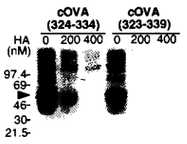

Fig. 4. The effect of herbimycin A on an in vitro immune-complex protein kinase assay of CD4-associated $p56^{lck}$. Thymocytes (10^7) and I-A^d transfected L cells (10^6) were cultured with the indicated concentration of herbimycin A (HA) for 2 hours. Then, the cOVA(324-334) or cOVA(323-339) peptide 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

Toshinori Nakayama,* Yuji Ueda, Hidehiro Yamada, Elizabeth W. Shores, Alfred Singer,† Carl H. June

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-

T. Nakayama, E. W. Shores, A. Singer, Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892.
Y. Ueda, H. Yamada, C. H. June, Immune Cell Biology Program, Naval Medical Research Institute, Bethesda, MD 20814.

*Present address: Department of Immunology, Faculty of Medicine, The University of Tokyo, 7-3-1 Hongo Bunkyo-ku, Tokyo 113, Japan.
†To whom correspondence should be addressed at Building 10 Room 4B-17, National Cancer Institute, Bethesda, MD 20892.

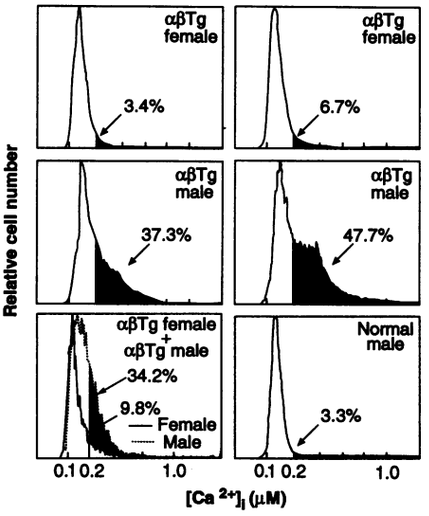

Fig. 1. Intracellular Ca^{2+} concentrations in unstimulated thymocytes from TCR-transgenic and normal mice as determined by flow cytometry (*12*). Unstimulated thymocytes from H-Y–specific TCRαβ transgenic (αβTg) male or female mice (*2*), as well as a transgene-negative normal littermate male mouse, were obtained and loaded with indo-1 to permit determination of their $[Ca^{2+}]_i$ (*12*). Upper and middle panels are histograms from two female and two male αβTg mice as indicated. Right lower panel is a histogram of a normal nontransgenic male littermate. Left lower panel is a cell mixing experiment in which unstimulated thymocytes from an αβTg male mouse were stained with a red dye [$DiIC_{18}$ (*5*)] and mixed with thymocytes from a female αβTg mouse. The cell mixture was loaded with indo-1 and warmed to 37°C, and steady-state Ca^{2+} histograms of the αβTg male and αβTg female thymocytes within the mixture were distinguished by gating on red fluorescence.

cytes have not yet been shown to transduce TCR signals in response to in vivo stimulation by endogenous ligands.

Consequently, we wished to determine if endogenous ligands stimulated developing thymocytes in vivo to mobilize intracellular Ca^{2+}. In preliminary experiments we used flow cytometry to measure the concentration of free intracellular Ca^{2+} ($[Ca^{2+}]_i$) in freshly prepared thymocytes from normal mice that had been loaded with the Ca^{2+}-sensitive dye indo-1. Even though these thymocytes had not been intentionally stimulated by any exogenous ligand, we found that approximately 3% had significant elevations of $[Ca^{2+}]_i$ with concentrations >0.2 μM (Fig. 1, right lower panel). Simultaneous determination of their CD4-CD8 phenotype revealed that thymocytes with a high Ca^{2+} concentration were present in each CD4-CD8 subset but that the majority were $CD4^+CD8^+$, the thymocyte subset in which most thymic selection events are thought to occur. To examine the relationship between $[Ca^{2+}]_i$ and intrathymic signaling, we used anti–H-Y TCRαβ transgenic (αβTg) mice (*2*). Most T cells in αβTg mice express the transgenic TCR whose ligand is the male antigen H-Y plus H-$2D^b$. The αβTg thymocytes in thymi of H-2^b female mice that express the MHC-restricting element D^b are positively selected but are negatively selected in thymi of H-2^b male mice that express both D^b and H-Y (*2*). The frequency of thymocytes with a high Ca^{2+} concentration was increased in negatively selecting male mice but not in female mice (Fig. 1). Because it is their TCR that permits αβTg thymocytes to distinguish between male and female thymi, this result indicates that elevations in intracellular Ca^{2+} in thymocytes from αβTg male mice result from engagement of

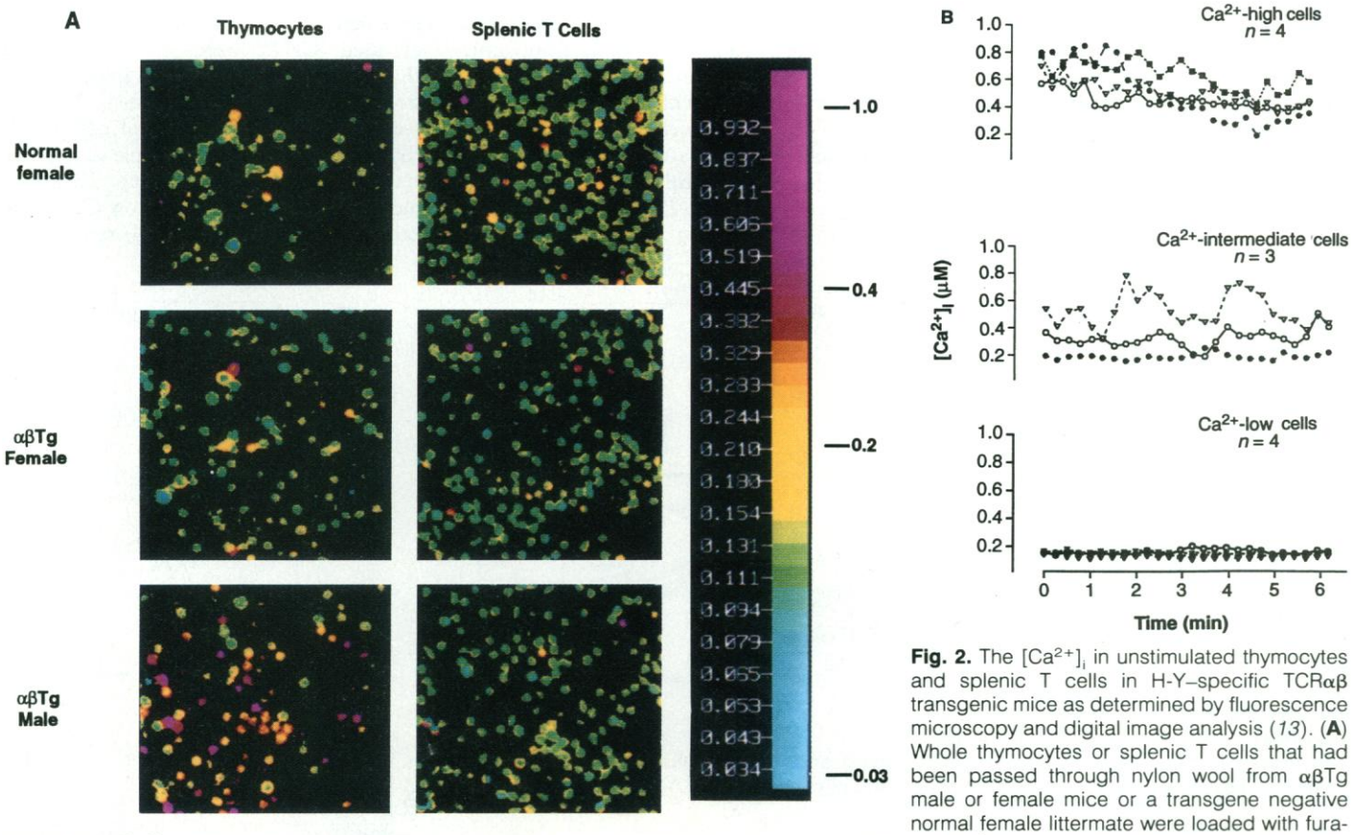

Fig. 2. The $[Ca^{2+}]_i$ in unstimulated thymocytes and splenic T cells in H-Y–specific TCRαβ transgenic mice as determined by fluorescence microscopy and digital image analysis (*13*). (**A**) Whole thymocytes or splenic T cells that had been passed through nylon wool from αβTg male or female mice or a transgene negative normal female littermate were loaded with fura-2, and their $[Ca^{2+}]_i$ was determined by video imaging. The hue of the cells in the picture represents $[Ca^{2+}]_i$, whose scale (micromolar) is shown on the right. (**B**) The $[Ca^{2+}]_i$ of representative individual cells from H-Y–specific TCRαβ transgenic male thymocytes was monitored every 15 s for 6 min at 37°C and was plotted separately on the basis of the initial $[Ca^{2+}]_i$, with Ca^{2+}-high cells >0.55 μM; Ca^{2+}-intermediate cells 0.2 μM to 0.55 μM; and Ca^{2+}-low cells <0.2 μM.

Table 1. $[Ca^{2+}]_i$ in αβTg male thymocytes expressing endogenous TCRα chains. Thymocytes from αβTg male mice were loaded with indo-1, and Ca^{2+} histograms were obtained (*12*). Alternatively, indo-1–loaded thymocytes were stained with MAb to the transgene idiotype (T3.70) and phycoerythrin-conjugated goat antibodies to mouse immunoglobulin G1. The cells were analyzed by flow cytometry with fluorescence excitation provided by dual argon ion lasers, one tuned for ultraviolet emission and the other tuned at 488 nm for phycoerythrin excitation. Ca^{2+} histograms of the id^- thymocytes were determined by electronic gating on phycoerythrin negative cells. Thymocytes with a high Ca^{2+} concentration (Ca^{2+}-high) were those with $[Ca^{2+}]_i$ >0.2 μM.

αβTg mouse	H-2	Ca^{2+}-high thymocytes		Id^- thymocytes
		Percent of total	Percent of id^-	Percent of total
3761	d	6	7	30
3764	b	18	4	10
3765	b	22	6	6

the transgenic TCRαβ by its antigenic ligand.

Although we thought that these results reflected in situ engagement of their transgenic TCRαβ, it is possible that thymocytes from H-2^b male mice might have stimulated each other during the in vitro assay because they expressed small amounts of the antigenic ligand D^b + H-Y on their cell surfaces. We assessed this possibility by mixing male and female thymocytes from H-2^b αβTg mice, loading the mixed cells with indo-1, and assessing the mixed cells for $[Ca^{2+}]_i$ (Fig. 1). To distinguish male from female thymocytes in the cell mixture, we stained the male thymocytes with a red surface dye before mixing them with female cells. The percentage of αβTg female thymocytes with elevated $[Ca^{2+}]_i$ was far less than that of αβTg male thymocytes in the same cell mixture, and the percentage of αβTg female thymocytes with elevated $[Ca^{2+}]_i$ was not different in the presence (9.8%) or absence (11%) of male thymocytes. Thus, elevations in $[Ca^{2+}]_i$ did not result from in vitro stimulation occurring during the assay.

To ascertain that elevations in $[Ca^{2+}]_i$ resulted from in vivo stimulation of each thymocyte's TCR and not from nonspecific lymphokines present in a negatively selecting thymus, we determined $[Ca^{2+}]_i$ in thymocytes that reside within a negatively selecting thymus but do not themselves express self-reactive TCR. In αβTg mice, virtually all T cells express the TCRβ transgene; however, a fraction of T cells express endogenously encoded TCRα chains rather than the TCRα transgene, and these T cells fail to be stained with the T3.70 anti-idiotypic reagent that is specific for the TCRαβ transgenic dimer (*7*). Because idiotype-negative (id^-) thymocytes do not express the TCRα transgene, they would not be specific for the D^b + H-Y antigenic ligand. In three individual αβTg male mice, the frequency of id^- thymocytes was highest in the nonselecting H-2^d thymus that did not express the D^b-selecting element for the TCRαβ transgenic receptor and was lowest in H-2^b mice that expressed the H-$2D^b$–selecting element (Table 1). More importantly, the frequency of id^- thymocytes with elevated $[Ca^{2+}]_i$ was not increased (Table 1). Thus, within a negatively selecting thymus, elevations in $[Ca^{2+}]_i$ occurred predominantly in the id^+ thymocyte population, indicating that elevations in $[Ca^{2+}]_i$ result from ligand-induced TCR-mediated stimulation of each thymocyte.

Elevations in $[Ca^{2+}]_i$ in individual thymocytes may be sustained or intermittent and oscillatory. Indeed, we found that the $[Ca^{2+}]_i$ elevations after TCR stimulation by cell-bound ligands were significantly prolonged relative to those resulting from TCR stimulation by soluble monoclonal antibody (MAb) and could be best appreciated by video microscopy in which the $[Ca^{2+}]_i$ in individual cells was determined over time. Consequently, resting thymocytes and splenic T cells from αβTg male and female mice were loaded with the Ca^{2+}-sensitive dye fura-2 and placed in short-term cultures in microscope chambers, and their $[Ca^{2+}]_i$ was determined by video imaging (Fig. 2A). As we had observed by flow cytometry, the frequency of unstimulated αβTg thymocytes with elevated $[Ca^{2+}]_i$ was increased in thymocytes from negatively selecting male mice. Moreover, this finding appeared to be developmentally regulated and organ-specific, as male T cells with elevated $[Ca^{2+}]_i$ were seen only in the thymus and not in peripheral spleen T cells from the same individual animals. We then recorded $[Ca^{2+}]_i$ in individual αβTg male thymocytes, obtaining multiple determinations from the same cells (Fig. 2B). Thymocytes that initially had a low Ca^{2+} concentration ($[Ca^{2+}]_i$ <0.2 μM) remained so

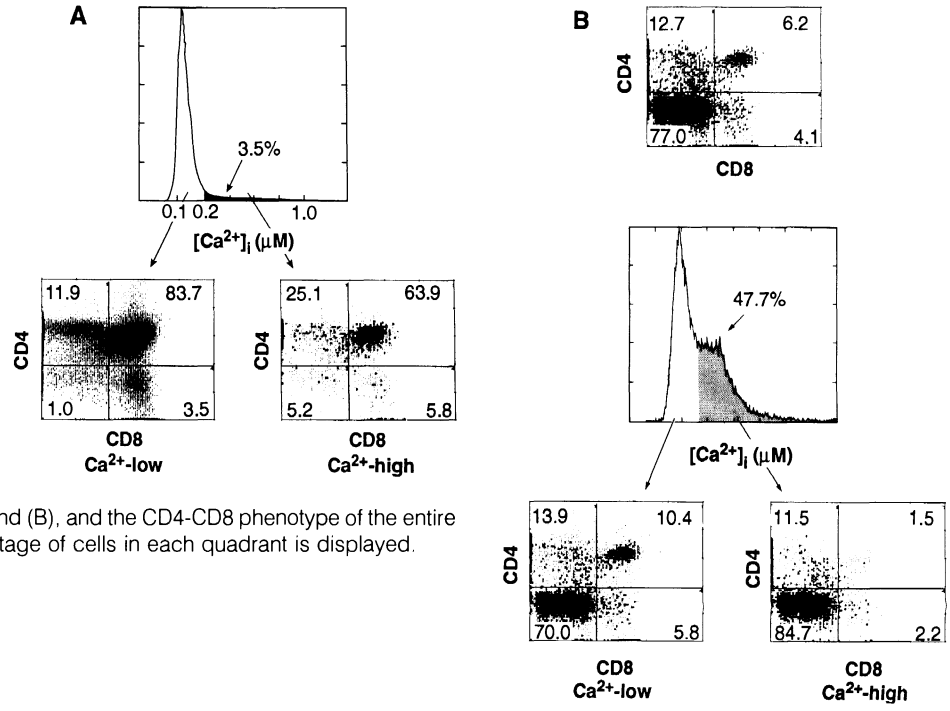

Fig. 3. CD4-CD8 phenotypes of normal and αβTg male thymocytes with elevated $[Ca^{2+}]_i$. Fresh thymocytes were prepared from young adult H-2^b normal (**A**) or αβTg (**B**) male mice. After loading with indo-1, thymocytes were stained at 4°C with phycoerythrin-conjugated anti-CD4 [MAb GK1.5 (*14*)] and fluorescein isothiocyanate–conjugated anti-CD8 [MAb 53-6.72 (*15*)] and then kept at 4°C until 10 min before measurement, when the cells were warmed to 37°C. Steady-state $[Ca^{2+}]_i$ histograms of unstimulated thymocytes were then determined by flow cytometry. The anti-CD4 and anti-CD8 staining antibodies did not affect the measurement of $[Ca^{2+}]_i$ in these thymocytes as identical measurements were obtained in parallel in the absence of anti-CD4 and anti-CD8 staining (*16*). CD4-CD8 profiles of thymocytes with elevated $[Ca^{2+}]_i$ (>0.2 μM) and baseline $[Ca^{2+}]_i$ (< 0.2 μM) are shown in (A) and (B), and the CD4-CD8 phenotype of the entire thymocyte population is shown in (B). The percentage of cells in each quadrant is displayed.

throughout the experiment, and thymocytes that initially had a high Ca^{2+} concentration ($[Ca^{2+}]_i$ >0.2 μM) remained so, although their $[Ca^{2+}]_i$ oscillated over time. Thus, observed elevations in $[Ca^{2+}]_i$ in thymocytes were not sporadic events of the entire population of thymocytes but rather indicated heterogeneity within the population of thymocytes. Moreover, the oscillations in $[Ca^{2+}]_i$ in thymocytes with large and moderate $[Ca^{2+}]_i$ indicated that these were not terminal elevations of $[Ca^{2+}]_i$ in moribund cells. Indeed, these thymocytes were viable cells by several criteria. (i) They excluded both Trypan blue and propidium iodide. (ii) They could be loaded with and retain indo-1 and fura-2, which requires intracellular esterase activity as well as intact plasma membranes. (iii) Their DNA was intact and not fragmented (*8*).

Finally, we assessed the CD4-CD8 phenotype of αβTg male thymocytes with elevated $[Ca^{2+}]_i$ (Fig. 3). Unlike most thymocytes with a high Ca^{2+} concentration from normal mice (Fig. 3A), thymocytes with a high Ca^{2+} concentration from αβTg male mice (Fig. 3B) were almost entirely $CD4^-CD8^-$, the thymocyte subset that is disproportionately increased in negatively selecting αβTg male mice (Fig. 3B) (*2*). MHC class I–restricted transgenic T cells undergoing negative selection can avoid clonal deletion by reducing their surface expression of CD8 (*7*), which provides an explanation for the increased frequency of $CD4^-CD8^-$ thymocytes in αβTg male mice. We have found that the $CD4^-CD8^-$ phenotype is a specific consequence of antigen encounter and negative selection among developing αβTg male thymocytes (*9*). Thus, the $CD4^-CD8^-$ phenotype of αβTg male thymocytes with elevated $[Ca^{2+}]_i$ is concordant with their having recently undergone and survived thymic negative selection.

Our data indicate that endogenous ligands do stimulate developing thymocytes to mobilize intracellular Ca^{2+} in vivo and that such intrathymic signaling events are evident in thymocytes expressing self-reactive TCR. In vivo elevations in $[Ca^{2+}]_i$ serve as markers of thymocytes whose TCRs have been stimulated by self ligands in the thymus and thus have been subjected to thymic negative selection. We presume that thymocytes that have been negatively selected in vivo to undergo apoptosis also have elevated $[Ca^{2+}]_i$, but most αβTg thymocytes with elevated $[Ca^{2+}]_i$ that are present at the time of measurement are cells that have survived negative selection. Finally, there was no definitive indication in our data that positive selection events in the thymus affected $[Ca^{2+}]_i$, but this issue requires further study.

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12. Thymocytes (1×10^7) were loaded with 1.8 μM indo-1 at 31°C for 30 min in 1 ml of Hanks solution supplemented with 1.0% fetal calf serum, 20 mM Hepes, and 5×10^{-5} M 2-mercaptoethanol (indo medium) (*10*). Cells were washed, incubated at 37°C for 10 min, and then assessed by flow cytometry at 800 cells per second (10^5 cells per histogram) (*10*). In the cell mixture experiment in Fig. 1, the male thymocytes were incubated with 30 μM $DiIC_{18}$ (*5*) for 5 min at 37°C, washed twice, and then mixed with equal numbers of female αβTg thymocytes. The cell mixture was loaded with indo-1, equilibrated at 37°C, and analyzed on a dual laser flow cytometer. Separate analysis of thymocytes from these mice loaded with indo-1 only, and not mixed together, showed that 40% of male and 11% of female αβTg thymocytes had $[Ca^{2+}]_i$ >0.2 μM.
13. The fluorescence digital image processing system used was similar to that of R. Y. Tsien and A. T. Harootunian (*11*). Hardware consisted of an FD5000 image processor (Gould), a Zeiss Axiovert microscope with a 1.3 numerical aperture ×40 objective, and a filter changer (Ludl Electronic Products, Hawthorne, NY) with excitation filters centered at 350 ± 10 nm and 380 ± 10 nm. The image processor and filter changer were interfaced to a Microvax host computer (Digital Equipment Corporation). Images were acquired through a charge-coupled device camera (Cohu, San Diego, CA) and image intensifier (Video Scope). The images were averaged, the background was subtracted, and a shading correction was applied (*11*).
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Selection of Drug-Resistant Bone Marrow Cells in Vivo After Retroviral Transfer of Human *MDR*1

Brian P. Sorrentino,* Stephen J. Brandt, David Bodine, Michael Gottesman, Ira Pastan, Amanda Cline, Arthur W. Nienhuis

Experiments were performed to determine if retroviral-mediated transfer of the human multidrug resistance 1 gene (*MDR*1) into murine bone marrow cells would confer drug resistance to the cells and whether the *MDR*1 gene could be used as a dominant selectable marker in vivo. When mice transplanted with bone marrow cells containing a transferred *MDR*1 gene were treated with the cytotoxic drug taxol, a substantial enrichment for transduced bone marrow cells was observed. This demonstration of positive selection establishes the ability to amplify clones of transduced hematopoietic cells in vivo and suggests possible applications in human therapy.

The failure of certain human tumors to respond to continued chemotherapy is frequently associated with enhanced expression of the multidrug resistance gene, *MDR*1 (*1*). The *MDR*1 gene product, a 170-kD transmembrane protein also known as P-glycoprotein, is an energy-dependent drug efflux pump. This protein extrudes many types of natural products from the cell, including frequently used chemotherapeutic agents, thereby conferring a drug-resistant phenotype to cells expressing this protein.

Bone marrow toxicity of chemotherapeutic drugs is often a dose-limiting side effect in the treatment of cancer patients. Transfer of the *MDR*1 gene to bone marrow cells would therefore be an attractive approach to overcome the myelosuppression associated with such drugs. Earlier studies with a line of transgenic mice containing human *MDR*1 coding sequences showed that these animals were protected from the myelosuppressive effects of a number of natural product chemotherapeutic agents (*2*). A recent study showed that transplan-