bromo-cyanide cleavage procedure was used because the leader sequence ended with a Met residue. The EIAV Tat protein was stored after lyophilization. Activity was tested after this procedure with a CAT assay (*5*).
7. Whereas the weak helix-forming tendency apparent in the NOESY cross-peak pattern does not materialize in all structure calculations, the protein forms stable helices simultaneously in the indicated regions (Fig. 1) in the presence of trifluoroethanol [H. Sticht *et al.*, *Eur. J. Biochem.* **218**, 973 (1993)].
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13. Such behavior would be reminiscent of the bZIP DNA binding motifs [D. Patak and P. Sigler, *Curr. Opin. Struct. Biol.* **2**, 115 (1992); (*14*)]. If sequence regions with a helix-forming tendency (Fig. 1) formed helices simultaneously, the protein would be of the helix-loop-helix-turn-helix type, as is observed in trifluoroethanol solution (*7*). This motif is known from homeodomain proteins [A. Laughon, *Biochemistry* **30**, 11357 (1991)]. Homeodomain Arg-Lys^2 and Arg-Lys^3 residues, which contribute to DNA minor grove recognition, would then correspond to Arg^4 and Arg^5 of EIAV Tat protein in TAR minor grove recognition [K. M. Weeks and D. M. Crothers, *Cell* **66**, 577 (1991)].
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16. Single-letter abbreviations for the amino acid residues are as follows: 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.
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20. Distance information was extracted from NOESY spectra with mixing times of 150 and 300 ms. Sequential ($|i–j| = 1$) to medium-range ($1 < |i–j| \leq 5$) NOEs were extracted from the 150-ms spectra to avoid spin diffusion effects. Long-range NOEs ($|i–j| > 5$) were extracted from both NOESY spectra as well as from ^{15}N-^{1}H 3D heteronuclear multiple-quantum coherence (HMQC) spectra. Nuclear magnetic resonance data were evaluated with the NDee program package (*21*); 429 intraresidual ($|i–j| = 0$), 250 sequential ($|i–j| = 1$), 146 medium-range ($|i–j| \leq 5$), and 34 long-range ($|i–j| > 5$) NOESY cross peaks could be assigned. A summary of the observed backbone cross peaks is given in Fig. 1. The structures were calculated from the NMR data according to the standard X-PLOR ab initio simulated annealing and refinement protocols with minor modifications. NOE cross peaks were grouped according to their intensity into three categories: strong (0.18 to 0.27 nm), medium (0.18 to 0.4 nm), and weak (0.18 to 0.55 nm). Starting from a template structure, 50 minimization steps were used, followed by 20-ps molecular dynamics at 1000 K with a soft-square NOE energy term (asymptote 0.1) and 10-ps molecular dynamics at 1000 K with increased weight on geometry and tilted asymptote (1.0) for the NOE energy term, cooling to 300 K in 50 K steps and 200 steps of minimization. Of the resulting 85 structures, the lowest energy structure was used as an input structure for a new round of ab initio calculation with identical protocol. The 11 structures from this second round of simulated annealing were subjected to molecular dynamics simulated annealing refinement, the energy function including an electrostatic term with a dielectric constant $\epsilon = 1$. Six structures were selected on the criteria of smallest number of NOE violations and lowest RMSD values and used for display in Fig. 2 and for calculation of the parameters in Table 1.
21. F. Herrmann *et al.*, unpublished results (the program is available on request).
22. We thank A. Gazit and A. Yaniv for their collaboration, and F. Herrmann for help with the ND program and artwork. Supported by the Fonds der Chemischen Industrie (FCI), an FCI grant (H.S. and D.W.), a MINERVA travel grant (D.W.), a grant from the Deutsche Forschungsgemeinschaft (P.R.), and the European Union BIOMED I–AIDS RESEARCH program.

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Anergic T Cells as Suppressor Cells in Vitro

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T cell–mediated suppression is an established phenomenon, but its underlying mechanisms are obscure. An in vitro system was used to test the possibility that anergic T cells can act as specific suppressor cells. Anergic human T cells caused inhibition of antigen-specific and allospecific T cell proliferation. In order for the inhibition to occur, the anergic T cells had to be specific for the same antigen-presenting cells (APCs) as the T cells that were suppressed. The mechanism of this suppression appears to be competition for the APC surface and for locally produced interleukin-2.

Evidence for T cell–mediated suppression has been derived from adoptive transfer systems in which T cells from an immunologically tolerant animal transfer tolerance to a naïve recipient animal if the T cells are injected together with specific antigen (*1*). Various models have been proposed to explain the mechanism of this T cell–mediated suppression. Suggestions that suppressor T cells represent a separate lineage and exert their effects through cascades of soluble factors have been discredited. Two current models exist. One model suggests that the effects are due to the suppressive effects of T cell–derived cytokines, such as interleukin-4 (IL-4), IL-10, or transforming growth factor–β (TGF-β), which inhibit the activation of IL-2–producing T cells (*2–4*). The other model proposes that antigen-specific T cells that have been rendered nonresponsive suppress other T cells that have the same specificity in a passive manner through competition for ligand and for cytokines such as IL-2 (*5*). We here provide in vitro evidence in support of the latter model.

Secretion of IL-2 by a subset of helper T lymphocytes (T_H1 cells) can be switched off as a result of partial signaling. This partial signaling can result from specific ligand recognition in the absence of costimulation (*6*), from receipt of full activation signals in the absence of IL-2–driven cell division (*7*), or from recognition of an altered ligand for which the T cell receptor has a lower affinity (*8*). T_H1 cells that have been turned off by these means are refractory to subsequent stimulation and are referred to as anergic. The induction of T cell anergy has been demonstrated in vitro (*6*, *7*, *9*) and in vivo (*10*). We test here whether or not anergic T cells have immunoregulatory effects on other T cells.

IL-2–secreting human T cell clones, specific for influenza hemagglutinin (HA) peptides [residues 100 through 115 (HC3) or 306 through 324 (HC6)] and restricted by HLA-DR1, were rendered anergic, by either incubation with soluble peptide in the absence of any added antigen-presenting cells (APCs) [as described (*9*)] or by incubation with immobilized antibody to CD3 (*11*). In response to the optimal stimulatory peptide concentration, these treatments led to >80% inhibition of T cell proliferation. The anergic T cells were added to cultures containing potentially reactive T cells, APCs, and antigen. Anergic T cells with the same specificity as the responsive T cells led to titratable inhibtion of proliferation (Fig. 1, A and B). This effect was specific because addition of an anergic clone with a different specificity (a third-party cell) caused less inhibition. No difference was seen in the degree of inhibition caused by T cells that had been rendered anergic by peptide or by antibody to CD3 (Fig. 1C).

Four possible mechanisms could contribute to this T cell–mediated suppression. First, it is possible that the anergic T cells, although unable to secrete IL-2, could be more lytic of the APC, thus depriving the responsive T cells of the opportunity to interact with ligand (*12*). Comparison of the lytic activity of the anergic and responsive T cells in a Cr release assay revealed that although the anergic T cells retained the ability to lyse antigen-bearing B cells, they were less efficient than the responsive T cells

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and caused no lysis of antigen-pulsed T cells.

A second possibility is that IL-2 was consumed because T cell anergy is accompanied by increased levels of IL-2 receptor expression (*13*) and of responsiveness to added IL-2 (*9*). In support of this possibility, inhibition by anergic cells could be reversed by addition of exogenous IL-2 (Fig. 1D). Furthermore, consumption of IL-2 could account for the weaker inhibition seen in the presence of third-party anergic T cells.

A third possible mechanism to account for the suppression of T cell reactivity is that the anergic T cells, although unable to secrete IL-2, do secrete a cytokine or cytokines that inhibit the potentially reactive T cells. Three candidate cytokines for such an effect are IL-4, IL-10 (*2*, *3*), and TGF-β (*4*). The weaker inhibition observed when a third-party anergic T cell was added suggested that this did not account for the phenomenon. However, this possibility was tested by the addition of recombinant IL-4 and IL-10 to cultures containing the responsive T cells (*14*). The responses of clones HC3 and HC6 were not inhibited by either cytokine when B cell lines were used as APCs; in fact, addition of IL-4 caused an increase in the proliferative response of clone HC3 (Fig. 2A). This APC-dependent difference in the effects of IL-10 has been attributed to the inhibition by IL-10 of the induction of the costimulatory molecule B7 in monocytes, but not in B cells (*3*). Because B cell lines were used as APCs in all the experiments shown here, these findings suggest that the secretion of IL-10 did not account for the observed inhibition. The addition of antibody to IL-10 into the cultures containing anergic T cells did not reverse the inhibition (Fig. 2, B and C).

We examined whether or not the suppression could be caused by TGF-β by adding monoclonal antibody to TGF-β to cultures containing anergic T cells. The monoclonal antibody did not reverse the inhibition caused by the presence of the specific anergic clone (Fig. 2, D and E). The partial inhibition of the response of clone HC3 that resulted from the addition of anergic HC6 was reversed in the presence of the antibody, suggesting that some of the effects of third-party anergic clones may be mediated by soluble factors such as TGF-β (Fig. 2D). The possibility that release of TGF-β by third-party anergic T cells could be increased in the presence of specific ligand was tested by coculture of clone HC3 with anergic HC6 in the presence of two populations of B cell APCs pulsed separately with the peptides for which clones HC3 and HC6 are specific. Under these circumstances, anergic HC6 cells caused ~30% inhibition of proliferation by HC3 cells (Fig. 2F). This inhibition was completely reversed by the addition of antibody to TGF-β. Again, no reversal of the specific inhibition caused by the HC6 clone was seen.

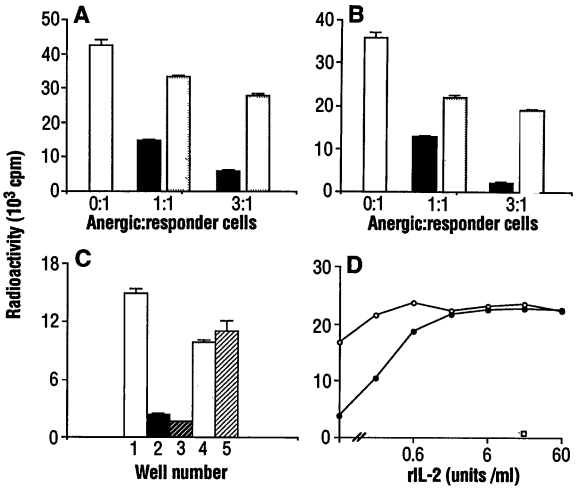

Fig. 1. Anergic T cells cause specific inhibition of proliferation by antigen-specific responder T cells. Wells contained (**A**) HC3 or (**B**) HC6 responder T cells (5 × 10^3 cells per well) and antigen-pulsed APCs (3 × 10^4 cells per well) alone (open bars) or together with added peptide-induced anergic cells with the same specificity (filled bars) or with a different specificity (shaded bars) from the responsive T cell clone at two ratios (*16*). (**C**) Wells contained: 1, HC3 responder T cells alone with antigen-pulsed APCs; 2, HC3 T cells rendered anergic with specific peptide; 3, HC3 T cells rendered anergic with antibody to CD3; 4, HC6 T cells rendered anergic with specific peptide; and 5, HC6 T cells rendered anergic with antibody to CD3. Wells 2 through 5 have a ratio of anergic to responder cells of 3:1. (**D**) The effects of culturing clone HC6 with antigen-pulsed APCs and increasing concentrations of recombinant IL-2 in the presence (closed circles) or absence (open circles) of three times as many anergic HC6 cells is shown. The single open square represents the response of HC6 to recombinant IL-2 (rIL-2) (10 U/ml) in the absence of antigen. The results are expressed as the mean (± SEM) of triplicate cultures minus background counts from wells with stimulator cells alone (<2000 cpm). SEMS were routinely <10%. All cytokine concentrations are plotted on a logarithmic scale.

The fourth possible mechanism is that anergic T cells exert their suppressive effects in a passive manner by competing with responsive cells for access to the APC membrane. To test this possibility, we added anergic T cells with specificity for one HA peptide to a proliferation assay containing a second T cell clone specific for a different peptide of the same antigen. The two peptides were displayed either on the same or on separate APC populations. The most inhibition was seen when the ligand for which the anergic T cells were specific was expressed on the same APC membrane as that for which the responsive T cells were specific (Fig. 3). A prediction that arises from this explanation is that increasing the number of APCs should overcome the inhibitory effect of the anergic

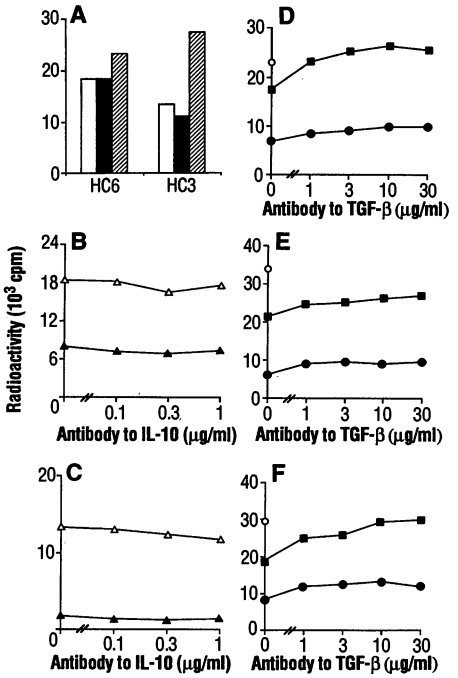

Fig. 2. The inhibitory effect of anergic T cells cannot be explained by the release of IL-4, IL-10, or TGF-β. Recombinant IL-10 or recombinant IL-4 (Genentech, Kent, United Kingdom) were titrated into cultures containing HC6 or HC3 cells (5 × 10^3 cells per well) and peptide-pulsed (10 μg/ml) DR1-expressing B-LCL cells (3 × 10^4 cells per well irradiated with 120 Gy of x-rays). Proliferation is shown in the absence of added cytokines (open bars) and in the presence either of IL-10 (filled bars) or of IL-4 (hatched bars) (each 100 U/ml). (**A**) The addition of purified monoclonal antibody to IL-10 did not reverse the inhibition caused by anergic T cells induced by antibody to CD3 for either HC6 (**B**) or HC3 (**C**). The ratio of anergic to responder cells was 3:1. The control responses of the two clones in the absence of added anergic cells are shown as open triangles. Monoclonal antibody to TGF-β (Genzyme) was added to cultures containing HC3 (**D**) or HC6 (**E**) responder T cells (5 × 10^3 cells per well) stimulated with peptide-pulsed (10 μg/ml) DR1–B-LCLs (3 × 10^4 cells per well) in the presence of anergic cells with the same specificity (filled circles) or different specificity (filled squares) as the responsive T cell clone. The responses of T cells to B-LCLs in the absence of anergic cells are shown as a single, open circle. (**F**) Two populations of B-LCLs were pulsed separately with peptide HA100–115 and HA306–324 and then added together to the culture wells. The experiment was performed as in (D). Results are expressed as the mean of triplicate cultures. SEMs were <10%. All cytokine concentrations are plotted on a logarithmic scale.

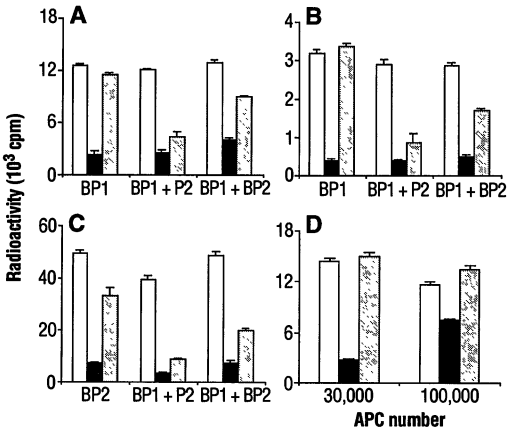

Fig. 3. Competition for the APC surface accounts for the inhibitory effects of unresponsive T cells. T cells rendered anergic with anti-CD3 with the same specificity (filled bars) or with a different specificity (shaded bars) as the responsive T cell clone were added to responder T cells (5 × 10^3 cells per well) at a ratio of 3:1. The controls (open bars) had no added anergic cells. Two peptides were used (P1 is HA306–324; P2 is HA100–115). Individual populations of B-LCLs were pulsed with either peptide separately (BP1 or BP2), with both peptides simultaneously (BP1 + P2), or with both peptides separately and then mixed (BP1 + BP2). (**A**) Responder T cell clone HC6. (**B**) NF4. (**C**) HC3. (**D**) The effect of titrating the number of APCs into cultures containing NF4 T cells (5 × 10^3 cells per well) with anergic NF4 (filled bars) or anergic HC3 (shaded bars) cells (1.5 × 10^4 cells per well) is shown. Results are expressed as the mean of triplicate cultures ± SEM.

cells; the data support this prediction (Fig. 3D). The mechanism that best fits these results is competition by the anergic T cells for the APC surface, coupled with competition for locally produced IL-2. The combination of these two forms of competition accounts for the degree of specificity of the observed suppression.

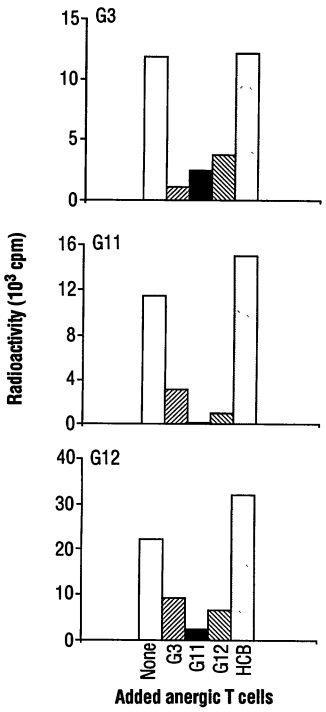

Fig. 4. Anergic alloreactive T cell clones, specific for DR1, inhibit the proliferation of DR1-specific T cells. Three DR1-specific T cell clones and a polyclonal T cell line specific for HA306–324 peptide and restricted by DR1 (HCB) were rendered anergic by overnight culture on immobilized antibody to CD3. The T cells were washed, irradiated (120 Gy), and then added, at a 3:1 ratio, as indicated, to responder alloreactive T cells (5 × 10^3) specific for DR1 in the presence of irradiated DR1–B-LCLs (3 × 10^4 cells per well) restricted for DR1. Results are expressed as the mean of triplicate cultures. SEMs were routinely <10%.

Therefore, a cohort of clonally expanded T cells that are rendered anergic should be able to exert suppressive effects on cells with different specificities, provided that the ligands are presented on the same APC. This mechanism could inhibit unwanted immune responses to self-antigens in the context of autoimmunity and to alloantigens in the context of transplantation. We examined the latter hypothesis by testing the suppressive effects of alloreactive T cells that had been rendered anergic. An allogenic major histocompatibility complex (MHC) molecule stimulates a large number of alloreactive T cells because it displays a wide array of peptides derived from cellular and serum proteins (*15*). Three anergic, anti-DR1, alloreactive T cell clones caused inhibition (Fig. 4), not only of responsive members of the same clone, but also of other anti-DR1 clones that are predicted to be specific for a different DR1 peptide complex on the surface of the allostimulator cells.

Suppression mediated by T cells has been described in experimental models of transplantation. T cells from a tolerant animal are able to transfer tolerance to a naïve animal when injected together with alloantigen (*1*), but the mechanism of this suppression has remained unclear. The results described here raise the possibility that anergic T cells, present in the tolerant host, may not be totally neutral but may be capable of exerting immunoregulatory effects. Such effects may not only be usable in the field of transplantation but may also play a physiological role in regulating self-immunity. If self-reactive T cells that escape the thymus become anergic by peripheral mechanisms, they may contribute to the maintenance of self-tolerance through competitive inhibitory effects.

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16. Human T cell clones, specific for HA residues 100 through 115 (clone HC3) or 306 through 324 (clones HC6 and NF4), were rendered unresponsive by overnight culture of accessory cell–free T cells in the presence of HA peptide HA100–115, HA peptide HA306–324 (both at a concentration of 10 μg/ml), or immobilized antibody to CD3 (2 μg/ml). After overnight incubation, the cells were washed, irradiated, and added to proliferation assays. For proliferation assays, wells were pulsed with 1 μCi of [^{3}H]thymidine (Amersham International, Amersham, United Kingdom) after 48 hours, and the cultures were harvested on glass fiber filters 18 hours later. Incorporation of [^{3}H]thymidine was measured by liquid scintillation spectroscopy.

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