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The Involvement of an ATP-Gated Ion Channel, P_{2X1}, in Thymocyte Apoptosis

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Summary

In the immune system, apoptosis is involved in intrathymic elimination of self-reactive thymocytes and in peripheral T cell tolerance to exogenous antigens. Here, we describe the role in T cell apoptosis of P_{2X1}, a nonselective cation channel activated by ATP. P_{2X1} molecules are up-regulated in thymocytes during dexamethasone-induced apoptosis, and antagonists to these receptors protect thymocytes from cell death. Moreover, P_{2X1} mRNA and protein levels increase in thymocytes induced to die in vivo by the superantigen staphylococcal enterotoxin B. In contrast, T cells undergoing apoptosis in the periphery do not express P_{2X1}. The demonstration that P_{2X1} ion channels play a role in the apoptosis of thymocytes but not peripheral T cells illustrates a novel mechanism contributing to thymocyte cell death and opens new possibilities for investigating clonal deletion in the thymus.

Introduction

For the immune system, programmed cell death, or apoptosis, is a critical event leading to selective lymphocyte development. For instance, thymocytes recognizing self-antigen are eliminated by this mechanism in order to delete autoreactive cells and ensure immune tolerance in the periphery. Apoptosis is known to be induced by antigenic stimulation via the T cell receptor (TCR) (von Boehmer et al., 1989; Fowlkes et al., 1988; Pircher et al., 1989; MacDonald and Lees, 1990) or through the glucocorticoid receptor in immature thymocytes (Wyllie, 1980; Cohen and Duke, 1984). However, only very recently, a third pathway has been proposed. This involves the lytic properties of extracellular ATP (Zheng et al., 1991; Pizzo et al., 1991). In the biochemical pathway leading to apoptosis, a key event is the elevation of intracellular calcium levels (Nicotera et al., 1989; McConkey et al., 1989a, 1989b, 1989c). This directly promotes the activation of endonucleases and DNA cleavage (Zhivotovsky et al., 1994). Extracellular ATP causes changes in the plasma membrane potential of thymocytes and calcium influx (Pizzo et al., 1991; Matkó et al., 1993). These observations suggest that the receptors for extracellular ATP could play an important role in apoptosis. To date, three types of ATP receptors, referred to as P2 purinoceptors, have been identified: ligand-gated ion channels (P_{2X}), G protein–coupled receptors (P_{2Y} and P_{2U}), and an ATP-gated pore (P_{2Z}) (Abbracchio and Burnstock, 1994). Among the receptors for extracellular ATP, P_{2X} receptors are nonselective cation channels with significant permeability to calcium (Burnstock, 1990; Bean, 1992). Several P_{2X} receptors have been cloned from excitable cell types such as neurons and smooth muscle cells (Valera et al., 1994; Brake et al., 1994; Buell et al., 1996a; Lewis et al., 1995; Chen et al., 1995; Surprenant et al., 1996). A possible role for one of these, P_{2X1}, in T cell apoptosis was highlighted by its sequence homology with a partial cDNA clone, RP-2, previously isolated by subtractive hybridization between apoptotic and nonapoptotic rat thymocytes (Owens et al., 1991).

In this study, we investigated the expression and function during T cell death of this newly cloned member of the P_{2X} family, P_{2X1}. Since the role of ATP receptors during the engagement of the antigen receptor on T cells has not been investigated, we have also examined the expression of P_{2X1} in thymocytes and lymphocytes from mice after staphylococcal enterotoxin B (SEB) injection. This research reveals that the ATP receptor P_{2X1} may be selectively involved in the process of immature thymocyte apoptosis, but not in that of peripheral T lymphocytes.

Results

Expression of P_{2X1} mRNA in Thymocytes during DEX-Induced Apoptosis

Since the partial sequence of P_{2X1} represented by RP-2 was cloned from immature thymocytes undergoing programmed cell death, we first determined whether P_{2X1} expression increased during apoptosis. To do this, we analyzed RNA from dexamethasone (DEX)-treated rat thymocytes by Northern blot using the rat vas deferens P_{2X1} cDNA probe. RNA from rat vas deferens was used as a positive control. In the vas deferens RNA, three major transcripts of 3.6, 2.6, and 1.8 kb and a minor one of 4.4 kb were detected. We have cloned these major transcripts and sequenced the corresponding cDNA. They result from variable length 3′ in the untranslated regions. Northern blot analysis confirmed that the three P_{2X1} mRNA transcripts (1.8, 3.1, and 4.4 kb) previously detected in rat thymus (Valera et al., 1994) increased in thymocytes that were induced to die by DEX (Figure 1A). Density scanning of the Northern blot and normalization with respect to β-actin expression confirmed that P_{2X1} mRNAs (1.8, 3.1, and 4.4 kb) are on average 8-fold more abundant after DEX treatment. These P_{2X1} transcripts were not detected before DEX treatment, as the dead and apoptotic cells naturally present in the thymus were initially removed by density sedimentation. The smallest mRNA (1.8 kb) seen in thymocytes corresponded in size

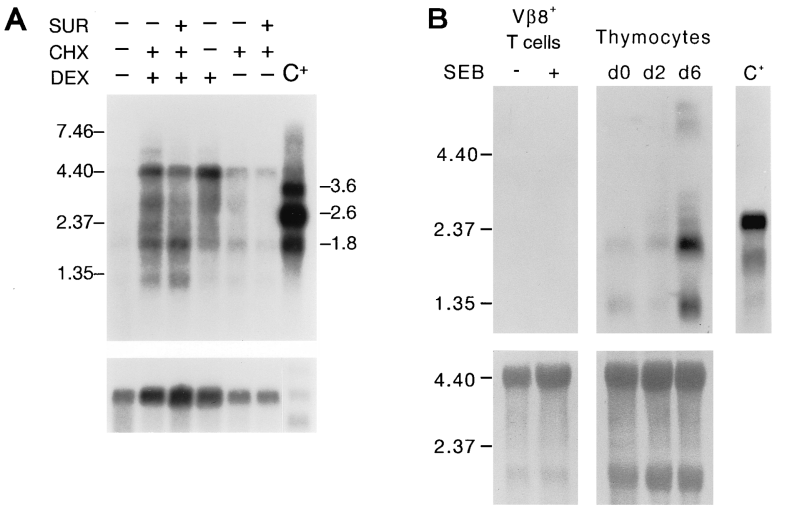

Figure 1. P_{2X1} mRNA Is Expressed in Apoptotic Thymocytes, but Not in Apoptotic Mature T Cells

(A) Northern blot assay for P_{2X1} mRNA was performed using 2 μg of poly(A)$^+$ RNA prepared from purified viable rat thymocytes incubated in the presence or absence of DEX (1 μM), 50 μg/ml cycloheximide (CHX), or 100 μM suramin (SUR) for 4 hr at 37°C. Positive control (C+) was obtained with 50 ng of poly(A)$^+$ RNA from rat vas deferens. Top, poly(A)$^+$ RNA hybridized with P_{2X1} [^{32}P]riboprobe; bottom, poly(A)$^+$ RNA hybridized with human actin [^{32}P]riboprobe.

(B) Northern blot assay for P_{2X1} mRNA was performed using 3.6 μg of total RNA isolated from V_β8.2-positive T lymphocytes without or with SEB-induced apoptosis in vitro (left panel), 5 μg of total RNA isolated from thymocytes before and after SEB injection (middle panel), and 0.2 μg of poly(A)$^+$ RNA from urinary bladder used as a positive control (right panel). In the top panels, RNAs were hybridized with a mouse urinary bladder P_{2X1} digoxigenin riboprobe. In the bottom panels, RNAs were stained with methylene blue.

and sequence (data not shown) to the P_{2X1} expressed in mouse urinary bladder (Valera et al., 1995). The size of one of the other transcripts differed slightly between the cells from the thymus and the cells from the vas deferens (3.1 instead of 2.6 kb, respectively), confirming previous observations (Valera et al., 1994). This may be due to differential splicing of the gene, but that remains to be proven. As observed for RP-2 (Owens et al., 1991), the accumulation of P_{2X1} mRNA was augmented when cycloheximide was added to the culture. Suramin, an antagonist of P_{2X1}, had no effect on P_{2X1} mRNA expression. The results from this experiment show that P_{2X1} is a death-associated mRNA that appears upon induction of a death program in thymocytes.

Among the thymocytes there are phenotypically distinct populations, immature ($CD4^-CD8^-$ and $CD4^+CD8^+$) and mature ($CD4^+CD8^-$ and $CD4^-CD8^+$) thymocytes. Immature double positive cells ($CD4^+CD8^+$) are the most sensitive to the induction of apoptosis by DEX. To correlate P_{2X1} induction with apoptosis, we have studied the expression of the P_{2X1} molecule in these thymocyte subsets after DEX treatment using an antibody directed against the C-terminus of P_{2X1}. Because this antibody reacts with an intracellular portion of the receptor, cytospins were performed with subpopulations of DEX-treated thymocytes sorted for CD4 and CD8 expression and then analyzed for P_{2X1} (Table 1). DEX treatment induced P_{2X1} molecule expression only in double positive immature ($CD4^+CD8^+$) thymocytes. A method for detecting cells with DNA breaks (ApopTag) confirmed that those immature thymocytes were undergoing apoptosis. In addition, we observed that apoptotic thymocytes sorted by flow cytometry expressed P_{2X1} molecules (Table 1). These data demonstrate a correlation between cell death and P_{2X1} molecule expression in immature thymocytes.

ATP Antagonists Rescue Thymocytes from Cell Death

Since the level of expression of P_{2X1} mRNA is increased in thymocytes undergoing apoptosis, we tested whether apoptosis is dependent on P_{2X1} receptor activation by extracellular ATP by adding exogenous ATP to thymocytes treated with a suboptimal concentration of DEX. As shown in Figure 2a, extracellular ATP has a biphasic effect on DEX-treated thymocytes. The enhancement in cell death was triggered by low concentrations of ATP (1–10 μM) and by higher amounts of ATP with a half-maximal inhibitory concentration (IC_{50}) of 100 μM. ADP acted similarly, although ADP was less potent at high concentrations (an IC_{50} of 400 μM), and UTP was inactive. Addition of apyrase, an enzyme that destroys extracellular ATP (Buell et al., 1996b), significantly decreased DEX-induced thymocyte apoptosis (Figure 2b), probably by degrading ATP released by dying cells present in the culture medium. These in vitro data support the role of ATP in DEX-induced thymocyte apoptosis.

Certain physiological and pharmacological properties of purinoceptors, which have been described previously (Humphrey et al., 1995), allow distinctions to be made between them. P_{2X1} receptors respond to αβ-methylene-ATP (α,βmeATP) and show a marked desensitization

Table 1. Expression of P_{2X1} Molecules on the Surface of Apoptotic Immature Thymocytes

	Apoptosis (%)		P_{2X1} Expression (%)	
Thymocytes	−DEX	+DEX	−DEX	+DEX
$CD4^-CD8^-$			–	7
$CD4^+CD8^+$	4	60	7.5	100
$CD4^+$ or $CD8^+$	4	18	6	4
FACS-sorted apoptotic thymocytes		100		100

Thymocytes were cultured for 6 hr without or with 1 μM DEX. CD4 and CD8 antigens were labeled with H129.19–phycoerythrin and 53-6.7–FITC (Pharmingen), respectively, and the four populations were sorted by flow cytometry. Apoptotic thymocytes were FACS sorted on the basis of the ratio between Hoechst 33342 and propidium iodide. Cytospins were performed with each sorted population, and P_{2X1} expression was analyzed using a rabbit antibody directed against the C-terminus of P_{2X1}. Apoptosis was determined using the ApopTag kit.

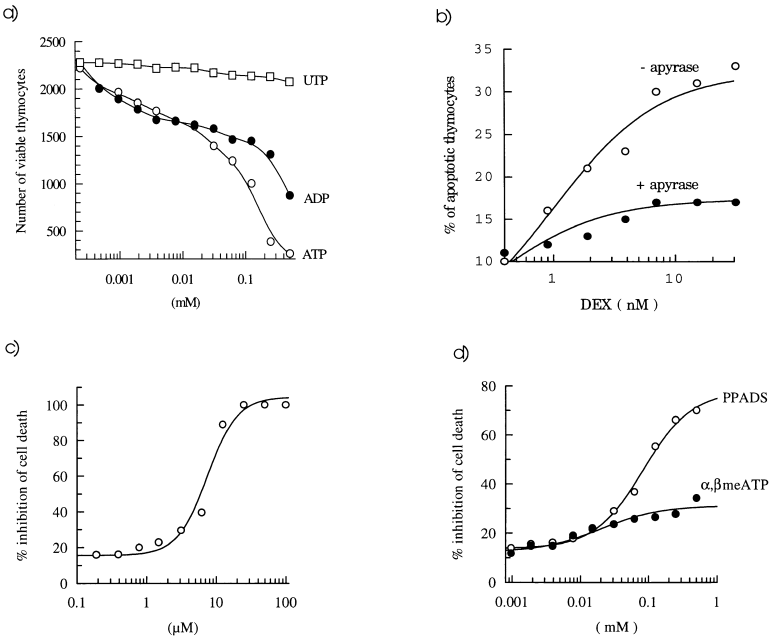

Figure 2. P_{2X1} Involvement in DEX-Induced Thymocyte Cell Death

Thymocyte suspensions were cultured at 2×10^6 cells per milliliter at 37°C over 16 hr as follows: (a) in the presence of DEX (5 nM) with ATP (open circles), ADP (closed circles), or UTP (open squares); (b) DEX-induced thymocyte apoptosis in the absence (open circles) or presence (closed circles) of apyrase (30 U/ml). Also shown is inhibition of DEX-induced thymocyte cell death by suramin (c), PPADS (open circles in [d]), and α,βmeATP (closed circles in [d]). These experiments (except [b]) were performed using a serum-free medium. Thymocytes were labeled with 10 μM bis-benzimide Hoechst 33342 and propidium iodide before cell viability analysis by FACS. After 16 hr of incubation at 37°C, 89 ± 8% of the thymocytes were alive, whereas in the presence of 5 nM of DEX, 50 ± 3% were alive, 25 ± 7% were apoptotic, and 20 ± 5% were dead (n = 4). In (a), results are expressed as the number of living thymocytes per 5×10^3 cells. In (c) and (d), the percent inhibition of cell death has been calculated based on the number of apoptotic and dead cells versus viable thymocytes. One representative experiment is shown.

(Valera et al., 1994; Kasakov and Burnstock, 1983). These receptors are also blocked by antagonists, including suramin (Leff et al., 1990) and pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) (Lambrecht et al., 1992). In our system, both antagonists, suramin (Figure 2c) and PPADS (Figure 2d), protected thymocytes from DEX-induced apoptosis in a dose-dependent manner (IC_{50} of 8 μM and 80 μM, respectively). The consequence of P_{2X1} activation by α,βmeATP was a partial inhibition of thymocyte cell death (Figure 2d). In the continued presence of receptor agonists (i.e., ATP or α,βmeATP), P_{2X1} receptors are desensitized. This implies a decrease in the channel function, but it is important to note that the channels remain active at a lower rate (Evans et al., 1995). It has been reported in many studies that responses mediated via P_{2X} purinoceptors are about 100 times more effectively evoked by stable analogs of ATP, such as α,βmeATP, than by ATP itself (Burnstock and Kennedy, 1985; Hoyle and Burnstock, 1991; Cusack, 1993). Also, in many tissues ATP-evoked responses are very short-lived, whereas those evoked by α,βmeATP are prolonged. This difference in stability between ATP and α,βmeATP may explain why ATP enhanced apoptosis while α,βmeATP inhibited it slightly. In addition, the partial agonist properties of α,β-meATP (Cusack, 1993) probably explain why the inhibition of cell death observed in the presence of α,βmeATP was not as impressive as that observed in the presence of the two antagonists, PPADS and suramin, which completely block the receptor. These results suggest a direct role for P_{2X1} in thymocyte apoptosis.

Correlation between P_{2X1} Expression and Cell Death In Vivo

To extend the in vitro finding that P_{2X1} is associated with immature thymocyte apoptosis, we used the bacterial superantigen SEB to determine whether P_{2X1} could be shown to play a role in clonal deletion of thymocytes and peripheral T cells in vivo. SEB is known to induce tolerance either by a rapid elimination by apoptosis (Kawabe and Ochi, 1991) or by functional inactivation of peripheral SEB-reactive V_β8-positive T cells (Rellahan et al., 1990). The levels of P_{2X1} mRNA in cells from lymph nodes and thymus were therefore assessed by Northern blot analysis either before or after SEB injection in mice. The amount of P_{2X1} mRNA (1.8 kb) was significantly increased in the thymus at day 6 (Figure 1B, middle). Interestingly, no induction of P_{2X1} mRNA was observed at any time in the lymph node cells (data not shown). The increase in P_{2X1} expression in the thymus raised the possibility that P_{2X1} purinoceptors may play a role when apoptosis is initiated by superantigen in vivo. Moreover, these data suggest a differential expression between thymocytes and peripheral T cells.

The lack of P_{2X1} expression in peripheral T cells was further investigated using TCR V_β8.2-positive transgenic mice. These SEB-reactive T lymphocytes are destined to die after a secondary in vitro stimulation with the superantigen, SEB (Ettinger et al., 1995). Northern blot analysis of P_{2X1} mRNA expression in V_β8.2-positive T cells after SEB-induced apoptosis in vitro revealed a lack of P_{2X1} mRNA expression (Figure 1B, left). These data demonstrate that P_{2X1} expression is linked to SEB-induced apoptosis in thymocytes, but not in peripheral T lymphocytes.

The time course of P_{2X1} expression after SEB injection was further evaluated in the thymus by immunohistochemistry. Tissue sections were double labeled with antibodies to P_{2X1} molecules and a T cell marker, Thy-1.2. P_{2X1}-positive T cells were only detected in the thymus (Figure 3) and not in the lymph nodes (data not shown). The majority of P_{2X1}-positive cells were also positive for the T cell marker, confirming the expression of P_{2X1} on thymocytes (Figures 3A–3F). The single labeling in red

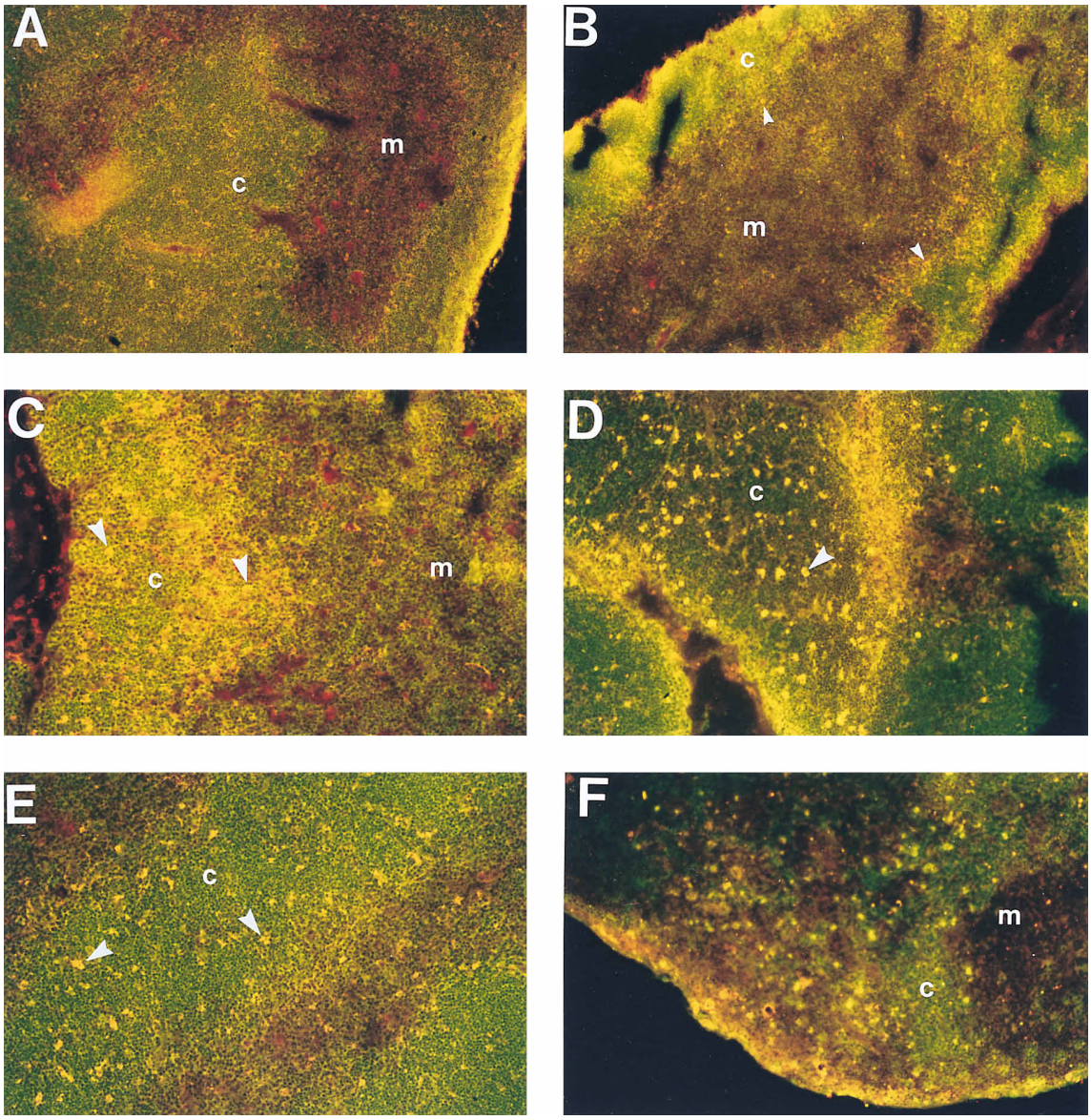

Figure 3. In Situ Detection of P_{2X1}-Expressing Thymocytes by Double Labeling of Thymic Sections following SEB Injection

Sections were sequentially labeled with anti-P_{2X1} antibody (rhodamine) and anti-Thy-1.2 monoclonal antibody, a T cell marker (FITC). The yellow labeling reveals thymocytes expressing P_{2X1}. (A) is before immunization; (B) is 2 days (magnification, ×10), (C) is 4 days, (D) is 6 days, (E) is 8 days, and (F) is 14 days after immunization (magnification, ×16). Thymic cortex (c) and medulla (m) are indicated; arrows indicate thymocytes expressing P_{2X1}.

observed in the thymus reflected the expression of P_{2X1} in small arteries and arterioles (Valera et al., 1994). The number of double-labeled cells in the thymus increased with time. Labeled cells accumulated in the cortex area at day 2 (Figure 3B). At day 4, these cells appeared to form a gradient, which peaked in the cortico–medullary junction (Figure 3C). From day 6, many of the P_{2X1}-positive cells appeared in the form of aggregates scattered in the cortex, possibly reflecting dying cells engulfed by macrophages (Surh and Sprent, 1994). P_{2X1} expression induced by SEB then decreased in a time-dependent fashion (Figures 3D–3F). The location of P_{2X1}-positive cells in the cortex of the thymus (Figure 3C) strongly suggests that these cells are immature thymocytes.

To confirm that the induction of P_{2X1} expression in the thymus was driven by the bacterial superantigen, we double labeled tissue sections with antibodies to P_{2X1} molecules and expressed the TCR $V_\beta 8$ domain by SEB-reactive T cells or, as a control, the TCR $V_\beta 6$ domain by SEB-unresponsive T cells. As seen in Figures 4B and 4C, 2 days after SEB injection $V_\beta 8$-positive, but not $V_\beta 6$-positive, T cells expressed P_{2X1}. Interestingly, not all $V_\beta 8$-positive thymocytes expressed P_{2X1}, as would be expected if apoptosis of mature thymocytes does not involve P_{2X1}. Moreover, the number of $V_\beta 8$-positive T cells in thymus could not account for all P_{2X1}-positive cells that were detected by immunohistochemistry, especially at day 4 after SEB injection (data not shown), suggesting

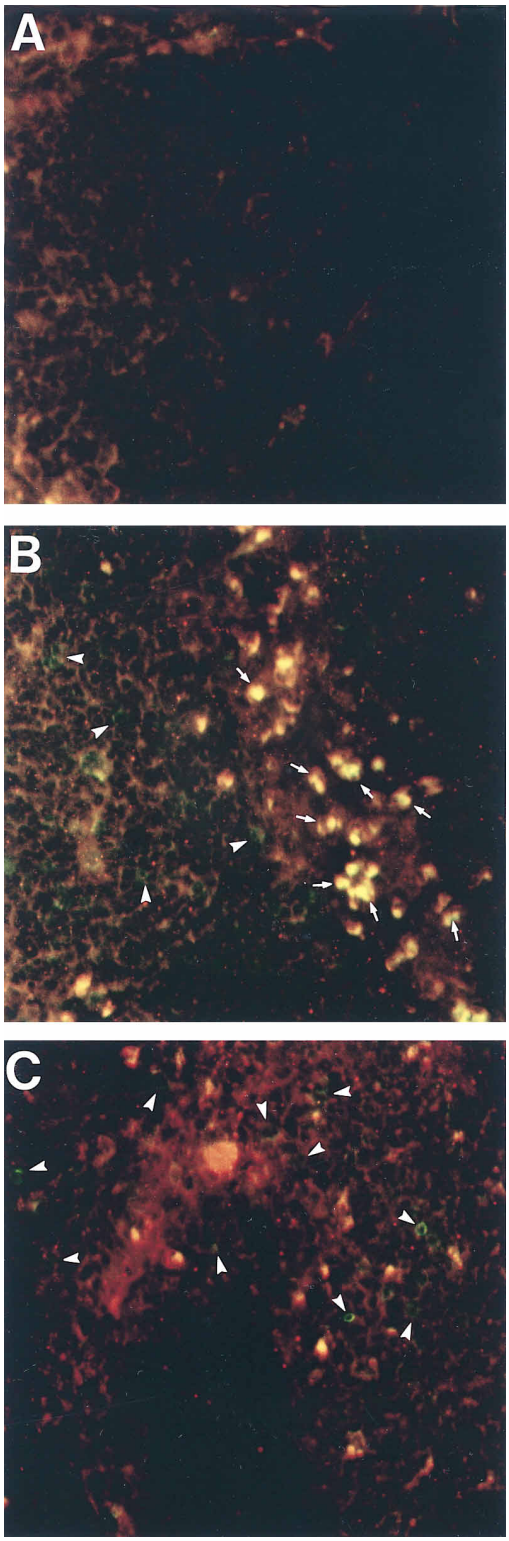

Figure 4. In Situ Detection of P_{2X1} Expression on $V_\beta 8$- and $V_\beta 6$-Positive Thymocytes following SEB Injection

Sections were sequentially labeled with anti-P_{2X1} antibody (rhodamine) and anti-$V_\beta 8$ (B) or anti-$V_\beta 6$ (C) TCR variable domain antibodies (FITC). The yellow labeling reveals $V_\beta 8$-positive T cells expressing P_{2X1} (arrows) 2 days after SEB injection. $V_\beta 8$- and $V_\beta 6$-positive thymocytes are labeled with green (arrowheads). Control (A) was done with rat and rabbit immunoglobulins as the first step. Magnification, ×40.

that there was a primary antigen-specific induction of P_{2X1} followed by a nonspecific effect, which was probably mediated by glucocorticoid.

Apoptosis was confirmed in the thymus and lymph nodes of SEB-injected mice using the ApopTag kit. A basal level of apoptosis in both the thymus and lymph nodes of nonimmunized mice was noted (Figures 5Aa and 5Ac). At 2 days after SEB injection, a significant increase in the number of apoptotic cells was observed in the T cell area of the lymph node section (Figure 5Ab). In the thymus, an increase in the number of apoptotic cells was also detected as early as day 2, with a maximum at day 8 (Figures 5Ad and 5B). As with the P_{2X1} labeling described above, over time individual apoptotic cells (day 2) appeared to be replaced by the formation of aggregates (day 6). By day 14, the number of apoptotic cells declined to the original basal level (Figure 5B). Quantification of apoptotic and P_{2X1}-positive cells in serial sections of thymus revealed that the number of apoptotic cells was still high at day 8, whereas the number of P_{2X1}-positive cells had already decreased. In vitro experiments showed that P_{2X1} expression was upregulated on apoptotic cells but decreased as the cells progress toward cell death (data not shown).

Discussion

In this study we suggest that P_{2X1}, a cation-selective ion channel activated by extracellular ATP, could be involved in thymocyte apoptosis. First, P_{2X1} mRNA and protein levels increase in thymocytes induced to die by DEX, a glucocorticoid, or by SEB, a superantigen acting through the TCR. Second, the proportion of apoptotic thymocytes correlates with the amount of ATP added to the culture. Addition of enzyme that degrades ATP or ATP antagonists protects thymocytes from apoptosis.

Pharmacological characterizations of P_{2X} purinoceptor subtypes, based on agonist potencies, have been made by whole-cell current recordings from dissociated cells (e.g., rat nodose neurons) and from cells transfected with various P_{2X} cDNA constructs. Among the seven reported P_{2X} purinoceptors, only P_{2X1} and P_{2X3} are activated and desensitized by α,βmeATP, but P_{2X3} molecules are not expressed in thymocytes (Lewis et al., 1995; Chen et al., 1995; Brake et al., 1994; Collo et al., 1996; Surprenant et al., 1996). These studies have also shown that several of these receptors, such as P_{2X4}, P_{2X6}, and P_{2X7}, are not antagonized by suramin (Buell et al., 1996a; Surprenant et al., 1996). The G protein–coupled ATP receptors P_{2U} and P_{2Y} are not desensitized by α,β-meATP (Abbracchio and Burnstock, 1994), and neither P_{2U} nor P_{2Z} receptors are antagonized by suramin (Abbracchio and Burnstock, 1994). In addition, P_{2U} receptors are activated by UTP. Our efforts to define agonist and antagonist properties of the purinoceptors linked to DEX-induced thymocyte apoptosis led us to the conclusion that P_{2X1} receptors may play a preferential role in thymocyte cell death, even though apoptosis is the end

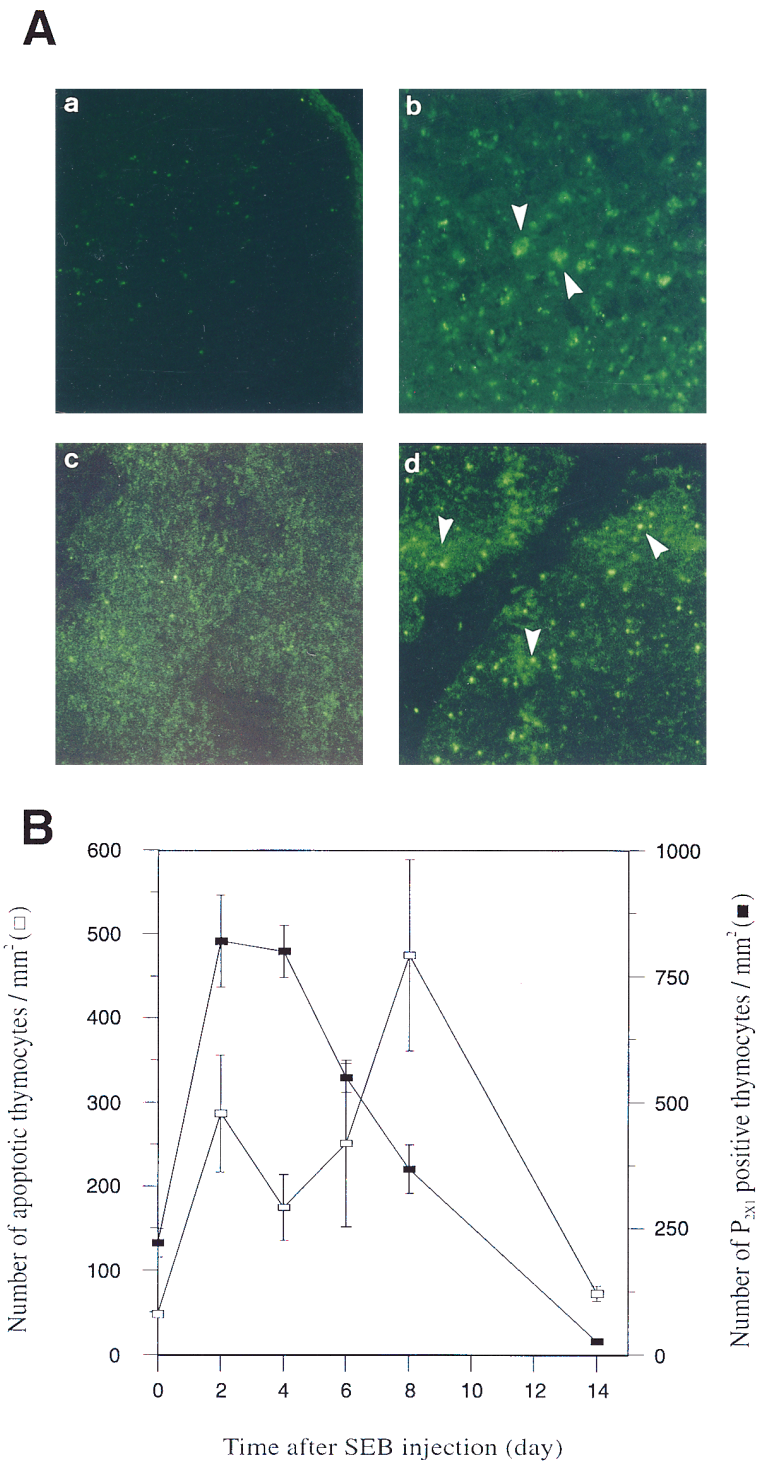

Figure 5. Apoptosis and P_{2X1} Expression in Thymus following SEB Injection

(A) In situ detection of apoptotic cells was performed in cryostat sections (5–10 μm) by direct labeling of degraded DNA with the Oncor ApopTag system according to the specifications of the manufacturer. Shown are lymph nodes (a and b) and thymus (c and d) from BALB/c mice before (a and c) and 2 days (b) or 8 days (d) after SEB injection. Cells with DNA fragmentation are labeled with FITC (arrows). Magnification, ×10).

(B) Quantification of apoptotic as well as P_{2X1}-positive cells in thymus sections. Labeled cells were counted under the microscope linked to the confocal. These data are derived from three independent experiments. The arithmetic means (±SEM) are shown for each timepoint after injection of 10 μg of SEB. Open squares represent apoptotic cells; closed squares represent P_{2X1}-positive cells.

of a cascade of events downstream from channel activation. However, higher concentrations of ATP were required in our study than would be expected if only P_{2X1} were expressed. The partial degradation of ATP and receptor desensitization that occur in these experiments may underlie such observations. In addition, as shown in Figure 2a, we observed a biphasic effect of extracellular ATP on thymocyte apoptosis. This result suggests the involvement of additional purinergic receptors with a lower affinity for ATP, such as P_{2X7} (Surprenant et al., 1996). P_{2X7} receptors, known functionally as P_{2Z} purinoceptors, are membrane pores constitutively expressed by murine T lymphocytes. P_{2X7} receptors are activated by high concentrations of ATP (0.1–1 mM) (Chused et al., 1996). Thus, the biphasic response to ATP seen in Figure 2a may suggest activation of P_{2X7} as well as P_{2X1} at high agonist concentrations.

Because intrathymic selection events are TCR specific, we have used SEB to show that the involvement of P_{2X1} molecules is not restricted to DEX-induced

apoptosis, but may also be implicated when apoptosis is initiated by antigen immunization. The induction of apoptosis by SEB in immature thymocytes has previously been proposed by Lin and colleagues (1992). Following intravenous administration of SEB, they documented a shrinkage of the thymus due to a reduction in cell number together with DNA degradation. The results reported here confirm the initial observation and provide in situ evidence of apoptosis occurring in the thymus after in vivo subcutaneous injection with SEB. Induction of apoptosis was tightly correlated with an increase in P_{2X1} expression among cortical thymocytes. We observed that both apoptosis and P_{2X1} expression were restricted to the same thymocyte subpopulation 2 days after SEB injection; dying SEB-reactive thymocytes ($V_\beta 8$ positive) expressed P_{2X1} molecules, while unresponsive thymocytes ($V_\beta 6$ positive) did not. The fact that we could detect P_{2X1} in cortical thymocytes (immature), but neither in medullar thymocytes (mature) nor in apoptotic peripheral T cells, suggests that P_{2X1} expression is lost during T cell maturation.

In addition to the initial SEB-specific apoptosis, we observed a nonspecific one, as only a subpopulation of P_{2X1}-positive cells expressed the $V_\beta 8$ domain of the TCR. Such a nonspecific cell death may be due to the elevation of endogenous glucocorticoid levels following antigen immunization (Gruber et al., 1994). Interestingly, mature thymocytes as well as T cells in the periphery are completely glucocorticoid resistant, although all have about the same number of glucocorticoid receptors per cell, with similar binding characteristics. It has been proposed that apoptosis induced by endogenous glucocorticoid could play a important role in the selection process during thymocyte maturation. One hypothesis is that among immature thymocytes, those that expressed a TCR without a particular affinity for self-MHC and remain unselected are of no use for the immune system and can be disposed of. These cells could activate the P_{2X1} gene in response to glucocorticoid, whereas a positive selection would involve a phenotypic shift, whereby P_{2X1} would no longer be expressed so that the cell would no longer be responsive to glucocorticoid. Another hypothesis raised was that cells with high affinity for MHC plus self-peptides (potentially harmful autoreactive cells) require a stimulation through both the TCR and glucocorticoid receptors to die. In this case, a defect in P_{2X1} function would result in resistance to glucocorticoid-induced apoptosis, leading to the survival of autoreactive T cells and the development of autoimmune diseases.

Our immunohistochemical analysis of the expression of the P_{2X1} molecule following SEB injection also suggests that P_{2X1} is not involved in either T cell activation or B cell apoptosis. If it had been involved in either of these processes, we would have detected P_{2X1} expression at days 2 and 14 post-SEB in the draining lymph nodes. Taken together, these results strongly suggest that the expression of P_{2X1} is restricted to apoptotic immature thymocytes.

Although direct evidence linking P_{2X1} function to thymocyte apoptosis in vivo has yet to be obtained, the identification of ATP receptors expressed in thymocyte subsets advances our understanding of the molecular mechanisms involved in the elimination of undesirable thymocytes and the connection between differentiation and apoptosis.

Experimental Procedures

Mice

We obtained 4- to 8-week-old female BALB/c mice from Iffa–CREDO (Lyon, France) and maintained them in our animal facility. The TCR $V_\beta 8.2$ transgenic mouse line (C57BL6.transgenic93/Ibm spf) (Uematsu et al., 1988) was provided by Dr. H. Bluethmann (Hoffmann–La Roche AG, Basel, Switzerland). These were backcrossed onto the BALB/c background. SEB (10 μg) from Toxin Technology (Sarasota, FL) was injected subcutaneously into each of the hind footpads. At various times, mice were killed and the popliteal lymph nodes and thymus were removed and frozen for immunohistochemistry or single cell suspensions were prepared.

Cell Cultures

Single cell suspensions were prepared by mechanical disruption of lymph nodes and thymus. Lymph node cells were resuspended in DMEM supplemented with 10% FCS, penicillin (50 U/ml), streptomycin (50 μg/ml), HEPES (10 mM), and 2-mercaptoethanol (5×10^{-5} M) and cultured at a final density of 2×10^6 cells per milliliter with 5 μg/ml SEB. On day 5, cells were collected, washed, and kept (2×10^6 cells per milliliter) in medium containing 5 U/ml of murine recombinant interleukin-2 (rIL-2) (Karasuyama and Melchers, 1988). By day 10, these cells had reverted to a resting phenotype as determined by forward- versus side-scatter parameters on a fluorescence-activated cell sorter FACS (FACStar Plus, Becton Dickinson, Errembodegem, Belgium), and more than 96% of the cells were $CD4^+V_\beta 8.2^+$. Viable cells were isolated by Ficoll density gradient centrifugation and restimulated with either 10 U/ml rIL-2 or 10 μg/ml SEB plus rIL-2. This secondary in vitro stimulation with SEB induced apoptosis of 45% of the cells within 24 hr, in contrast with the 15% found in the presence of rIL-2 alone.

Because FCS contains adenosine 5′-triphosphate (ATP) presumably released during blood platelet activation (Schermerhorn et al., 1994), thymocytes were resuspended in a serum-free medium (Blasey et al., 1995), reducing the effect of extracellular ATP in controls. Thymocytes were incubated at 2×10^6 per milliliter in 24-well Costar plates (2 ml per well) at 37°C in the absence or presence of DEX (5 nM) with ATP, adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), uridine 5′-triphosphate (UTP), α,βme-ATP, PPADS, or suramin. DEX-induced apoptosis in the absence or presence of apyrase (30 U/ml) was done in DMEM supplemented with 10% FCS. In the experiment performed to investigate the effect of ATP degradation by apyrase on thymocyte apoptosis, the FCS was the source of extracellular ATP. These compounds were purchased from Sigma (St. Louis, MO) except for suramin and PPADS, which were obtained from Bayer (Zurich, Switzerland).

Flow Cytometric Analysis of Apoptotic Cells

After 16 hr in culture, thymocytes were labeled for 30 min at 37°C with 10 μM bis-benzimide Hoechst 33342 (Sigma). After two washes with cold PBS containing 0.2% BSA and 0.02% NaN_3, cells were resuspended at 10^6 per milliliter with 2 μg/ml propidium iodide (Sigma). Using a protocol adapted from Belloc et al. (1994), we performed the analysis of apoptotic cells with a FACStar Plus (Becton Dickinson).

Northern Blotting

RNA and poly(A)$^+$ RNA were prepared as described elsewhere (Valera et al., 1994). Samples were quantified by measuring the OD at 260 nm and staining the membrane with methylene blue. The RNA was fractionated on a 1% agarose–6% formaldehyde gel and electroblotted to a noncharged nylon membrane (BDH). Hybridization was done with a digoxigenin–UTP-labeled riboprobe (100 ng/ml) corresponding to the entire mouse P_{2X1} sequence (Valera et al., 1994) according to the specifications of the manufacturer (Boehringer Mannheim). For the radioactive Northern blot analysis, all procedures and reagents have been described previously (Valera et al., 1994). P_{2X1} probes can be obtained from G. B.

Immunohistochemistry

Freshly isolated lymphoid tissues were immersed in OCT compound (Miles, Naperville, IL), rapidly frozen on dry ice, and sectioned at 5 μm. For detection of apoptosis in situ, sections were processed according to the specifications of the manufacturer (ApopTag, Oncor). For immunolabeling, sections were air dried, fixed in ethanol, and rehydrated in PBS before saturation with PBS, 0.1% BSA containing 10% mouse serum. Sections were incubated sequentially, first with anti-P_{2X1} antibody, a rabbit polyclonal antibody raised against the C-terminal part of P_{2X1} (a gift from Dr. R. P. Elde, University of Minnesota; Vulchanova et al., 1996) and, second, with a rhodamine-labeled donkey anti-rabbit antibody (Jackson ImmunoResearch). In addition to anti-P_{2X1} antibody labeling, sections were incubated first with a rat anti-Thy-1.2 monoclonal antibody (T24; 15 μg/ml) (Dennert et al., 1995) or anti-$V_\beta 8$ (KJ-16) (Haskins et al., 1984) or anti-$V_\beta 6$ (44.22.1) (Acha-orbea et al., 1985) and, second, with a fluorescein isothiocyanate (FITC)-labeled mouse anti-rat $(Fab')_2$ antibody (Jackson ImmunoResearch). Specificity controls included incubating sections with the following: first, the rabbit anti-P_{2X1} antibody followed by the mouse anti-rat FITC; or, second, the anti-Thy.1-2 followed by the rhodamine-labeled donkey anti-rabbit antibody; and, third, rabbit serum followed by the rhodamine-labeled donkey anti-rabbit antibody. None of these three combinations produced a positive signal.

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