extracted from the indicated tissues of a young mink, as described (*21*).

24. TGF-β–treated or untreated subconfluent Mv1Lu cells were detached from petri dishes by treatment with trypsin (0.05%) and 0.02 mM EDTA in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline and allowed to recover in growth medium supplemented with 10 mM Hepes (pH 7.4) with gentle swirling for at least 1 hour at 37°C. After washing, the cells were resuspended in complete KRH medium [125 mM NaCl, 5 mM KCl, 12 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 6 mM glucose, 10 mM Hepes (NaOH buffer, pH 7.4)] loaded with fura-2, and analyzed in a Perkin-Elmer LS-5B fluorimeter as described (*5–7*). Cells ($5 \pm 1 \times 10^5$) suspended in complete KRH medium were first incubated for 10 min, with or without ryanodine (10 μM) (dashed or continuous lines, respectively). Cells in Fig. 3, A and B, were then switched to a Ca^{2+}-free KRH medium by addition of excess EGTA (3 mM) and challenged with Bk (0.05 μM) and with Tg (0.1 μM) where indicated (*6, 7*). Cells in Fig. 3, C and D, were treated with fetal calf serum (0.5%) for 10 min to induce, by means of a large increase in $[Ca^{2+}]_i$, the activation of the RyR and promote the effect of the alkaloid. The cells were collected by centrifugation, washed, and resuspended in the same Ca^{2+}-containing KRH medium, and incubation was continued for 15 min to assure refilling of unaffected Ca^{2+} pools and recovery from serum stimulation. Excess EGTA (3 mM) was then added, followed 2 min later by the addition of Bk (1 μM) and then 3 to 4 min later by the addition of Iono (0.5 μM), where indicated. Data represent the results of eight to ten experiments. The $[Ca^{2+}]_i$ calibration values are shown on the left-hand side of the traces.

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No Requirement for p56^{lck} in the Antigen-Stimulated Clonal Deletion of Thymocytes

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Activation of protein-tyrosine kinases (PTKs) is required for signal transduction during T cell activation, although the pathway used during thymic selection is unknown. An in vitro system was established in which T cell receptor transgenic thymocytes underwent clonal deletion in response to peptide antigen. The effects of two PTK-specific inhibitors, herbimycin A and genistein, on the clonal deletion of immature thymocytes and the activation of mature thymocytes were examined. Clonal deletion occurred while T cell activation was inhibited and when no p56^{lck} activity was evident. Thus, p56^{lck} is not required for the antigen-stimulated step of clonal deletion of immature thymocytes, and negative selection proceeds via a distinct pathway.

Because many growth factor receptors have cytoplasmic tyrosine kinase domains, PTKs are thought to participate in cell growth. The T cell antigen receptor (TCR) complex, which is not itself a PTK, functionally associates with at least two Src-like PTKs, p56^{lck} and p59^{fyn} (*1*). A model has been proposed in which the activation of PTK is the essential first step of biochemical change during T cell activation. Stimulation of the TCR results in the tyrosine phosphorylation of several intracellular substrates within 5 s, whereas the hydrolysis of phosphatidylinositides (PI) does not occur until approximately 30 to 40 s after stimulation (*2*). Furthermore, stimulation of the TCR induces tyrosine phosphorylation of phospholipase C (PLC)–γ1, presumably through the activation of PTKs (*3*). PLC-γ1 catalytic activity itself may be augmented by tyrosine phosphorylation of PLC-γ1 (*4*). The PLC-γ1 catalyzes the breakdown of PI, leading to the generation of inositol trisphosphate (IP_3), which has been implicated in the elevation of intracellular Ca^{2+} levels, and diacylglycerol, a potent activator of protein kinase C. Indeed, treatment of T cells with both phorbol esters (to activate protein kinase C) and Ca^{2+} ionophores (to increase cytosolic Ca^{2+}) can mimic phenotypic changes observed with T cell activation. Two potent PTK-specific inhibitors with distinct modes of action, herbimycin A (*5*) and genistein (*6*), inhibit PI turnover induced by TCR stimulation and result in the failure of T cell activation (*7, 8*). Lastly, p56^{lck} and p59^{fyn} are both required for T cell activation (*9, 10*). Thus, the activation of PTKs is required for signal transduction during T cell activation through the TCR.

Within the thymus, T cells undergo a stringent process of positive and negative selection during development to emerge as self-tolerant, major histocompatibility complex (MHC)–restricted functional cells (*11*). Although the selection process is mediated by the TCR, which also mediates T cell activation, the signal transduction pathway in the thymic selection process is unknown. We investigated whether the signal transduction pathway in the thymic selection of immature T cells is similar to that in T cell activation of mature T cells. Using mice transgenic for a TCR from a T cell hybridoma DO11.10 that recognizes chicken ovalbumin in the context of the MHC class II molecule I-A^d, we showed that exposure of immature thymocytes to self antigen [cOVA(323-339) peptide derived from chicken ovalbumin] induces intrathymic apoptosis in vivo (*12*). Thus, negative selection occurs by apoptosis. With the same TCR transgenic mice, we developed an in vitro clonal deletion model in which both antigen-induced apoptosis of immature CD4$^+$CD8$^+$TCR^{lo} transgenic thymocytes and antigen-induced activation of mature CD4$^+$CD8$^-$ transgenic thymocytes could be observed simultaneously (*13*). A similar in vitro clonal deletion system was reported (*14*). Here, we investigated the signal transduction mechanism of clonal deletion by using pharmacologic agents.

Transgenic thymocytes were cultured with I-A^{d+} antigen-presenting cells in the presence of cOVA(323-339) peptide or cOVA(324-334) peptide (control peptide) with the use of the in vitro system (*13*). The cOVA(324-334) peptide binds to I-A^d (*15*) but does not activate the transgenic TCR (*12*). Three-color analysis of the cultured thymocytes was done with monoclonal antibodies (MAbs) to CD4, CD8, and the transgenic TCR (KJ1-26, a clonotype-specific MAb). The number of mature CD4$^+$CD8$^-$ thymocytes present at the end of 20 hours of culture was similar to the number present at the beginning of culture (Fig. 1, A and B). However, greater than 95% of the immature CD4$^+$CD8$^+$KJ1-26^{lo} thymocytes died after 20 hours of culture in the presence of cOVA(323-339) peptide (Fig. 1, C and D). The presence of cOVA(324-334) control peptide had virtually no effect on clonal deletion or T cell activation. DNA fragmentation was augmented by the cOVA(323-339) peptide as early as 5 hours after culture (Fig. 2), although thymocytes showed background spontaneous DNA fragmentation when cultured in vitro with the cOVA(324-334) peptide. To define the potential role of PTKs in thymocyte clonal deletion and activation, we added either herbimycin A or genistein to the in vitro deletion experiments. Neither herbimycin A nor genistein blocked the clonal deletion of CD4$^+$CD8$^+$KJ1-26^{lo} thymocytes induced by the cOVA(323-339) peptide (Fig. 1, C and D). The DNA fragmentation induced by the cOVA(323-339) peptide was also not inhibited (Fig. 2).

Although no change could be seen in the number of mature CD4$^+$CD8$^-$ thymocytes with or without cOVA(323-339) peptide (Fig. 1, A and B), the addition of cOVA(323-339) peptide for 20 hours acti-

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vated the $CD4^+CD8^-$ thymocytes, as shown by the presence of blastic cells among only the $CD4^+CD8^-$ population. Gating on the data collected in the experiment described in Fig. 1 yielded the percentage of blastic cells (cells larger than unstimulated cells) in $CD4^+CD8^-$ thymocytes (Fig. 3A). Herbimycin A and genistein inhibited the appearance of the blastic cells in a concentration-dependent manner, with 400 nM herbimycin A and 37 μM (10 μg/ml) genistein virtually eliminating blastogenesis.

We examined DNA synthesis, an independent marker of T cell activation, using a similar culture system (*16*) (Fig. 3B). Both herbimycin A and genistein clearly inhibited [^{3}H]thymidine uptake by thymocytes. These results indicate that herbimycin A and genistein inhibit the activation of T cells but do not affect the signaling pathway leading to clonal deletion. The inhibitor concentrations that were used are consistent with other studies (*17*). A 12- to 16-hour treatment of herbimycin A is required to inhibit TCR-induced increases in substrate tyrosine phosphorylation (*7*). However, 2 hours of prior treatment was sufficient to inhibit blastogenesis and [^{3}H]thymidine incorporation in our system. The reason for this discrepancy is not clear, but differences in the type of cells or methods of stimulation used might be responsible. In some cases, the effect of treatment with herbimycin A appears within 1 hour (*18*). Clonal deletion was still observed with 16 hours of prior treatment with herbimycin A (*19*). Because T cells require only 15 min of treatment with genistein before antigen is added to block the activation process (*8*), it is unlikely that the herbimycin A treatment time was not sufficient to inhibit clonal deletion. Although these drugs showed partial cytotoxicity to

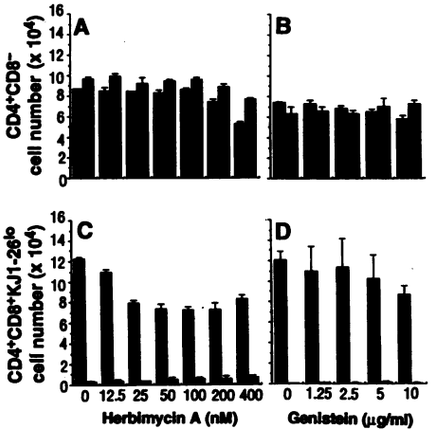

Fig. 1. The effect of herbimycin A and genistein on in vitro clonal deletion; the method has been described in detail elsewhere (*13*). Briefly, 1 × 10^6 thymocytes from a DO10 transgenic mouse were cultured with 10^5 A20 cells (a B cell lymphoma line) as a source of antigen-presenting cells in triplicate, 200-μl cultures [2-mercaptoethanol–free RPMI medium supplemented with 10% FCS (*21*)]. Herbimycin A and genistein were obtained from Y. Uehara and Biomol (Plymouth Meeting, Pennsylvania), respectively. Herbimycin A (**A** and **C**) or genistein (**B** and **D**) was added to yield the indicated final concentrations. After 2 hours, cOVA(323-339) (hatched bars) or cOVA(324-334) (black bars) was added to a final concentration of 1 μM. The thymocytes were cultured for 20 hours after the addition of peptide. Percentages of the $CD4^+CD8^-$ and $CD4^+CD8^+$ thymocytes are approximately 20 and 70%, respectively. Sixty to 70% of the $CD4^+CD8^+$ thymocytes expressed transgenic TCR ($KJ1\text{-}26^+$). Fluorescence-activated cell sorter profiles of the thymocytes are shown elsewhere (*13*). Approximately 30% of $CD4^+CD8^+$ thymocytes died spontaneously during the 20-hour culture with the cOVA(324-334) peptide. Half of the thymocytes were analyzed by flow cytometry (FACScan; Becton Dickinson). In our three-color analysis we used fluorescein isothiocyanate–conjugated anti-CD8α (clone 53-6.7; Becton Dickinson), phycoerythrin-conjugated anti-CD4 (clone GK1.5; Becton Dickinson), and biotinylated KJ1-26 and streptavidin-Red613 (Gibco; BRL). A20 cells were easily eliminated by forward scatter (FSC) gating. Markers were placed to define the boundaries for the $CD4^+CD8^+$ population, and then the number of the $CD4^+CD8^-$ cells (A and B) and $CD4^+CD8^+KJ1\text{-}26^{lo}$ cells (C and D) was calculated. The results shown represent the mean and the standard deviation of the calculated cell number. The experiment was repeated more than five times with similar results.

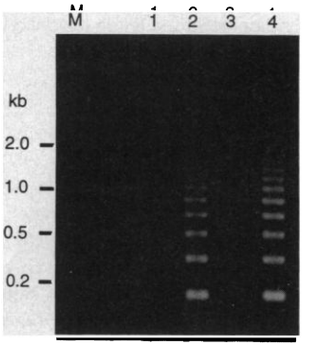

Fig. 2. DNA fragmentation induced in thymocytes by peptide antigen in an in vitro clonal deletion system. Thymocytes and A20 cells were cultured as described (Fig. 1), with either 0.02% dimethyl sulfoxide as vehicle control (lanes 1 and 2) or genistein (10 μg/ml) (lanes 3 and 4) for 15 min. Then, cOVA(324-334) (lanes 1 and 3) or cOVA(323-339) (lanes 2 and 4) was added to a final concentration of 1 μM. Cells were harvested after 5 hours of culture, because DNA fragmentation occurs earlier than the decrease of the $CD4^+CD8^+$ population. Fragmented DNA was prepared from 5 × 10^5 thymocytes as described (*22*), and samples were separated by electrophoresis on a 1.5% agarose gel containing ethidium bromide (0.5 μg/ml). Size markers (1-kb ladder marker; Gibco; BRL) are shown in lane M. The experiment was repeated three times with similar results.

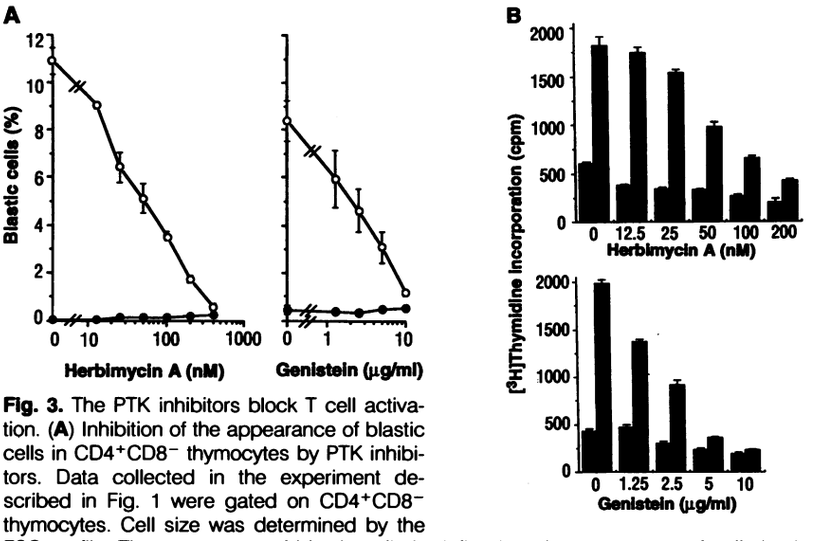

Fig. 3. The PTK inhibitors block T cell activation. (**A**) Inhibition of the appearance of blastic cells in $CD4^+CD8^-$ thymocytes by PTK inhibitors. Data collected in the experiment described in Fig. 1 were gated on $CD4^+CD8^-$ thymocytes. Cell size was determined by the FSC profile. The percentage of blastic cells is defined as the percentage of cells having values greater than 170 FSC units, the upper limit of FSC for unstimulated thymocytes (*13*). The percentage of blastic cells with cOVA(323-339) (open circles) or with cOVA(324-334) (closed circles) at the indicated concentrations of PTK inhibitors is shown. The results represent the mean and the standard deviation. (**B**) Inhibition of [^{3}H]thymidine incorporation of thymocytes by PTK inhibitors. DO10 transgenic thymocytes (5 × 10^5) were cultured with the indicated concentrations of herbimycin A or genistein for 2 hours on 2 × 10^5 $I\text{-}A^d$ transfected L cells (*16*) that had been treated with mitomycin C (50 μg/ml) (Sigma) for 30 min. The cOVA(323-339) (hatched bars) or cOVA(324-334) (black bars) peptide was added to a final concentration of 1 μM. Triplicate, 200-μl cultures were each pulsed with 5 μCi of [^{3}H]thymidine for 14 hours of a 20-hour culture. Floating cells were harvested, and the incorporation of [^{3}H]thymidine (cpm) was determined. The results represent the mean and the standard deviation of the incorporated cpm.

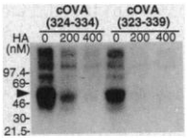

Fig. 4. The effect of herbimycin A on an in vitro immune-complex protein kinase assay of CD4-associated $p56^{lck}$. Thymocytes (10^7) and I-A^d transfected L cells (10^6) were cultured with the indicated concentration of herbimycin A (HA) for 2 hours. Then, the cOVA(324-334) or cOVA(323-339) peptide was added to a final concentration of 1 μM. After an additional 20 hours of culture, floating cells were harvested and solubilized in Nonidet P-40 buffer as described (*20*). Immunoprecipitation with anti-CD4 antibody (GK1.5) and an in vitro protein kinase assay were performed as described (*20*). Samples corresponding to 7.5×10^6 cells with cOVA(324-334) and to 2.5×10^6 cells with cOVA(323-339) were subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis and autoradiography (*23*). The arrowhead indicates the position of $p56^{lck}$. Molecular size markers are indicated at the left in kilodaltons.

thymocytes at higher concentrations, cOVA(323-339) and cOVA(324-334) had different deleting abilities (*19*).

Because the amount of $p59^{fyn}$ is small at the $CD4^+CD8^+$ stage when clonal deletion mainly occurs (*9*), $p56^{lck}$ may be more likely to be involved than $p59^{fyn}$ if PTK activity were involved in signaling leading to clonal deletion. To examine whether the PTK inhibitor blocked $p56^{lck}$ activity, we treated thymocytes with herbimycin A and lysed them after 20 hours. Immune complex kinase assays were performed as described (*20*). Consistent with our previous results (Fig. 3A), 400 nM herbimycin A almost completely blocked the autophosphorylation of $p56^{lck}$ (Fig. 4), although clonal deletion continued to occur. With antiphosphotyrosine immunoblot, we examined the phosphotyrosine profile in whole lysates of thymocytes during the in vitro clonal deletion system. No significant changes in the amount of phosphotyrosine were detected with or without the peptide (*19*).

Thus far, little is known about whether the difference between the signaling of clonal deletion and T cell activation is qualitative or quantitative. Here, we demonstrated that clonal deletion is independent of $p56^{lck}$, which suggests that the signaling machinery of clonal deletion is qualitatively distinct from that of T cell activation. Although other putative PTKs resistant to both herbimycin A and genistein might be involved in clonal deletion, we favor the possibility that the signaling pathway of negative selection is independent of PTK activity because such resistant PTKs have not been identified. Thus, the TCR may associate with another signaling mechanism independent of PTKs that is important in negative selection.

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In Vivo Calcium Elevations in Thymocytes with T Cell Receptors That Are Specific for Self Ligands

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Selection of the T cell receptor (TCR) repertoire in the thymus probably involves TCR-mediated signals transduced in developing thymocytes after interaction with thymic stromal cells bearing self ligands. TCR-transduced signals should have identifiable consequences that would distinguish thymocytes whose TCRs have been engaged by self ligands from those whose TCRs have not. Among thymocytes expressing a transgenic TCR of defined specificity, a large number had elevated intracellular calcium concentrations but only when resident in a negatively selecting thymus in which their self ligand was expressed. Thus, developing thymocytes are stimulated by endogenous ligands in vivo to mobilize intracellular calcium, and increased intracellular calcium concentrations may reflect the consequences of intrathymic signaling associated with thymic negative selection.

The developmental fate of individual T cells maturing in the thymus is determined by the specificity of the T cell antigen receptors they express. The maturation of thymocytes expressing TCRs with potential reactivity with self ligands is aborted by a process referred to as negative selection (*1*). Conversely, the maturation of thymocytes expressing TCRs that are potentially reactive with foreign antigens presented by self major histocompatibility complex (MHC)–encoded molecules is promoted by a process called positive selection (*2*, *3*). Because intrathymic selection events are TCR-specific, they are probably a consequence of receptor-mediated signals stimulated by interaction with thymic stromal cells bearing self ligands (*4*). As thymocytes mature, the TCRs that they express become competent to transduce signals mobilizing intracellular Ca^{2+} (*5*, *6*). However, developing thymo-

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