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Extracellular Nicotinamide Adenine Dinucleotide Induces T Cell Apoptosis In Vivo and In Vitro¹

Zhang-Xu Liu, Olga Azhipa, Shigefumi Okamoto, Sugantha Govindarajan, and Gunther Dennert²

Incubation of mouse T cells expressing the cell surface enzyme ADP ribosyltransferase with nicotinamide adenine dinucleotide (NAD) had been reported to cause ADP ribosylation of cell surface molecules, inhibition of transmembrane signaling, and suppression of immune responses. In this study, we analyze the reasons for these effects and report that contact of T cells with NAD causes cell death. Naive T cells when incubated with NAD and adoptively transferred into semiallogeneic mice fail to cause graft-vs-host disease, and when injected into syngeneic, T cell-deficient recipients do not reconstitute these mice. Rather, they accumulate in the liver, leading to an increase of apoptotic lymphocytes in this organ. Similar effects are induced by injection of NAD, shown to cause a dramatic increase of apoptotic CD3⁺, CD4⁺, and CD8⁺ cells in the liver. Consistent with this, in vitro incubation of naive T cells with NAD is shown to induce apoptosis. In contrast, no cell death is demonstrable when T cells are activated before incubation with NAD. It is concluded that ecto-NAD, as substrate of ADP ribosyltransferase, acts on naive, but not on activated CD69⁺ T cells. *The Journal of Immunology*, 2001, 167: 4942–4947.

Posttranslational modification of proteins involved in signaling constitutes an important mechanism in cell regulation. While this is well established for transmission of intracellular signals, it has yet to be clearly documented for signals received on the cell surface. Evidence for existence of such a mechanism has emerged from the demonstration of mono-ADP ribosyltransferases (ADPRT)³ on mammalian cells (1). ADPRTs introduce posttranslational modifications into proteins by transferring ADP ribose from nicotinamide adenine dinucleotide (NAD) to arginine or cysteine residues. Five ADPRTs (ART1–5) have been cloned from mouse, rat, rabbit, and human tissues and shown to be 25- to 40-kDa, GPI-anchored proteins, with exception of ART5 (2–4). In the rat, there exist two ART2 alleles, previously known as T cell differentiation marker RT6. Interestingly, T cells expressing RT6 (ART2) were reported to induce resistance to autoimmune diabetes in diabetes-prone BB/W rats (5, 6). In the mouse, two ART2 genes, ART2a and ART2b, have been described, besides ART1, ART3, ART4, and ART5, all of which may be expressed on lymphocytes, with exception of ART3 and ART5 (3, 4, 7–9). There is therefore a large family of enzymes capable of ADP ribosylating proteins on various cell types, including lymphocytes. This emerging picture is complicated by the fact that there are strain variations in expression of these enzymes. Thus, ART2a is not expressed in C57BL/6 mice, and ART2b is not expressed in NZW mice (10, 11). Reminiscent of observations in rats, in mice a correlation

between ART2 transcript levels in diabetes-prone nonobese diabetic/Lt mice and control nonobese nondiabetic/Lt mice has been reported, pointing to a role of ADPRT in regulation of autoimmune diabetes (12).

Because of the many genes coding for ADPRTs (3, 13–15) and lack of Abs specific for these molecules, much work with lymphocytes has been accomplished by using enzyme assays to identify respective molecules and to study their function. Results showed that ADPRT activity is expressed on T cells, but not B cells (16, 17). Upon incubation with NAD, cell surface proteins on T cells are ADP ribosylated, and responses to activation signals, transmitted by the TCR, are inhibited (18–20). These observations then raise the question as to how precisely ADP ribosylation modulates T cell functions. Experiments revealed that the enzyme attaches ADP ribose to arginine residues of CD45, CD44, CD43, CD8, and LFA-1 (18, 19), in turn posing the question as to how modification of these molecules regulates TCR signaling. Using T cell lines, expressing ADPRT, evidence was obtained that ADP ribosylation of cell surface molecules inhibits association of receptors into a signal-transmitting cluster (19, 20). The speculation was hence put forward that ADP ribosylation of coreceptors inhibits TCR signaling by altering receptor association (19, 20). Such changes in receptor interaction could have various effects. Receptors could fail to transmit stimulatory signals or generate negative signals, leading to anergy or cell death.

In this communication, we report that treatment of mouse T cells with NAD before injection into animals results in their inability to mediate immune functions and to reconstitute recipients. Moreover, injection of NAD into animals causes accumulation of apoptotic T cells in the liver. It is shown that incubation of naive, but not activated T cells with NAD induces cell death in vitro, explaining the effects NAD exerts in vivo.

Materials and Methods

Assays for graft-vs-host disease (GVHD), marrow stem cell proliferation, T cell proliferation, and homing

To induce GVHD, spleen cells from C57BL/6 (H-2^b) mice were injected i.v. into B6D2F₁ (H-2^{dx^b}) mice. All mice were obtained from The Jackson Laboratory (Bar Harbor, ME). After 10 days, spleens were harvested, weights determined, and spleen indexes, i.e., spleen weight increases over

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³ Abbreviations used in this paper: ADPRT, ADP ribosyltransferase; CFUS, CFU spleen; GVHD, graft-vs-host disease; NAD, nicotinamide adenine dinucleotide; PI, propidium iodide.

controls, calculated. Spleen cells were assayed for presence of CTL by cytotoxicity assay on P815 targets (H-2^d) or cultured with C3H (H-2^k) spleen stimulator cells for 5 days, followed by cytotoxicity assay on C1.18.4 targets (H-2^k).

Bone marrow was assayed for the number of CFU spleen (CFUS) (21) after treatment with NAD. Marrow was harvested from long bones and incubated in RPMI 1640 medium, with or without 1 mM NAD, at 37°C for 3 h. After washing, aliquots of 10⁶ cells were injected i.v. into lethally irradiated syngeneic recipients. After 8 days, spleens were harvested and stained with Bouin's solution, and colonies were counted (21).

To assay the proliferative activity of naive T cells, splenic T cells were isolated by nylon wool adherence from C57BL/6 Thy-1.2 mice. Purified T cells were incubated in RPMI 1640 medium, with or without 1 mM NAD for 3 h at 37°C. After washing, 10⁷ T cells per mouse were injected i.v. into T cell-deficient recipients. The recipients were adult-thymectomized C57BL/6 Thy-1.1 mice (The Jackson Laboratory) that had been lethally irradiated and reconstituted with 10⁷ bone marrow cells, depleted of T cells by treatment with anti-Thy-1 Ab and complement, 1 day before T cell injection. Four weeks later, spleens were harvested and assayed for Thy-1.2-staining cells by FACS analysis.

To assay homing of T cells (20), nylon wool-purified splenic T cells were incubated in RPMI 1640 medium, with or without 1 mM NAD, at 37°C for 3 h, labeled with ⁵¹Cr, and injected i.v. into syngeneic recipients at a dose of 5 × 10⁶ cells per recipient. After 1 h, animals were sacrificed, spleens and livers were harvested, and radioactivity was determined in a gamma counter. The percentage of radioactivity recovered from organs was plotted, setting results from untreated cells as 100%.

Histology

To visualize apoptotic lymphocytes in the liver, liver tissue was fixed in 10% neutral buffered Formalin and embedded in paraffin. Five-micrometer sections were affixed to slides, deparaffinized. Apoptotic cells were visualized by the TUNEL assay, using the in situ cell death detection kit purchased from Boehringer Mannheim (Indianapolis, IN).

Lymphocyte purification and fluorometric analysis and tissue culture procedures

T cells were purified from spleen cells by nylon wool adherence (20). B cells were isolated from spleen cells by panning spleen cells on plates coated with anti-Ig Abs (21). The resulting cell populations were assayed for purity by fluorometric staining. For FACS analysis, 10⁶ cells were stained with mAbs at a concentration of 1 μg per 100 μl PBS containing 0.2% BSA (Boehringer Mannheim) and 0.05% sodium azide for 30 min on ice (17). The following Abs were used: FITC-conjugated anti-Thy-1.2, anti-CD62L, PE-conjugated anti-CD3, anti-CD4, anti-CD8, and Cy-Chrome-conjugated anti-CD69, all purchased from BD PharMingen (San Diego, CA). Apoptotic cells were detected by the annexin V fluos staining kit (Roche, Indianapolis, IN) (22). In all analyses, dead cells were gated out using propidium iodide (PI) staining; therefore, all data represent results from live cells. FACS analysis was performed on a FACStar^{Plus} (BD Biosciences, Mountain View, CA).

To assay CTL responses, spleen cells were harvested 10 days after induction of GVHD and cultured with irradiated C3H stimulator cells in complete RPMI 1640 medium, containing 10% FCS for 5 days in 24-well plates (20). To prepare C1.18.4 (H-2^k) targets, cells were labeled with 100 μCi Na₂⁵¹CrO₄ (DuPont, Boston, MA) in 5% FCS RPMI 1640 for 120 min at 37°C. Ex vivo CTL assays on H-2^d target P815 were performed with spleen cells from mice undergoing GVHD.

To stimulate T cells in vitro, nylon wool-purified T cells were cultured on anti-CD3-coated tissue culture plates. Plates were incubated with a 1/1000 dilution of anti-CD3 Ab (500AA2 ascites) overnight to absorb the Ab. After washing the plates, T cells were added in complete RPMI 1640 medium and incubated for 24 h. Cells were harvested and then cultured in the presence or absence of 1 mM NAD for 24 h and analyzed for activation by CD69 staining and for cell death by annexin V staining.

Results

Spleen cells treated with NAD lose ability to induce GVHD

Previous experiments had shown that incubating T cells, expressing ADPRT, with NAD leads to ADP ribosylation of cell surface molecules (16–18, 23, 24) and concomitant failure to respond to activation signals (19, 20). These findings raised the question as to what are the long-term effects of cell surface ADP ribosylation on in vivo T cell responses. To examine this, spleen cells from B6 (H-2^b) mice were incubated with NAD and then injected into

B6D2F₁ (H-2^{b×d}) recipients to assay ability to induce GVHD. Fig. 1A shows that infusion of untreated B6 spleen cells into B6D2F₁ mice induces cell dose-dependent symptoms of GVHD, demonstrable by increased spleen weights. Preincubation of donor cells with NAD causes almost complete inhibition of ability to induce splenomegaly (Fig. 1A).

A hallmark of GVHD is the induction of donor CTL with specificity for recipient MHC Ags that are not shared between the two. Thus, spleen cells from F₁ mice, undergoing GVHD, contain B6 CTL able to lyse H-2^d targets (Fig. 1B). Consistent with results in Fig. 1A, spleen cells from mice, injected with NAD-treated B6 cells, are not able to lyse H-2^d targets (Fig. 1B). Therefore, NAD-treated B6 T cells fail to generate CTL specific for H-2^d targets when injected into F₁ recipients. A prediction therefore is that in animals, infused with NAD-treated B6 cells, the function of recipient T cells should be preserved. Indeed, Fig. 1C shows that spleen cells from F₁ mice injected with NAD-treated B6 cells mount a perfectly normal CTL response to third-party H-2^k stimulator cells in vitro. In contrast, spleen cells from mice injected with untreated B6 cells do not respond, because recipient T cells had been inactivated by action of B6 donor CTL, recognizing H-2^d Ags. These results show that spleen cells from F₁ mice, infused with NAD-treated parental B6 T cells, retain immune responsiveness.

NAD-incubated T cells are not able to reconstitute T cell-deficient mice

The finding that NAD-incubated T cells fail to induce GVHD points to the possibility that NAD inhibits T cell proliferation. To examine this, NAD-treated T cells were adoptively transferred into T cell-deficient syngeneic recipients to assay their survival and expansion. B6 Thy-1.1⁺ mice were thymectomized, lethally irradiated, and reconstituted with syngeneic bone marrow. Twenty-four hours later, animals were adoptively transferred with 10⁷ purified, control, or NAD-treated B6 Thy-1.2⁺ T cells. Mice were sacrificed 4 wk later, and spleens were analyzed for Thy-1.2⁺ T

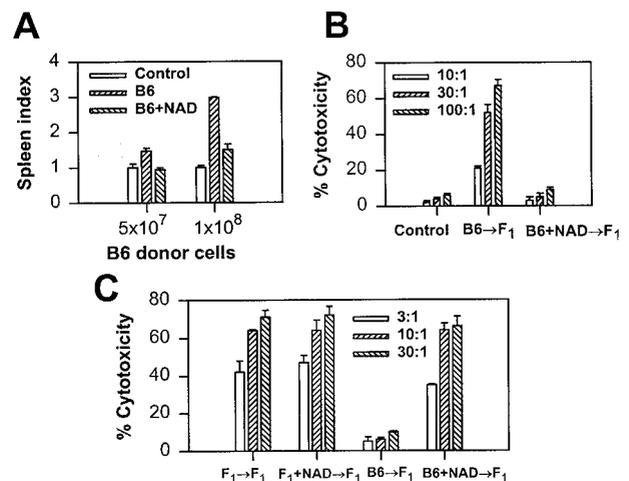


FIGURE 1. NAD treatment of donor T cells inhibits induction of GVHD in F₁ hybrid mice. *A*, B6 spleen cells were cultured with or without 1 mM NAD for 3 h and then injected i.v. into groups of four BDF₁ recipients at the cell concentrations indicated. On day 10, animals were sacrificed, spleen weights determined, and indexes calculated. *B*, Animals were prepared as in *A*, and spleen cells were harvested on day 10, at which time they were assayed for cytolytic activity on P815 (H-2^d) targets at the E:T ratios indicated. *C*, Animals were prepared as in *A*. Spleen cells were harvested on day 10 and cultured with C3H stimulator cells for 5 days, at which time cytolytic activity was assayed on H-2^k target C1.18.4 at the E:T ratios indicated. Results presented are from one of three repeat experiments.

cells by FACS analysis. Results in Fig. 2A show that mice that had been injected with NAD-treated T cells do not contain a detectable number of Thy-1.2⁺ T cells. In contrast, spleen cells from animals reconstituted with untreated T cells contain 3.5% Thy-1.2-staining T cells. Therefore, incubation of T cells with NAD before adoptive transfer into T cell-deficient mice interferes with their ability to reconstitute the T cell compartment of these mice.

The inhibitory effect of NAD on T cells could be due to toxicity, independent of expression of ADPRT. To examine this, effects of NAD on cells, known to lack ADPRT, were investigated. Assay of bone marrow from B6 mice for presence of ADPRT activity, using ADPRT enzyme assays, as well as labeling with radioactive NAD (16), failed to demonstrate any ADPRT activity on these cells (data not shown). Therefore, bone marrow cells appeared to be suitable to examine effects of NAD on cells not expressing ADPRT. To this end, bone marrow cells were incubated with NAD and then injected into syngeneic, lethally irradiated recipients. Results in Fig. 2B show that NAD-treated marrow generates the same number of CFUS as marrow not treated with NAD. Therefore, the inhibitory effect of NAD on T cells is not seen in bone marrow stem cells, devoid of ADPRT.

Injection of NAD-treated T cells leads to accumulation of apoptotic lymphocytes in the liver

The observation that NAD-treated T cells do not induce GVHD and are not able to repopulate spleens of T cell-depleted mice raises the possibility that NAD induces T cell anergy or apoptosis. Interestingly, our previous experiments had shown that NAD-treated T cells, when injected into mice, fail to home to lymphoid organs (20). This pointed to the possibility that aberrant homing is responsible for failure of T cells to respond to and to thrive in recipient animals. An important question, therefore, is where do NAD-treated T cells migrate? Results in Fig. 3A show that incubating purified T cells with increasing concentrations of NAD,

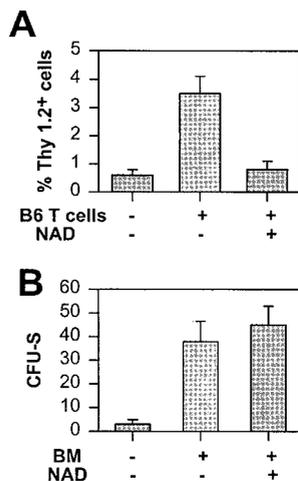


FIGURE 2. NAD inhibits T cells, but not bone marrow stem cells. *A*, Groups of four, adult-thymectomized, B6 Thy-1.1 mice were lethally irradiated and reconstituted with syngeneic T cell-free bone marrow. One day later, purified splenic T cells from B6 Thy-1.2 mice were incubated with 1 mM NAD for 3 h at 37°C, before i.v. injection at a dose of 10^7 T cells per mouse, as indicated. Four weeks later, animals were sacrificed and spleens were assayed for the percentage of Thy-1.2⁺ cells by FACS. The percentage of Thy-1.2-staining cells per spleen is plotted from one of two similar experiments. *B*, B6 bone marrow was treated or not treated with 1 mM NAD for 3 h and injected i.v. at a dose of 10^6 cells per mouse into groups of three lethally irradiated animals. CFUS were determined on day 8. Results from two experiments are shown.

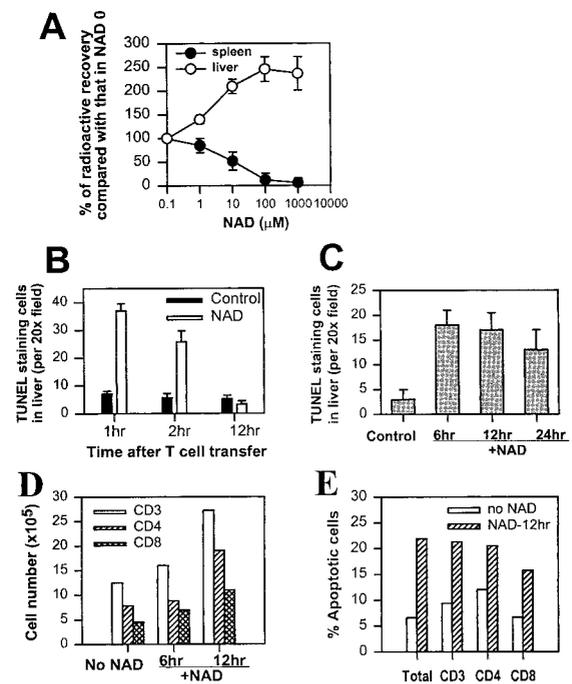


FIGURE 3. NAD induces accumulation of T cells and T cell death in the liver. *A*, B6 T were cultured with or without 1 mM NAD for 3 h and labeled with ^{51}Cr for the last 60 min of the incubation. After washing, cells were injected i.v. into sets of three B6 recipients at a dose of 5×10^6 cells per animal. After 1 h, mice were sacrificed, and spleens and livers were recovered for determination of radioactivity. Radioactivity in organs of mice injected with untreated cells was set at 100%. Shown are data from one of two similar experiments. *B*, Injection of NAD-treated T cells leads to an increase of apoptotic cells in the liver. B6 T cells were incubated with or without 1 mM NAD for 3 h and then injected i.v. at a dose of 10^7 cells per mouse into groups of three B6 recipients. After 1, 2, and 12 h, mice were sacrificed, and liver sections were prepared and assayed for TUNEL-staining lymphocytes. Plotted are the means of TUNEL-staining lymphocytes per section in a $\times 20$ field from multiple sections. The data shown are from one of two similar experiments. *C*, NAD injection induces lymphocyte death in the liver. Groups of three B6 mice were injected i.v. with PBS (control) or 1 mg NAD in PBS. At the times indicated, livers were harvested and sections assayed for TUNEL-staining lymphocytes. The number of TUNEL-staining lymphocytes per $\times 20$ field is plotted, as well as SD values derived from multiple sections. The data shown are from one of two similar experiments. *D*, NAD injection induces an increase of T cells in the liver. Groups of three B6 mice were injected i.v. with PBS (No NAD) or 1 mg NAD in PBS, and livers were harvested at 6 and 12 h. Mononuclear cells were isolated from pooled livers from these groups; stained for CD3, CD4, and CD8; and analyzed by FACS. The calculated number of cells per liver is plotted. The data shown are from one of two similar experiments. *E*, NAD injection induces death of T cell in the liver. Groups of three B6 mice were injected i.v. with PBS (No NAD) or 1 mg NAD in PBS, and livers were harvested after 12 h. Mononuclear cells were isolated from pooled livers from these groups, stained for T cell markers as well as annexin V, and analyzed by FACS. The data shown are from one of two similar experiments.

before injection, leads to a gradual decrease of T cell homing to the spleen and a reciprocal increase of cells in the liver. Therefore, the inability of NAD-treated T cells to induce GVHD could be due to sequestration of T cells to the liver, which in turn raises the question as to why T cells migrate to this organ.

Published data have shown that T cells, undergoing cell death, are captured in the liver (25). Therefore, the observation that NAD-treated T cells accumulate in this organ could indicate that they undergo apoptosis. To examine this, purified T cells were

incubated with NAD, injected into mice and liver sections assayed by TUNEL staining for apoptotic lymphocytes. Results in Fig. 3B show that livers from mice injected with untreated T cells contain 5–7 TUNEL-staining cells per field at all time points. In contrast, liver sections from mice injected with NAD-treated T cells contain a mean of 37 staining cells at the 1-h time point, 27 at 2 h, but only 3 at the 12-h time point. These data suggest that NAD-treated T cells die in the liver or, less likely, induce cell death in companion lymphocytes.

NAD injection induces accumulation of apoptotic T cells in the liver

The observation that *in vitro* treatment of T cells with NAD leads to accumulation of apoptotic lymphocytes in the liver raises the question as to whether NAD has similar effects *in vivo*. To find out, mice were injected with NAD, and liver sections were assayed for apoptotic lymphocytes at various times thereafter. Results in Fig. 3C show that livers from NAD-injected mice contain a significantly higher number of TUNEL-staining lymphocytes at the 6-, 12-, and 24-h time points than PBS-injected controls.

To examine the phenotype of the apoptotic cells, liver mononuclear cells from a pool of three livers were isolated, stained with Abs, and analyzed by FACS. Fig. 3D shows that NAD injection causes a small increase in the total number of CD3⁺ cells per liver at the 6-h time point and a more significant increase of CD3⁺, CD4⁺, and CD8⁺ cells at 12 h. To examine whether this increase consists of apoptotic cells, lymphocytes were stained for annexin V. Results in Fig. 3E show that the majority of annexin V-staining cells at the 12-h time point are T cells, both CD4⁺ and CD8⁺ cells. Therefore, injection of NAD into naive animals induces rapid accumulation of apoptotic CD4⁺ and CD8⁺ T cells in the liver.

NAD induces apoptosis of T cells, but not B cells in vitro

The finding that NAD-treated T cells do not reconstitute T cell-deficient mice and that injection of NAD induces T cell death *in vivo* suggests that NAD induces T cell apoptosis. To directly show this, purified, splenic T cells were incubated with various amounts of NAD for various times and assayed for apoptosis by annexin V staining. Results in Fig. 4A reveal a small increase of annexin V-staining cells as early as 3 h after addition of 1000 μ M NAD, reaching a substantial level by 24 h. Titration of NAD for the 24-h time point reveals a very small increase of cell death at 10 μ M NAD, an intermediate effect at 100 μ M NAD, and a high effect at 1000 μ M NAD (Fig. 4C). In contrast to the results with T cells, purified, splenic B cells, previously shown to lack detectable cell surface ADPRT (20), when incubated with 1000 μ M NAD, show no increase in annexin V-staining cells (Fig. 4B).

NAD induces apoptosis of naive, but not activated T cells

The finding that NAD-treated B6 T cells when injected into F₁ mice do not respond (Fig. 1) suggests that naive T cells are sensitive to NAD-induced cell death. Splenic T cells from unprimed mice express CD62L, but not CD69, and are therefore predominantly naive T cells (26, 27) (Fig. 5A). Incubation of these cells with NAD causes an increase of annexin V-staining cells in the PI-negative population from 5% to 33% (Fig. 5B). The annexin V-staining cells are contained in the CD3⁺ cell population, which increases from 11% to 36% in the presence of NAD (Fig. 5C). Therefore, cell death is induced by NAD in the naive CD62L⁺CD69⁻ T cell population. To examine whether cell death is induced in both the CD4⁺ and CD8⁺ cell population, double staining for CD4, CD8, and annexin V was done. Fig. 5C shows that annexin V staining in the CD4⁺ population increases from 7 to 18%, and in the CD8⁺ population from 3 to 15%. Therefore,

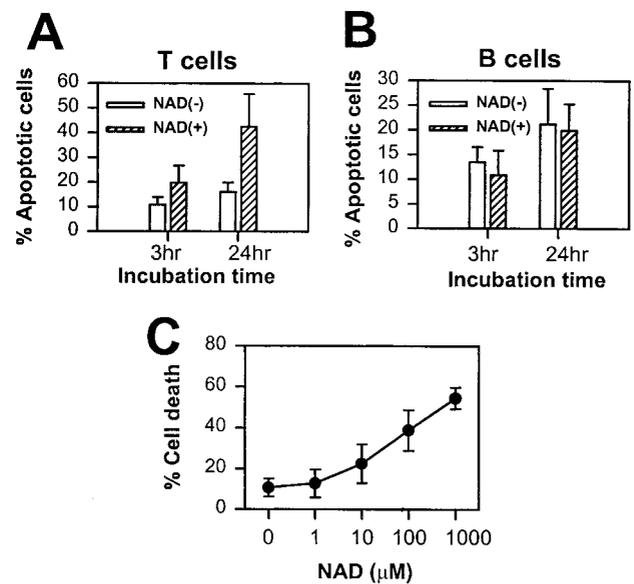


FIGURE 4. NAD induces apoptosis in T cells, but not B cells. *A*, B6 T cells were cultured for the times indicated in 1 mM NAD at 37°C in complete medium. At the times indicated, cells were harvested and assayed for annexin V staining by FACS. The mean percentage of annexin V-staining cells is plotted from a total of nine independent experiments, including SD values. *B*, B6 B cells were cultured with NAD and assayed as in *A*. The data shown are means from three independent experiments, including SD values. *C*, B6 T cells were cultured with various concentrations of NAD, as in *A*. After 24 h, cells were harvested and assayed for annexin V staining by FACS. The mean percentage of annexin V-staining cells from three independent experiments is plotted, including SD values.

both CD4⁺ and CD8⁺ cells are induced for cell death in the presence of NAD.

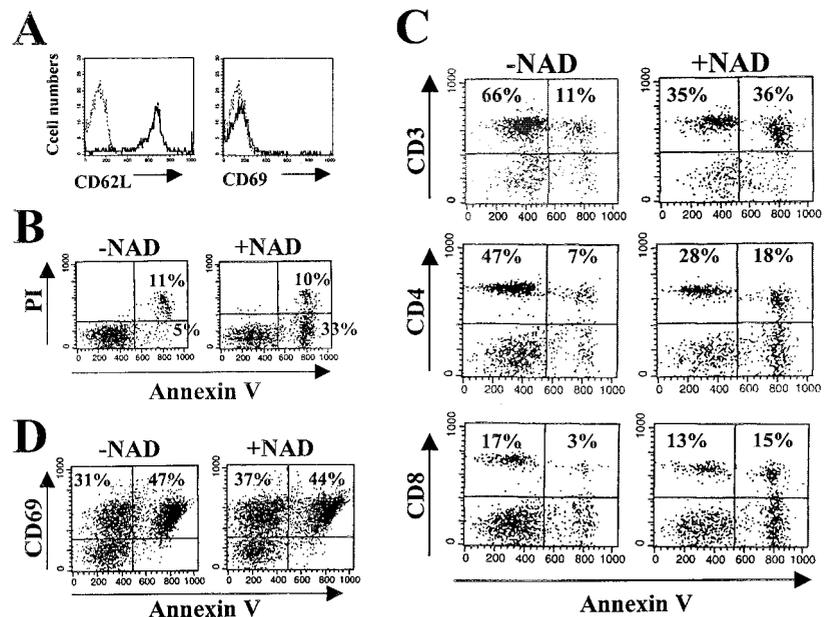
Previous experiments had shown that activation of T cells by TCR cross-linking leads to a rapid release of ADPRT from the T cell surface (17, 24). One would therefore predict that activated T cells are resistant to NAD-induced apoptosis. To examine this, T cells were first incubated on anti-CD3-coated tissue culture plates, followed by incubation with NAD. Results in Fig. 5D show that T cells stimulated on anti-CD3-coated plates for 24 h contain 78% CD69⁺ cells. Among these, 47% are apoptotic and stain with annexin V after a 24-h incubation without NAD. Inclusion of NAD into the culture medium does not increase the percentage of annexin V-staining cells in the CD69⁺ population. Therefore, activated, CD69⁺ cells do not undergo increased cell death when incubated with NAD.

Discussion

Results presented in this communication show that T cells treated with NAD accumulate in the liver as early as 1 h after injection, and TUNEL staining suggests that the injected cells undergo apoptosis in the liver. Because the liver is known as a graveyard for dying T cells (25), these results support the notion that *in vitro* treatment of T cells with NAD induces an apoptotic signal, resulting in accumulation of dying T cells in the liver. In agreement, functional assays show that NAD-treated T cells do not induce GVHD and, when injected into T cell-deficient mice, fail to expand.

The striking efficiency with which NAD inactivates T cells *in vitro* raised the question as to whether NAD can exert similar effects *in vivo*. We show that NAD concentrations *in vitro*, as low as 1–10 μ M, may exert effects on T cell homing *in vivo* (20). It was therefore possible that injection of NAD leads to transient

FIGURE 5. NAD induces apoptosis in naive T cells. **A**, Purified splenic B6 T cells were analyzed by FACS for expression of CD62L and CD69. The dotted lines represent nonstained control cells. **B**, The cells from **A** were cultured for 24 h with or without 1 mM NAD in complete medium, stained with PI and annexin V, and analyzed by FACS. Analysis of the live cell population is shown. **C**, B6 T cells were cultured for 24 h with or without 1 mM NAD in complete medium. Cells were then stained for CD3, CD4, CD8, and annexin V, and analyzed by FACS. **D**, B6 T cells were first cultured for 24 h on anti-CD3-coated tissue culture plates. Cells were harvested and then recultured for 24 h with or without 1 mM NAD in complete medium. Cells were stained for CD69 and annexin V and analyzed by FACS. Data shown in **B**, **C**, and **D** are from one of three independent and similar experiments.



NAD levels able to induce demonstrable effects *in vivo*. Indeed, we show that injection of NAD causes a significant increase of TUNEL-staining lymphocytes in the liver. FACS analysis of mononuclear cells, isolated from livers of NAD-injected mice, revealed that apoptotic cells are in both the CD4⁺ and CD8⁺ cell populations. Therefore, injection of NAD can lead to concentrations, capable of inducing sequestration of T cells, undergoing apoptosis, in the liver.

An interesting question then is whether NAD has effects on both naive and activated T cells. We show that NAD-treated naive T cells fail to induce GVHD in F₁ hybrid mice and, when injected into T cell-depleted syngeneic mice, do not reconstitute these mice. These data are consistent with the notion that naive T cells are inactivated by NAD incubation, a conclusion that is also supported by *in vitro* data. Incubation of naive T cells with NAD leads to death in the CD62L⁺CD69⁻ cell population. In contrast, no death was demonstrable in CD69⁺ cells, activated by TCR cross-linking. It therefore appears that naive, but not activated T cells are sensitive to the NAD-induced apoptosis mechanism. This result is not unexpected, as activation of T cells had been shown to cause release of ADPRT from the cell surface, thereby depriving cells of the enzyme, responsible for modification of cell surface molecules (17–20, 24). Our experiments showing that T cells, but not B cells and hemopoietic stem cells, are sensitive to NAD-induced apoptosis are consistent with this finding, as neither B cells nor bone marrow cells express ADPRT (20, 23, and unpublished results). Therefore, there is a correlation between expression of ADPRT and sensitivity to NAD-induced cell death.

A molecule that is also expressed on lymphoid cells and utilizes NAD as substrate is CD38. CD38 mediates NADase and ADP ribosyltransferase activity, and is commonly expressed on B cells, hemopoietic stem cells, and activated, but not naive T cells (28–30). Given this distribution, it is highly unlikely that CD38 causes NAD-induced apoptosis, as no cell death is demonstrable in B cells, hemopoietic stem cells, and activated T cells. It is possible, however, that CD38 counteracts the action of ADPRT by removing NAD substrate from the extracellular space via its NADase activity.

An intriguing question is how ADP ribosylation of cell surface molecules, mediated by ADPRT, induces apoptosis. We had previously reported that in T cells in which cell surface molecules are

ADP ribosylated, Ab-induced receptor capping and transmembrane signaling are inhibited (19, 20). It is therefore possible that changes in receptor association, caused by the modification, initiate the apoptotic signal. Recently, results were reported suggesting that ADP ribosylation of CD38 can induce cell death in various cell types (30). The demonstration in this study that NAD induces cell death in naive T cells that do not express CD38 (data not shown) makes this mechanism of ADPRT action unlikely.

The finding that NAD induces cell death *in vitro* and *in vivo* raises the more general question as to whether this pathway is utilized by the immune system to limit T cell responses. Published data have shown that elimination of RT6 (ART2)-expressing T cells in BB-DR rats induces diabetes (5), pointing to an immunoregulatory function of RT6. An unresolved question then is how does intracellular NAD reach an enzyme expressed on the cell surface? NAD is present not only in nucleated cells, but also in erythrocytes in concentrations approaching 1 mM (31). Hence, trauma and inflammation, associated with cell lysis, could result in levels of ecto-NAD, able to suppress T cell functions. Under normal conditions, NAD released from lysing cells is removed from the extracellular space by cell-bound and free NADases, of which CD38 is one. The steady state concentration of NAD in the serum is 0.1 μM (32), which is below the *K_m* of ADPRTs. NAD released from lysing cells, exceeding this base level during inflammation, could therefore serve to limit the destructive action of autoreactive T cells, infiltrating the site of tissue injury.

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