thymus dependent phenomenon. As in the nonthymectomized mice (Fig. 3E), $CD4^+$ cells with $V_\beta6$ TCRs disappeared (Fig. 3F), demonstrating extrathymic clonal elimination of mature $CD4^+$ T cells with self-reactive receptors. Some $CD8^+$ T cells with $V_\beta6$ receptors remain in the thymectomized mice (Fig. 3F); perhaps these cells represent a population that does not recognize Mls-1^a in association with $E_\alpha E_\beta$ or is dependent on $CD4^+$ cells for recognition of Mls. The lack of elimination of these extrathymic mature single positive $CD8^+$ T cells contrasts with intrathymic clonal deletion that occurs at the $CD4^+CD8^+$ stage of T cell development (*1–4, 30*) and therefore affects both CD4 and CD8 subsets.

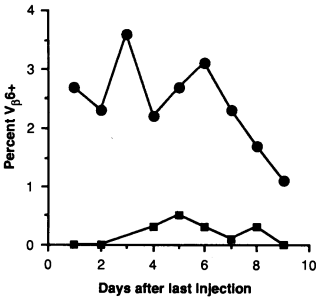

Fig. 4. Kinetics of disappearance of $V_\beta6$ expressing T cells after termination of anti-E_α treatment. Squares, control; circles, anti-Eα–treated mice were treated as described in Fig. 1 with anti-E_α MAb for 14 days, and $V_\beta6$ expression was analyzed by flow cytometry on whole lymph node cells on the days indicated, after treatment with anti-E_α had been stopped. Expression of $E_\alpha E_\beta$ can first be detected again in the spleens and lymph nodes on day 7 after the last injection of anti-E_α. Data shown are normalized to the percent of αβ TCR expressing T cells in the lymph nodes and are representative of three independent experiments.

Our data with anti-E_α–treated mice that were thymectomized and allowed to reexpress $E_\alpha E_\beta$ show that mature peripheral T cells with self-reactive receptors are subject to extrathymic clonal elimination. Where and how extrathymic autoreactive cells are eliminated remains to be elucidated. The in vitro expansion of $V_\beta6$ T cells upon antigenic stimulation suggests that the in vivo disappearance observed among peripheral $V_\beta6$ T cells does not reflect a property inherent to specific signalling properties of certain T cell receptors, as also reported for T cells recognizing staphylococcal enterotoxins (*31*). Thus, these findings do not provide support for the suggestions (*10*) that tolerance is dictated by the developmental stage at which T cells encounter self antigens. Rather, the consequences of antigenic stimulation may be determined by the manner of antigen presentation. In vitro stimulation may cause expansion because costimulatory signals required for lymphokine production (*32*) are provided, whereas in vivo exposure to self antigens apparently elicits signals leading to clonal elimination. While the mechanisms responsible for the disappearance of potentially self-reactive peripheral T cells therefore remain to be determined, this process can clearly be employed for maintenance of self tolerance.

REFERENCES AND NOTES

1. P. Kisielow, U. D. Bluthmann, U. Staerz, M. Steinmetz, H. von Boehmer, *Nature* **333**, 742 (1988).
2. J. W. Kappler, U. Staerz, J. White, P. Marrack, *ibid.*

332, 174 (1988); H. R. MacDonald *et al.*, *ibid.*, p. 40; H. Pirchner, K. Burke, R. Lang, H. Hengartner, R. M. Zingernagel, *ibid.* **342**, 559 (1989); J. White *et al.*, *Cell* **56**, 27 (1989).
3. J. W. Kappler *et al.*, *Cell* **49**, 263 (1987); J. W. Kappler, N. Roehm, P. Marrack, *ibid.* **49**, 273 (1987); J. Bill, O. Kanagawa, D. L. Woodland, E. Palmer, *J. Exp. Med.* **169**, 405 (1989); D. L. Woodland, P. Happ, J. Bill, E. Palmer, *Science* **247**, 964 (1990).
4. W. Sha *et al.*, *Nature* **336**, 73 (1988); L. J. Berg, B. Fazekas de St. Groth, A. M. Pullen, M. M. Davis, *ibid.* **340**, 559 (1989).
5. H. Smith, I. M Chen, R. Kubo, K. S. K. Tung, *Science* **245**, 749 (1989); R. J. Hodes, S. Sharrow, A. Solomon, *ibid.* **246**, 1041 (1989).
6. A. M. Fry, L. A. Jones, A. M. Kruisbeek, L. A. Matis, *ibid.*, p. 1044; L. A. Jones, L. T. Chin, G. Merriam, L. A. Nelson, A. M. Kruisbeek, *J. Exp. Med.* **172**, 1277 (1990).
7. F. Ramsdell, T. Lantz, B. J. Fowlkes, *Science* **246**, 1038 (1989); J. L. Roberts, S. O. Sharrow, A. Singer, *J. Exp. Med.* **171**, 935 (1990).
8. G. Morohan, J. Allison, J. F. A. P. Miller, *Nature* **339**, 622 (1989); K. M. Murphy, C. T. Weaver, M. Elish, P. M. Allen, D. Loh, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 10034 (1989); D. Lo *et al.*, *Cell* **53**, 159 (1989); J. Markmann *et al.*, *Nature* **336**, 476 (1988).
9. H. von Boehmer and K. Schubiger, *Eur. J. Immunol.* **14**, 1048 (1984); H. von Boehmer and K. Hafen, *Nature* **320**, 626 (1986); A. R. Ready, E. J. Jenkinson, R. Kingston, J. J. T. Owen, *ibid.* **310**, 231 (1984); R. K. Jordan, J. H. Robinson, N. A. Hopkinson, K. C. House, A. L. Bentley, *ibid.* **314**, 454 (1985); J. Miller, L. Daitch, S. Rath, E. Seleing, *J. Immunol.* **144**, 334 (1990); J. Boehme *et al.*, *Science* **244**, 1179 (1989).
10. C. A. Smith, G. T. Williams, R. Kingston, E. J. Jenkinson, J. T. T. Owen, *Nature* **337**, 181 (1989); M. Mercep, A. M. Weissman, S. J. Frank, R. D. Klausner, J. A. Ashwell, *Science* **246**, 1162 (1989).
11. P. Marrack *et al.*, *Cell* **53**, 627 (1988).
12. D. E. Speiser, R. Schneider, H. Hengartner, H. R. MacDonald, R. M. Zingernagel, *J. Exp. Med.* **170**, 595 (1989); D. E. Speiser, R. K. Lees, H. Hengartner, R. M. Zinkernagel, H. R. MacDonald, *ibid.*, p. 2165.
13. S. R. Webb and J. Sprent, *ibid.* **171**, 953 (1990); H. R. MacDonald, R. K. Lees, Y. Chvatchko, *ibid.*, p. 1381.
14. S. Qin, S. Cobbold, R. Benjamin, H. Waldmann, *ibid.* **169**, 779 (1989); H. Rammensee, R. Kroschewski, B. Frangoulis, *Nature* **339**, 541 (1989).
15. C. S. David and G. Anderson, in *Transgeneic Mice and Mutants in MHC Research*, I. K. Egorov and C. S. David, Eds. (Springer-Verlag, Berlin, 1990), pp. 229–236.
16. C. A. Janeway, Jr., E. A. Lerner, J. M. Jason, B. Jones, *Immunogenetics* **10**, 481 (1980); C. A. Janeway, Jr., and M. E. Katz, *J. Immunol.* **134**, 2057, (1985); D. H. Lynch, R. E. Gress, B. W. Needleman, S. A. Rosenberg, R. J. Hodes, *ibid.*, p. 2071 (1985); A. L. Glasebrook and F. W. Fitch, *J. Exp. Med.* **151**, 876 (1980); R. Abe and R. J. Hodes, *J. Immunol.* **140**, 4132 (1988); S. MacPhail and O. Stutman, *Immunogenetics* **24**, 139 (1986); K. A. Wall, M. I. Lorber, M. Loken, S. McClatchey, F. W. Fitch, *J. Immunol.* **131**, 1056 (1983); S. MacPhail and O. Stutman, *Eur. J. Immunol.* **14**, 318 (1984).
17. A. M. Kruisbeek *et al.*, *J. Exp. Med.* **161**, 1029 (1985).
18. S. Marusic-Galesic, D. L. Longo, A. M. Kruisbeek, *Nature* **333**, 180 (1988).
19. L. A. Jones and A. M. Kruisbeek, unpublished data.
20. L. A. Jones, J. C. Zuniga-Pflucker, J. S. Fine, D. L. Longo, A. M. Kruisbeek, *Progress Immunol.* **VII**, 289 (1989) (blocking of deletion of $V_\beta 11$ cells); treatment with anti-E_α also prevents deletion of $V_\beta 5$-expressing T cells in the strains used here, as well as in BALB/c and C3H/HeJ mice. Both $V_\beta 11$ and $V_\beta 5$ T cells are deleted when recognizing self antigens (cotolerogens) in association with $E_\alpha E_\beta$ (*3*).
21. N. S. Liao, J. Maltzman, D. H. Raulet, *J. Exp. Med.* **170**, 135 (1989).
22. M. S. Vacchio and R. J. Hodes, *ibid.*, p. 1335.
23. Maximal responsiveness was reached with anti-$V_\beta 6$ at 50 μg/ml with spleen cells from other mouse strains: for BALB/c (H-2^d, Mls-1^b): 40,000 cpm; for DBA/1(H-2^q, Mls-1^a but nondeleting for $V_\beta 6$): 19,000 cpm; compare to 17,000 cpm for anti-E_α–treated mice. Note that DBA/1 mice are not only nondeleting, but apparently also nontolerizing for $V_\beta 6$-expressing T cells, presumably because $A_\alpha{}^q A_\beta{}^q$ molecules are unable to present Mls-1^a (*15*).
24. Supernatants from the cultures shown in Fig. 2, A and B, were diluted 1:2, 1:4 and 1:8 and tested for their ability to support proliferation of a subline of CTLL that responds to both IL-2 and IL-4; CTLL proliferation was observed in a dose dependent fashion only with supernatants from T cells from anti-E_α–treated mice, with cultures giving maximal proliferation in Fig. 2, A and B, yielding maximal proliferation of CTLL cells as well.
25. Control untreated mice, although unresponsive to anti-$V_\beta 6$ cross-linking, did respond by proliferation and lymphokine production to TCR cross-linking with control MAbs to $V_\beta 14$ and pan MAbs to αβ TCR in a dose dependent fashion.
26. Peripheral T cells from anti-E_α–treated (DBA/2 × C57B1/6)F_1 mice not only respond to spleen cells from DBA/2 (H-2^d, Mls-1^a) and C57B1/6 (H-2^b, Mls-1^b) mice, as shown in Fig. 2C, but also to spleen cells from BALB/c (H-2^d, Mls-1^b) and B10.D2 (H-2^d, Mls-1^b) mice. When stimulator cells express Mls-1^a, $V_\beta 6$ T cells constitute part of the responding population (*27*). Thus responses to Mls-1^b stimulator cells probably reflect representation of Mls-1^a gene products derived from the responder cells on class II MHC molecules of the stimulator cells; the degree of responsiveness is consistent with this hypothesis: the H-2^b haplotype is weakly stimulatory for Mls-1^a whereas H-2^d is highly stimulatory (*15*). In addition, the responding cells in these MLCs included T cells responding to cotolerogens presented in association with $E_\alpha E_\beta$ (*3*) that also were not deleted in anti-E_α–treated mice (*20*); the response to BALB/c and B10.D2 stimulator cells may include such T cells.
27. T cell enriched spleen cells from anti-E_α treated mice were cultured for 5 days at 3×10^6 cells per well of 24-well plates with 3×10^6 mitomycin C-treated syngeneic (DBA/2) or allogeneic (C3H/HeJ) stimulator cells. On days 5 and 8 of culture, viable cells were purified by centrifugation on Lympholyte M and analyzed for V_β expression by flow cytometry. Allogeneic stimulation did not enrich for $V_\beta 6^+$ T cells compared to analysis before culture. Stimulation with syngeneic Mls-1^a (DBA/2) expressing cells resulted in a three-fold enhancement of $V_\beta 6^+$ T cells by day 5 that further expanded to 25% of the αβ TCR expressing cells by day 8.
28. Mice were analyzed by two-color flow cytometry for IL-2 receptor (IL-2R) and $V_\beta 6$ or IL-2R and αβ TCR at each of the time points indicated in Fig. 4; control and treated mice did not differ. In addition, $V_\beta 6^+$ T cells did not increase in size (indicating blast formation) by forward scatter.
29. J. Sprent, *Cell. Immunol.* **21**, 278 (1976).
30. B. J. Fowlkes, R. H. Schwartz, D. M. Pardoll, *Nature* **334**, 620 (1988); H. R. MacDonald, H. Hengartner, T. Pedrazzini, *ibid.* **335**, 174 (1988).
31. B. L. Rellahan, L. A. Jones, A. M. Kruisbeek, A. M. Fry, L. A. Matis, *J. Exp. Med.* **172**, 1091 (1990).
32. R. H. Schwartz, *Science* **248**, 1349 (1990).
33. MAbs used: 14-4-4 (anti-E_α), K. Ozato, N. Mayer, D. H. Sachs, *J. Immunol.* **124**, 533 (1980); GK1.5 (anti-CD4), Dialynas *et al.*, *ibid.* **131**, 2445 (1983); H57-597 (anti-pan αβ TCR), R. T. Kubo *et al.*, *ibid.* **142**, 2736 (1989); J11D (anti-HSA), J. Bruce, F. Symington, T. McKern, J. Sprent, *ibid.* **127**, 2496 (1981); RR4-7 (anti-Vβ6), O. Kanagawa, E. Palmer, J. Bill, *Cell. Immunol.* **119**, 214 (1989); MKD6 (anti-A^d_β), J. W. Kappler, B. Skidmore, J. White, P. Marrack, *J. Exp. Med.* **153**, 1198 (1981).
34. We thank R. Kubo, O. Kanagawa, and D. H. Raulet for providing MAbs to TCRs, F. Hausman and D. Stephany for expert and cheerful flow cytometry analysis, and R. N. Germain for many stimulating discussions and critical reading of the manuscript. L. T. C. is an HHMI/NIH Research Scholar.

23 August 1990; accepted 6 November 1990

Hemolin: An Insect-Immune Protein Belonging to the Immunoglobulin Superfamily

SHAO-CONG SUN, INGRID LINDSTRÖM, HANS G. BOMAN, INGRID FAYE, OTTO SCHMIDT*

Insects have an efficient defense system against infections. Their antibacterial immune proteins have been well characterized. However, the molecular mechanisms by which insects recognize foreignness are not yet known. Data are presented showing that hemolin (previously named P4), a bacteria-inducible hemolymph protein of the giant silk moth *Hyalophora cecropia*, belongs to the immunoglobulin superfamily. Functional analyses indicate that hemolin is one of the first hemolymph components to bind to the bacterial surface, taking part in a protein complex formation that is likely to initiate the immune response.

INSECTS ARE WELL ADAPTED TO DIverse environments and defend themselves against many parasites and microorganisms by highly effective cellular (*1*) and humoral immune mechanisms (*2*). It has been generally accepted that these reactions do not depend on immunoglobulins (Igs) (*3*) although attempts have been made to identify antibodies in insects (*4*). Some insect proteins (*5–7*) have been reported to contain Ig-like domains (*8*). However, these proteins do not seem to be involved in the immune response, but rather they play a role in neural cell interaction.

Antibacterial proteins have been identified in the giant silk moth, *Hyalophora cecropia*, and these proteins constitute an important part of the humoral immune defense. A set of proteins induced by bacterial infection

Department of Microbiology, University of Stockholm, S-106 91 Stockholm, Sweden.

*On leave from Biologie III, Universität Freiburg, D-78 Freiburg, FRG.