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Immunology

p53 expression is required for thymocyte apoptosis induced by adenosine deaminase deficiency

(T-cell development/severe combined immunodeficiency/*bcl-2* gene)

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ABSTRACT **Adenosine deaminase (ADA, EC 3.5.4.4) is a ubiquitous enzyme in the purine catabolic pathway. In contrast to the widespread tissue distribution of this enzyme, inherited ADA deficiency in human results in a tissue-specific severe combined immunodeficiency. To explain the molecular basis for this remarkable tissue specificity, we have used a genetic approach to study ADA deficiency. We demonstrate that ADA deficiency causes depletion of $CD8^{low}$ transitional and $CD4^+CD8^+$ double-positive thymocytes by an apoptotic mechanism. This effect is mediated by a p53-dependent pathway, since p53-deficient mice are resistant to the apoptosis induced by ADA deficiency. DNA damage, known to be caused by the abnormal accumulation of dATP in ADA deficiency, is therefore responsible for the ablation of T-cell development and for the immunodeficiency. The two thymocyte subsets most susceptible to apoptosis induced by ADA deficiency are also the two thymocyte subsets with the lowest levels of *bcl-2* expression. We show that thymocytes from transgenic mice that overexpress *bcl-2* in the thymus are rescued from apoptosis induced by ADA deficiency. Thus, the tissue specificity of the pathological effects of ADA deficiency is due to the low *bcl-2* expression in $CD8^{low}$ transitional and $CD4^+CD8^+$ double-positive thymocytes.**

Inherited deficiency of adenosine deaminase (ADA) results in severe combined immunodeficiency disease in humans (1). ADA is a housekeeping enzyme in the purine degradation pathway that deaminates adenosine and deoxyadenosine (dAdo) to yield inosine and deoxyinosine, respectively. Two metabolic pathways affected by ADA deficiency may contribute to the immunodeficiency observed in ADA-deficient patients. One pathway involves abnormal accumulation of dATP, the phosphorylated end product of the ADA substrate dAdo (2). The second metabolic consequence of the accumulation of dAdo in ADA deficiency is inactivation of the enzyme *S*-adenosylhomocysteine hydrolase, resulting in the accumulation of its substrate, with consequent inhibition of specific *S*-adenosylmethionine-dependent methylation of nucleic acids, proteins, and lipids (3).

Studies using cultured T cells and potent ADA inhibitors such as deoxycoformycin (DC) have demonstrated that the accumulation of dATP leads to inhibition of ribonucleotide reductase and to an imbalance in dNTP levels (4). This imbalance causes inhibition of both replicative DNA synthesis (4, 5) and DNA repair (6) processes in T lymphocytes.

Clinical observations of ADA-deficient patients suggest that the development of T lymphocytes is affected at an intrathymic stage of differentiation (7). Consistent with these observations, thymocytes have increased ability to accumulate high levels of dATP from extracellular dAdo in the presence of ADA inhibitors (8). The ability of thymocytes to accumulate dATP may be explained by developmental stage- and tissue-specific regulation of the relevant purine metabolic enzymes (9).

These observations imply that in the course of intrathymic T-cell development, thymocytes become specifically sensitive to the metabolic consequences of ADA deficiency. However, the precise stage(s) in thymocyte differentiation that is affected in ADA deficiency, as well as the molecular and cellular mechanisms that abrogate intrathymic T-cell differentiation and cause immune dysfunction, is still unknown. In the present work, we have used a murine model for ADA deficiency and the potent ADA inhibitor DC (10) to answer these questions.

MATERIALS AND METHODS

Animals and *in Vivo* Injections. C57BL/6 mice were used as controls. p53-knockout (homozygous p53 −/−) mice were purchased from Genpharm (Mountain View, CA). Mice overexpressing the *bcl-2* transgene in T cells (11) were obtained from Hung-Sia Teh (University of British Columbia, Vancouver). Both the *bcl-2* transgenic and the p53 −/− mice were on a C57BL/6 background. C57BL/6 mice were purchased from The Jackson Laboratory and used at 4–6 weeks of age. DC (Pentostatin; gift of Parke–Davis) was injected intraperitoneally from day 1 to day 3 at 300 μg/day per mouse (10). Analysis was performed 24 hr after the last injection.

Cell Isolation and Culture. $CD4^-$ thymocytes (>99% pure) were isolated by negative selection using avidin-activated magnetic beads (Advanced Magnetics, Cambridge, MA) to deplete thymocytes that had bound biotin-conjugated anti-CD4 monoclonal antibody (mAb) (PharMingen). $CD3^-CD4^-CD8^-$ (triple-negative, TN) or $CD3^{low}CD4^-CD8^{low}$ (transitional) thymocytes were sorted from $CD4^-$ cells reacted with fluorescein isothiocyanate (FITC)-conjugated anti-CD3 mAb (PharMingen) followed by biotin-conjugated anti-CD8 mAb (PharMingen) developed by streptavidin Cy-chrome (PharMingen). $CD4^+CD8^+$ (double-positive, DP) and $CD4^+CD8^-$ (single-positive, SP) thymocytes were sorted from total thymocytes reacted with FITC-conjugated anti-CD8 mAb (PharMingen) followed by phycoerythrin (PE)-conjugated anti-CD4 mAb (PharMingen). Sorted populations were cultured at 37°C for 14 or 16 hr in the presence or absence of DC (20 μM) and/or dAdo (60 μM).

Total thymocytes or fractionated subpopulations were cultured for 14 hr (unless otherwise indicated) at 0.5–5 × 10^6 cells per ml at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 10 mM Hepes, and 50 μM 2-mercaptoethanol.

Flow Cytometry. Flow cytometry was performed with a dual-laser FACScan or a dual-laser FACStar Plus (Becton Dickinson). For three-color analysis, thymocytes (2 × 10^6) were reacted sequentially with FITC-conjugated anti-CD3 mAb for 45 min, PE-conjugated anti-CD4 mAb for 25 min, and by biotin-conjugated anti-CD8 mAb for 20 min developed by streptavidin Cy-Chrome for 20 min. Control antibodies in-

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Abbreviations: ADA, adenosine deaminase; DC, deoxycoformycin; DP, double-positive; SP, single-positive; TN, triple-negative.

cluded FITC-conjugated anti-Leu-4 mAb, PE-conjugated anti-Leu-4 mAb, and biotin-conjugated anti-Leu-1 mAb (Becton Dickinson). All fluorescence data were collected with logarithmic amplification from 50,000–90,000 viable cells as determined by forward and side scatter intensity.

Flow Cytometric Analysis of Subdiploid DNA. Total thymocytes or isolated thymic subpopulations were washed once in isotonic phosphate-buffered saline after culture in single-cell suspension. Cells were then fixed at 2×10^6 cells per ml in 70% ethanol for 1–12 hr at 4°C. Pellets were then resuspended in 50 μl of 0.01% (wt/vol) propidium iodide (Sigma) with 0.6% (vol/vol) Nonidet P-40 followed by 500 μl of 0.2% (wt/vol) RNase (bovine pancreatic type II; Sigma). After staining with propidium iodide, nuclei were then analyzed by flow cytometry and frequencies of subdiploid DNA were determined.

RESULTS

During normal intrathymic maturation, T cells progress through distinct differentiation stages defined by cell surface expression of the T-cell receptor/CD3 complex and the CD4 and CD8 coreceptors. Developing thymocytes can be divided according to their order of appearance during differentiation into four subsets: TN ($CD3^-CD4^-CD8^-$), transitional ($CD3^{low}CD4^-CD8^{low}$), DP ($CD4^+CD8^+$), and SP ($CD4^+$ or $CD8^+$) (12).

To define the differentiation stage at which ADA deficiency affects thymocyte development, we injected the ADA inhibitor DC into 3-week-old C57BL/6 mice and analyzed its effect on thymocyte development. Three-color fluorescence analysis (Fig. 1) showed that the first thymocyte subset affected after DC injection was the transitional $CD3^{low}CD4^-CD8^{low}$ subpopulation, which by 24 hr was reduced to 24% that of the control saline-injected mice and by day 3, to 4% that of the control mice. The products of transitional thymocytes, the DP thymocytes, decreased to 30% and 5% of control at 2 and 3 days, respectively, following DC injection. The mature SP $CD4^+$ cells were essentially unaffected in the first 2 days and slightly decreased after 3 days, most likely as a result of depletion of their DP precursor cell population. The number of immature TN cells was reduced only moderately after 2 and 3 days (70% and 42% of control). *In vitro* experiments using purified thymocyte subpopulations isolated by cell sorting gave similar results (data not shown).

Our finding that inhibition of ADA activity exerts its effect on $CD8^{low}$ and DP cells raises the possibility that the depletion of these thymocytes in ADA deficiency is due to induction of apoptosis. To test this hypothesis, total thymocytes were cultured for 14 hr in the presence or absence of various concentrations of dAdo and DC and analyzed for apoptosis by flow cytometry. The combination of dAdo and DC was needed to induce thymocyte apoptosis, and the maximal effect was observed at 60 μM dAdo and 20 μM DC, the highest con-

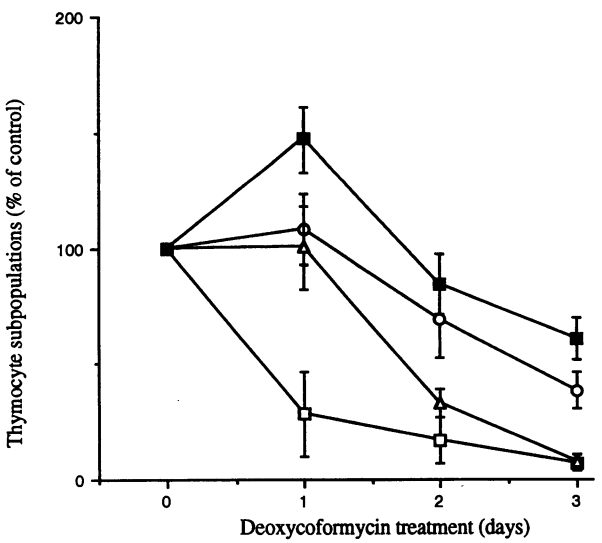

FIG. 1. Effect of inhibition of ADA activity on the frequencies of thymocyte subsets *in vivo*. Thymocytes derived from C57BL/6 mice were treated for 1–3 days with DC. Recovered thymuses from DC-treated mice and saline-treated control mice were analyzed by three-color flow cytometry for the frequency of TN ($CD3^-CD4^-CD8^-$) (○), transitional ($CD3^{low}CD4^-CD8^{low}$) (□), DP ($CD4^+CD8^+$) (△), and SP ($CD4^+CD8^-$) (■) thymocytes. Frequencies of each subpopulation are expressed as percentages of number of cells in control mice, based on the total thymocytes recovered at each time point. Individual mice were analyzed (at least two mice per group). Results are means of five independent experiments. Vertical bars represent the standard deviation.

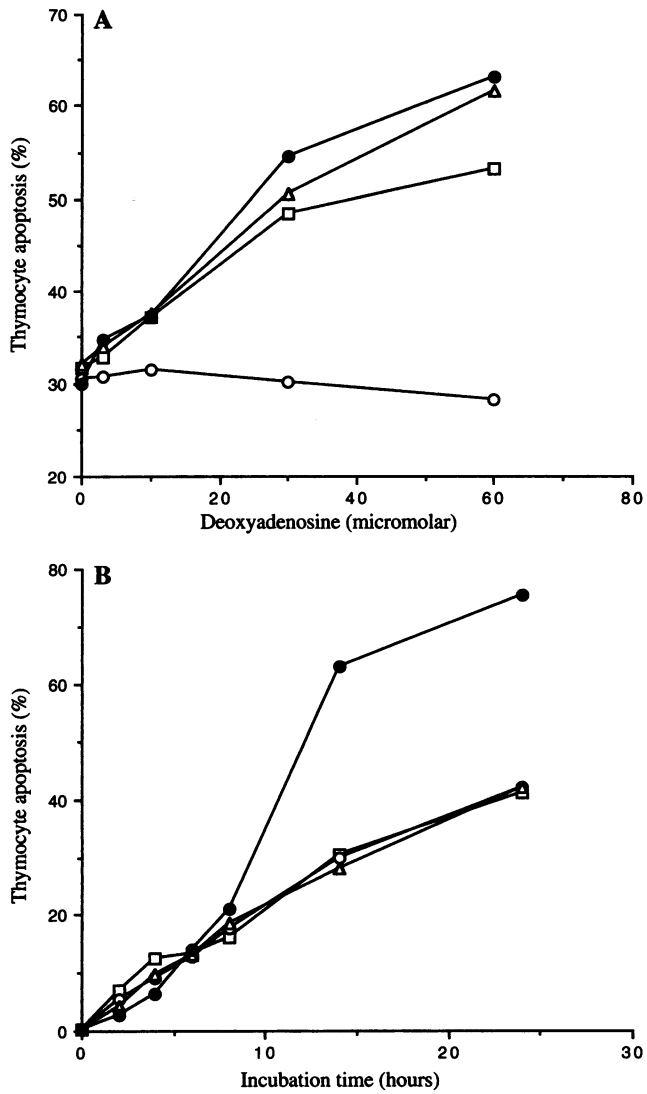

FIG. 2. Thymocyte apoptosis is induced by dAdo in the presence of the ADA inhibitor DC *in vitro*. Thymocytes (5×10^5 cells per ml) were incubated for the indicated times at 37°C in RPMI 1640 medium in the presence or absence the indicated concentrations of dAdo and DC. The frequencies of apoptotic thymocytes were determined by flow cytometry. (*A*) For determination of the dose response of thymocyte apoptosis to dAdo, thymocytes were incubated for 14 hr in the presence of 0, 2.5, 10, 30, or 60 μM dAdo without DC (○) or with 3 μM (□), 10 μM (△), or 20 μM (●) DC. (*B*) Time course of thymocyte apoptosis in the absence of dAdo or DC (○) or in the presence of 60 μM dAdo alone (□), 20 μM DC alone (△), or 60 μM dAdo in combination with 20 μM DC (●).

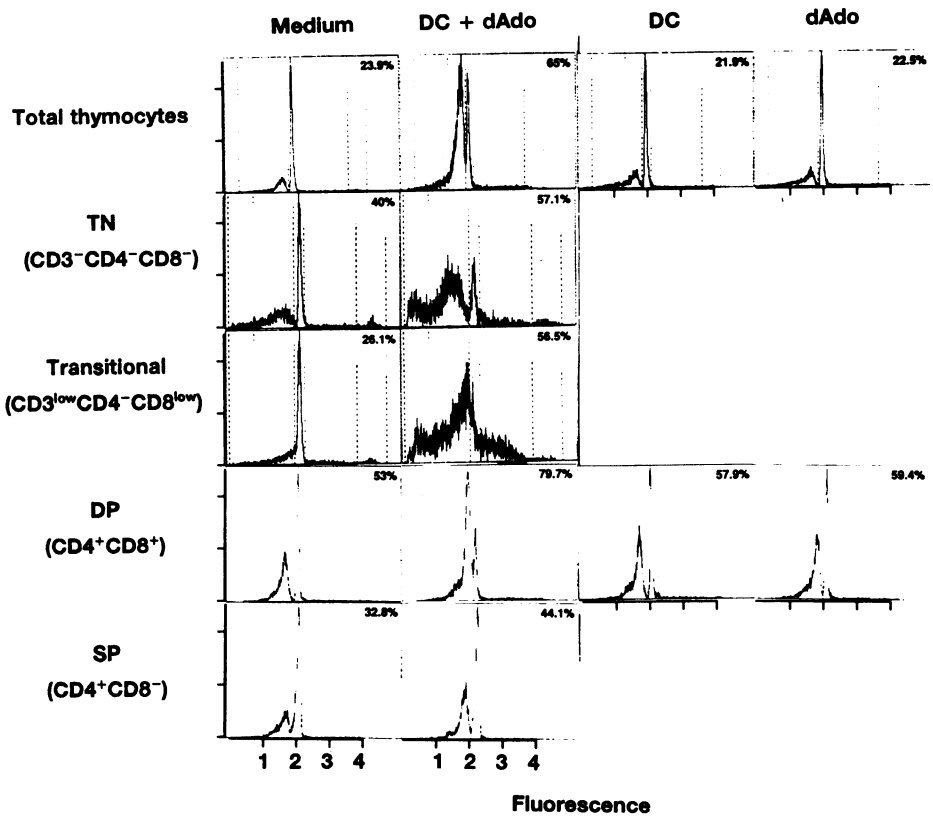

FIG. 3. ADA inhibition induces apoptosis in $CD3^{low}CD4^{-}CD8^{low}$ transitional and $CD4^{+}CD8^{+}$ DP thymocytes. Subdiploid DNA frequencies of total thymocytes and isolated thymocyte subsets purified by fluorescence-activated cell sorting are shown. Cells were cultured for 16 hr in single-cell suspension in the presence or absence of DC (20 μM) and/or dAdo (60 μM) and then lysed. Nuclei were stained with propidium iodide for subdiploid DNA quantification by flow cytometry. The effective concentration of DC and dAdo and the optimal time of treatment were determined in separate experiments. Percentages represent the proportion of subdiploid, apoptotic cells. Results are from a single experiment with thymocytes obtained from five mice. Similar results were obtained in three additional independent experiments.

centrations tested (Fig. 2*A*). The need for the addition of the ADA substrate in *in vitro* experiments arises because the density of cultured thymocytes is much lower than *in vivo* and thus the dAdo that is released from intracellular sources may not reach a high enough concentration to allow dATP accumulation *in vitro*. The maximum effect of dAdo added in combination with DC was observed between 14 and 24 hr, whereas no increase in apoptosis above background was observed with either compound added alone (Fig. 2*B*).

To identify the thymocyte subpopulation that is affected by inhibition of ADA, we purified TN, $CD8^{low}$ transitional, DP, and mature SP $CD4^{+}$ thymocyte subpopulations by cell sorting. These subpopulations were cultured overnight at 37°C in the presence or absence of the ADA inhibitor DC and the ADA substrate dAdo. Inhibition of ADA activity caused cell death by apoptosis in two of the thymocyte subpopulations: the $CD8^{low}$ transitional cells and the DP cells (Fig. 3). In contrast, mature SP $CD4^{+}$ T cells were unaffected. The cultured TN cells showed an intermediate frequency of apoptotic cells between DP and SP cells. dAdo or DC added separately had no effect (Fig. 3), in agreement with the reported level of dATP accumulation in thymocytes *in vitro* (8). In addition, incubation in the presence of (*Z*)-5′-fluoro-4′,5′-didehydro-5′-deoxyadenosine, an adenosine analog that inhibits the methylation pathway affected in ADA deficiency but does not cause accumulation of dATP (13), failed to induce apoptosis in thymocytes (data not shown). Thus, ADA inhibition caused cell death by apoptosis predominately in $CD8^{low}$ transitional and DP thymocytes, in correlation with dATP accumulation and independent of the inhibition of methylation.

Apoptosis can be part of normal development or, alternatively, can be induced by a variety of agents that cause DNA damage (14). Following DNA damage p53 expression is induced and the activated p53 protein can either induce cell cycle arrest or activate cell apoptosis (15). Expression of the p53 protein is a requirement for the induction of apoptosis following DNA damage by γ irradiation (16). Since the accumulation of dATP can inhibit DNA repair and cause DNA damage in T lymphocytes (6), it is possible that ADA inhibition also induces apoptosis via a p53-dependent pathway. To test this hypothesis, we examined the requirement for p53 to mediate the apoptosis induced by ADA deficiency in the murine model. To that end, we studied the effect of DC and dAdo on thymocytes derived from mice carrying a germline disruption of the p53 gene and compared them with thymocytes from wild-type control mice (Fig. 4). Whereas cultures derived from wild-type mice showed a high frequency of apoptotic cells, no significant increase in apoptotic cells was detected in p53-deficient mice. We conclude that the absence of p53 prevents the effects of ADA inhibition.

p53-dependent apoptosis mediated by the adenovirus E1A protein can be blocked by simultaneous expression of the *bcl-2* gene (17). During intrathymic T-cell development the expression of *bcl-2* is tightly regulated; early TN thymocytes express *bcl-2* but lose its expression with further differentiation to the $CD8^{low}$ and DP stages. Following selection to SP cells, mature thymocytes regain *bcl-2* expression (18). This pattern of *bcl-2* expression is inversely correlated with susceptibility to apoptosis that occurs during T-cell selection (19) and in response to various other agents (19). Since inhibition of ADA causes apoptosis in the same thymocyte populations, and since this process is dependent upon p53 expression, we examined whether it could also be prevented by the expression of the *bcl-2* transgene. Indeed, thymocytes from *bcl-2* transgenic mice

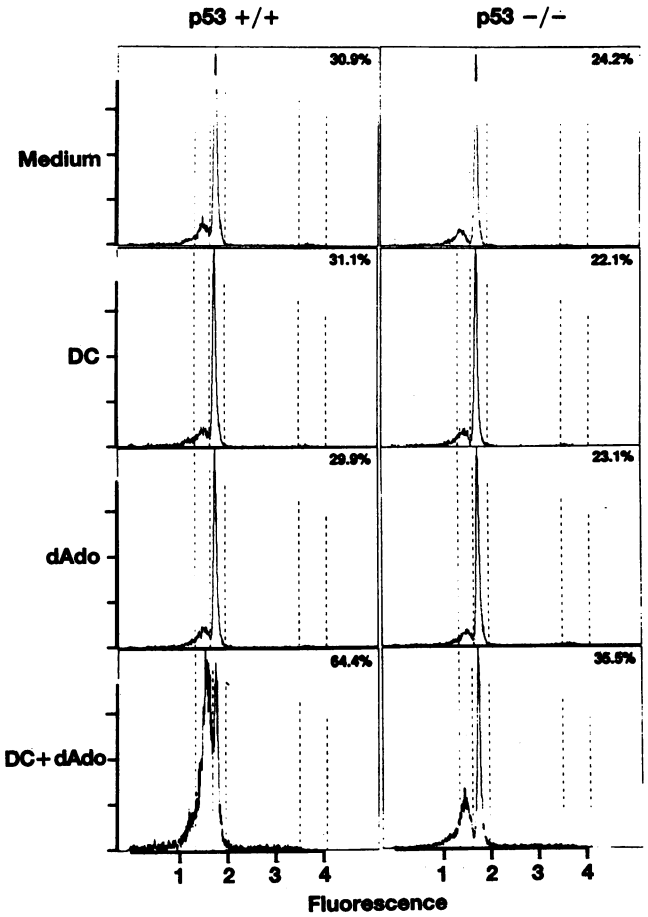

FIG. 4. p53 is required for apoptosis induced by ADA inhibition in thymocytes. Frequencies of cells with subdiploid DNA derived from thymocyte cultures of p53 −/− and wild-type control mice were measured. Thymocytes were cultured for 14 hr in single-cell suspension in the presence or absence of DC (20 μM) and/or dAdo (60 μM). Recovered cells were then lysed, and nuclei were stained with propidium iodide and analyzed by flow cytometry for the presence of subdiploid DNA. Percentages represent the proportion of subdiploid, apoptotic cells. Results are from a single experiment with two wild-type and two p53 −/− mice. Three additional experiments gave similar results.

were resistant to apoptosis induced by the ADA inhibitor DC added in combination with the ADA substrate dAdo at concentrations that induced apoptosis in the majority of thymocytes from wild-type control mice (Fig. 5). Thus, overexpression of *bcl-2* rescues thymocytes from apoptosis induced by ADA deficiency.

DISCUSSION

Here we describe findings that provide an explanation for the T-cell and developmental specificity observed in ADA deficiency. We found that ADA deficiency selectively affected thymocytes at the $CD8^{low}$ transitional and $CD4^+CD8^+$ DP stages of intrathymic differentiation (Fig. 1). These differentiation stages coincide with rearrangement of T-cell antigen receptor genes and with clonal selection, biological processes that are unique to the immune system. The characteristics of thymocytes at these two differentiation stages include low expression of *bcl-2* (18) and, as a result, increased sensitivity to apoptosis (20), a property required for clonal selection. This characteristic exposes thymocytes to apoptosis due to lack of ADA activity as demonstrated in this study (Fig. 1), resulting in immunodeficiency.

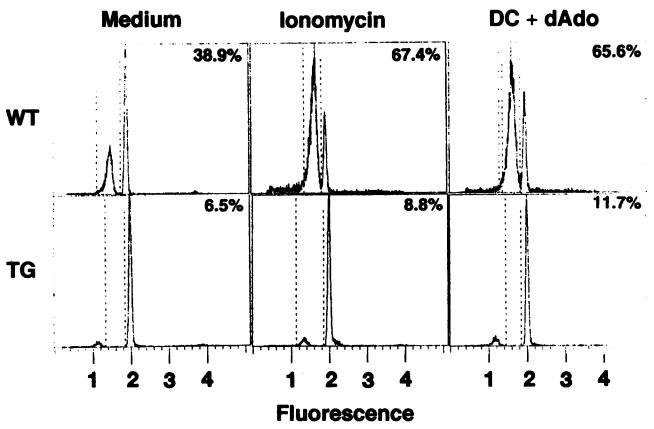

FIG. 5. *bcl-2* overexpression in transgenic mice rescues thymocytes from apoptosis mediated by inhibition of ADA activity. Frequencies of cells with subdiploid DNA derived from thymocyte cultures of *bcl-2* transgenic (TG) and wild-type control (WT) mice are shown. Thymocytes were cultured for 14 hr in single-cell suspensions in the presence or absence of ionomycin (0.05 μM) or DC (20 μM) plus dAdo (60 μM). Recovered cells were lysed, and nuclei were stained with propidium iodide and analyzed by flow cytometry for relative content of subdiploid DNA. Percentages represent the proportion of subdiploid, apoptotic cells. Results are from a single experiment with two wild-type and two *bcl-2* transgenic mice. Seven additional experiments gave similar results. Average percentages of apoptotic cells and their standard deviations were as follows: wild-type control, 34.8 ± 6.8; wild-type with DC plus dAdo, 70.1 ± 6.6; *bcl-2* transgenic control, 6.6 ± 0.55; *bcl-2* with DC plus dAdo, 8.3 ± 2.6.

Apoptosis can be induced by various extracellular or metabolic signals (14). In the case of ADA deficiency two metabolic pathways are largely affected, deoxynucleotide metabolism, due to dATP accumulation (4), and various methylation pathways, due to inhibition of *S*-adenosylhomocysteine hydrolase activity (3). Inhibition of methylation alone by a nonphosphorylatable dAdo analog does not cause apoptosis in thymocytes (data not shown). Thus, the accumulation of dATP due to lack of ADA activity is solely responsible for the apoptosis observed in thymocyte populations.

The biochemical mechanism(s) by which *bcl-2* protects against apoptosis by various agents is unclear. Recently, it has been shown that *bcl-2* interferes with the accumulation of dATP and thus prevents the onset of apoptosis in a pre-B-cell line that overproduces *bcl-2* (21). A similar mechanism may thus explain the protection from dATP-induced DNA damage in thymocytes from transgenic mice that overexpress *bcl-2*.

The absence of ADA activity in thymocytes results in dATP accumulation that causes depletion of the three other dNTP pools (8). As a result of depletion of dNTP pools, DNA repair is inhibited, and T lymphocytes accumulate DNA breaks (6). Apoptosis triggered by DNA damage in thymocytes is specifically dependent on the expression of the p53 protein (16). Our observation that inhibition of ADA activity also triggers an apoptotic process that is dependent on p53 expression (Fig. 4) supports the hypothesis that DNA damage caused by ADA deficiency is responsible for the induction of apoptosis in immature thymocytes.

The tissue and developmental-stage specificity of ADA deficiency is the result of two contributing factors. First, deoxynucleotide metabolism in the thymus has unique properties, such as increased capacity to phosphorylate dAdo and decreased ability to degrade deoxynucleotides (8). As a result, thymocytes accumulate much higher levels of dATP in the absence of ADA activity than other tissues (8). Second, $CD8^{low}$ and DP thymocytes seem to be more sensitive to apoptosis induced by accumulated dATP, due to lack of expression of *bcl-2* at these stages of differentiation (18).

In conclusion, in this study we have used a genetic approach in a murine model to examine the mechanism by which ADA deficiency causes immunodeficiency. We provide evidence for the involvement of a p53-dependent apoptotic mechanism that causes cell death of immature $CD8^{low}$ and DP thymocytes in ADA deficiency. This apoptosis is triggered by the accumulation of dATP and can be prevented by the overexpression of *bcl-2* transgene.

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