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Rapid Induction of Naive T Cell Apoptosis by Ecto-Nicotinamide Adenine Dinucleotidase: Requirement for Mono(ADP-Ribosyl)Transferase 2 and a Downstream Effector

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Lymphocytes express a number of NAD-metabolizing ectoenzymes, including mono(ADP-ribosyl)transferases (ART) and ADP ribosylcyclases. These enzymes may regulate lymphocyte functions following the release of NAD in injured or inflammatory tissues. We report here that extracellular NAD induces apoptosis in BALB/c splenic T cells with an IC₅₀ of 3–5 μM. Annexin V staining of cells was observed already 10 min after treatment with NAD in the absence of any additional signal. Removal of GPI-anchored cell surface proteins by phosphatidylinositol-specific phospholipase C treatment rendered cells resistant to NAD-mediated apoptosis. RT-PCR analyses revealed that resting BALB/c T cells expressed the genes for GPI-anchored ART2.1 and ART2.2 but not ART1. ART2-specific antisera blocked radiolabeling of cell surface proteins with both [³²P]NAD and NAD-mediated apoptosis. Further analyses revealed that natural knockout mice for Art2a found to exert a regulatory role in models for autoimmune insulin-dependent diabetes mellitus. Indeed, diabetes prone BB-DP rats have reduced ART2 expression, and the transfer of ART2+ T cells from their BB-DR-resistant counterparts confers protection. Failure to develop this T cell subset is thus one of the genetically determined factors leading to enhanced susceptibility for autoimmune diabetes (8).

The first evidence supporting a role of ART in the immune system came from experiments in rats. In this species, there is a single locus encoding ART2 with two alleles (ART2a and ART2b), both expressed as 25- to 35-kDa GPI-anchored maturation markers on the surface of T lymphocytes (7). ART2+ T cells have been found to exert a regulatory role in models for autoimmune insulin-dependent diabetes mellitus. Indeed, diabetes prone BB-DP rats have reduced ART2 expression, and the transfer of ART2+ T cells from their BB-DR-resistant counterparts confers protection. Failure to develop this T cell subset is thus one of the genetically determined factors leading to enhanced susceptibility for autoimmune diabetes (8).

While NAD concentrations in the cytoplasm lie in the millimolar range, concentrations of extracellular NAD are usually submicromolar in serum. However, NAD can be released into the extracellular environment by lytic and nonlytic mechanisms, e.g., following cell damage or acute cell death in injured and inflammatory tissues, and could participate in the regulation of immune functions. Indeed, lymphocytes express a number of membrane-bound NAD-metabolizing ectoenzymes, including CD38 and NAD-dependent ADP ribosyltransferases.

Mono(ADP-ribosyl)transferases (ARTs)³ catalyze a posttranslational modification of proteins by transferring the ADP-ribose moiety of NAD to specific amino acids, e.g., arginine residues, on target proteins. These enzymes have well-known regulatory functions in the prokaryotic world and usually inactivate the function of target proteins (1). They also correspond to various bacterial toxins that have potent effects on mammalian cells (2). More recently, several ARTs have been cloned from different tissues. Five groups of ART-encoding genes, denoted ART1 to ART5, have been identified in the mouse, rat, rabbit, and human, based on sequence homology and genomic organization (3). ART1–ART4 isoforms correspond to 30- to 40-kDa GPI-anchored cell surface ARTs, whereas ART5 is not GPI-linked and may be secreted (4, 5). Their biological function is not fully understood, but they seem to play an important role in the regulation of myogenesis or long-term potentiation in hippocampal neurons, for instance, or in the regulation of lymphocyte functions (5, 6).

The mouse homologue of the rat ART2 gene has been tandemly duplicated to produce two loci (Art2a and Art2b) with multiple alleles (9, 10). In mice, ART activity has also been detected on lymphoid cells, including T cell lymphomas (11, 12), splenocytes (13, 14), and activated CTL (15) and is believed to participate in the regulation of cell functions. A 35-kDa GPI-anchored ART was found to mediate inhibition of proliferation and cytotoxic activity of CTL cell lines in the presence of extracellular NAD (16). A similar NAD-mediated inhibitory effect has more recently been reported
on the proliferation of normal peripheral T cells in vitro (17). ARTs encoded by Art1, Art2a, Art2b, Art3, Art4, and Art5 can potentially mediate these effects, although neither Art5 nor Art3 are expressed on normal lymphoid cells (5, 18, 19). ART1 is a GPI-anchored protein that has been considered a candidate ART responsible for this regulation because it was cloned from T cell lymphomas (20). However, ART1 is expressed at low levels in lymphoid tissues and predominates in heart and skeletal muscles (21). By contrast, Art2a and Art2b mRNAs are expressed at higher level in lymphoid tissues (9, 10). Moreover, the recent development of specific mAbs has allowed us to demonstrate that ART2a is selectively present on mature T lymphocytes from most mouse strains although at different levels (22).

Deficient expression of ART2.1 and ART2.2 has been reported in several inbred mice. In C57BL/6, the Art2a sequence contains a stop codon leading to the synthesis of a truncated protein with no enzymatic activity (23). Reciprocally, NZW mice suffer from a deletion of the Art2b gene (24). Like in rats, defective expression of ART2s has been proposed as one of the factors influencing the onset and/or progression of autoimmune diseases (24, 25).

To date, most experiments concerning the role of ARTs and the effect of extracellular NAD on T lymphocytes have been performed in C57BL/6 mice that express ART2.2 at a very high level but lack functional ART2.1 on their surface (22–24). The potential role of differential ART expression on NAD-induced regulation of immune functions has not been investigated. Experiments reported herein address this issue by comparing the effect of extracellular NAD on T lymphocytes from appropriate inbred mice. We demonstrate that NAD triggers rapid induction of apoptosis in normal peripheral T cells from BALB/c and (C57BL/6 × NZW)/F1 mice but not from natural knockout mice for ART2a (C57BL/6) or ART2b (NZW).

**Materials and Methods**

**Reagents**

FITC-conjugated anti-CD3ε (145-2C11) and PE-conjugated anti-B20 (RA3-6B2) mAbs used for fluorescence staining were purchased from BD PharMingen (San Diego, CA). FITC-conjugated anti-CD8α (CT-CD8a) and PE-conjugated anti-CD4 (CT-CD4) were obtained from Caltag Laboratories (South San Francisco, CA).

**Animals**

BALB/c and C57BL/6 mice were maintained in the animal facilities of the Institut Jacques Monod (Paris, France) and of the University Hospital (Hamburg, Germany). NZW/OlaHsd mice were purchased from Harlan (Oxon, U.K.). F1 hybrids from C57BL/6 females mated with NZW males were bred and are designated elsewhere as B6NW/F. The offspring of the reciprocal mating were also tested and gave similar results (not shown). The Wistar rat R8 and the outbred rabbit 12760 were obtained from and reciprocal mating were also tested and gave similar results (not shown).

**Antibodies**

Preimmune sera were prepared from blood obtained by retroorbital bleeding from rat R8 and by draining of the ear vein from rabbit 12760 before ballistic DNA immunization with 8–12 shots (1 mg DNA coated on 1-μm gold particles) of pME.ART2.2, an expression vector for GPI-anchored, membrane-tethered ART2.2-specific rat IgG2a mAb (designated Nika-102) was generated following fusion of R8 splenocytes with Sp2/0 myeloma cells as described previously (22).

**Cell preparation and proliferation assay**

Splenocyte suspensions were prepared aseptically in cold PBS containing 10% heat-inactivated FCS. Erythrocytes were removed by treatment with NH₄Cl (160 mM, 3 min). Cells were then washed, resuspended in complete RPMI 1640 medium containing 5 × 10⁻³ 2-ME, 0.2 mM glutamine, 1 mM pyruvate, 20 mM HEPES, and 10% FBS (all culture reagents were from Life Technologies, Grand Island, NY). T cells were purified by two passages over nylon wool columns. Purified T cells contained >80% living T cells and <10% B cells as determined by flow cytometry. For in vitro stimulation, spleen cells or purified T cells were cultured for 2 days at 37°C in 96-well flat-bottom tissue plates (2 × 10⁵ cells/well) either coated with 10 μg/ml anti-CD3ε (145-2C11) mAb or in the presence of 3 μg/ml Con A, 10 ng/ml PMA plus 100 ng/ml ionomycin (ICN, Costa Mesa, CA). After 8 h, cells were harvested (Filtermate 196; Packard, Meriden, CT), and the radioactivity incorporated into DNA was counted (TopCount; Packard).

**Immunofluorescence analysis, detection of apoptotic cells, and treatment with phosphatidylinositolspecific phospholipase C (PI-PLC)**

Staining procedures were used for immunofluorescence staining. Briefly, 1 × 10⁶ cells were washed and resuspended in FACS buffer (CellWash; Becton Dickinson, Mountain View, CA). They were then resuspended in 100 μl FACS buffer containing 1 μg of each mAb. After 30 min of incubation at 4°C, cells were washed twice and resuspended in 300 μl FACS buffer after analysis by flow cytometry on a FACSCalibur (Becton Dickinson).

**Detection of apoptotic cells was performed by annexin V/propidium iodide (PI) staining as previously described (26).** A second method was used to detect and quantify apoptosis based on TUNEL of DNA strand breaks (27). Briefly, cells were washed twice in PBS containing 1% BSA and fixed with 4% paraformaldehyde for 30 min at room temperature. Cells were washed at 4°C and permeabilized with sodium citrate buffer containing 0.1% Triton X-100 for 2 min on ice. After washing, cells were incubated with FITC-dUTP in the presence of TdT for 1 h at 37°C using the Cell Death Detection kit (Roche Diagnostic Systems). Following incubation and washing, cells were counterstained with PI (0.05 μg/ml) in FACS buffer containing 200 μg/ml DNase-free RNase (Roche Diagnostic Systems). After 30 min of incubation at room temperature, cells were analyzed by flow cytometry.

In some experiments, lymphocytes were treated with PI-PLC before incubation with NAD and quantification of apoptosis using annexin V/PI staining. Cells were incubated for 30 min at 37°C in RPMI 1640 (3 × 10⁷ cells/ml) containing 10 μM PI-PLC (Sigma). They were then suspended at 1 × 10⁶ cells/ml in complete RPMI 1640 and incubated 1 h at 37°C with or without NAD before detection of apoptosis.

**ADP-ribosylation of proteins**

ADP-ribosylation of membrane proteins was analyzed by SDS-PAGE after incubation of cells with [³²P]NAD as previously described (17) with some modifications. Briefly, 2 × 10⁷ purified T lymphocytes were incubated in 100 μl complete RPMI 1640 medium containing 10 μCi [³²P]NAD (350 μCi/mmol; ICN Pharmaceuticals), 1 mM ADP-ribose (Sigma), 100 μM cold NAD, and 1 μM protease inhibitor cocktail (P8340; Sigma) at 37°C for 1 h. T lymphocytes were then washed three times in cold RPMI 1640 to remove unbound radioactivity. Crude cell lysates were obtained by suspension of cell pellets in lysis buffer (PBS containing 1% Nonidet P-40, 1 mM EDTA, 1 mM PMSF, 1 mM iodoacetamide, pH 5.4), followed by incubation on ice for 30 min. Insoluble material was pelleted by centrifugation (12,000 × g for 4 min) at 4°C. Supernatants were collected. Proteins were separated by SDS-PAGE (10% gel), and dried gels were exposed to x-ray films at −70°C for 4 days using intensifying screens.

**RT-PCR analysis of Art1, Art2a, and Art2b expression**

The relative expression of the Ab1, Ab2a, and Ab2b genes was analyzed by semi-quantitative RT-PCR analysis. Total RNA was extracted from either 2 × 10⁷ purified T cells from BALB/c, C57BL/6, NZW, and B6NWF₆ mice or BALB/c skeletal muscle using the RNA Plus kit (Quantum- Appligene, Strasbourg, France). cDNA was synthesized from 3 μg of total RNA in a volume of 33 μl using the first-strand cDNA synthesis kit and oligo(dT)₁₄ as primers (Amersham Pharmacia Biotech, Uppsala, Sweden) according to manufacturer’s recommendations. The cDNA concentration of all samples was adjusted after pilot PCR for β-actin using forward 5′-TTGAAGATCTTGCCATCCATGAAAAAC-3′ and reverse 5′-TA AACGCGACTGCAGTACAGTCCG-3′ primers. PCR were conducted
in 20-μl reaction volumes containing 1 μl cDNA, 1× PCR buffer, 2.5 U Taq polymerase, 1.5 mM MgCl₂ (all from Bioline, London, U.K.), 100 μM dNTPs (Roche Diagnostic Systems) and 5 pmol of each primer. PCR was performed on a PTC-200 thermocycler (MJ Research, Cambridge, MA) for 30–40 cycles of 92°C, 60°C, and 72°C, 1 min each. Specific primers were derived from separate exons in nonconserved regions of Art1, Art2a, and Art2b deduced from the published sequences (GenBank accession numbers X95825 (Art1); X52991 (Art2a), and X87612 (Art2b)). Enzymatic digestion confirmed the specificity of all PCR products (not shown). The following primers were used: for Art1, forward ADPRT: 5'-TGCTGCTCCTA CACGCCAAC-3' and reverse 5'-TCAACATCGGGTAACTGCT-3' or forward ADRT 5'-GAGCCACCCAGCTCCTGAGGT-3' and reverse 5'-AGTTGACAGCAGCTTCAGTC-3'; for Art2a, forward Arta2 5'-ATC CACAGAAGCCTTAACTGAG-3' and reverse 5'-CTACGGCTCAG CAAGAGTAA-3'; for Art2b, forward Art2b 5'-CCTCGCTATAGATTIT TAACAG-3' and reverse 5'-CTACGGCTCAGCAAGAGTAA-3'. PCR products were analyzed on 1% agarose gels and ethidium bromide staining.

Results
Expression of ART isoforms in T lymphocytes
Before analyzing the effect of extracellular NAD on lymphocytes, we determined the expression of Art2a-, Art2b-, and Art1-encoded ART isoforms on nylon-purified T cells from BALB/c, C57BL/6, NZW, and B6NWF1 mice by RT-PCR. As illustrated in Fig. 1, Art2a mRNA was present in T cells from BALB/c mice and, at an apparently lower level, in cells from NZW and B6NWF1 animals. In C57BL/6, a low Art2a signal was detected, but this sequence contains an in-frame stop codon as reported previously (23). Art2b was transcribed at high level in C57BL/6 mice but at lower level in BALB/c and in B6NWF1 hybrids. This was consistent with results obtained by staining with ART2.2-specific Nika-102 mAb as already reported (22). As expected, Art2b transcripts were absent in ART2.2-deficient NZW mice (24).

Importantly, Art1-specific transcripts were not detected in T lymphocytes from any of the strains tested, even though in two different pairs of primers were used. By contrast, they were readily amplified from skeletal muscle of BALB/c mice. Although it has been observed that transfection of Art2b transcripts were absent in ART2.2-deficient NZW mice (24).

Ecto-NAD selectively inhibits T cell activation
It has previously been reported that extracellular NAD suppresses the proliferation of C57BL/6 mouse T cells. We wondered whether the differential expression of Art2a and Art2b would influence the sensitivity to NAD-mediated suppressive effects on T cell function. To address this question, we first compared the effects of extracellular NAD on spleen cell proliferation induced by Con A in BALB/c and C57BL/6 mice (Fig. 2A). In both strains, cell proliferation was strongly inhibited by low concentrations of NAD with an IC₅₀ in the range of 3–5 μM. The proliferative response was also blocked by NAD in B6NWF1 mice, whereas NZW T cells were resistant. The effect of NAD on BALB/c splenocytes was specific to T cells, as previously reported for C57BL/6 lymphocytes (17), because LPS-induced B cell proliferation was not affected (Fig. 2B). We further tested the effect of NAD on the proliferation of nylon-purified T cells induced by Con A, PHA, anti-CD3 mAb, or PMA-ionomycin. Fig. 2C shows that NAD inhibited the response irrespective of the activation pathway, including the direct stimulation of cytoplasmic protein kinase C by PMA and ionomycin. This indicated that the effect of NAD, at least in BALB/c mice, was not caused by an early blockage of the transmembrane signaling pathway.

Inhibition of cell proliferation could potentially reflect a direct toxic effect of ecto-NAD on T lymphocytes. This was tested by incubating nylon-purified T cells from the different strains with 10 μM NAD. Cell viability was followed over 48 h by staining with

![FIGURE 2. Effect of NAD on lymphocyte proliferation and viability. Cells were cultured in presence of NAD, and [³H]Tdr incorporation was measured on day 2 (A–C). A, Splenic cells from BALB/c, C57BL/6, NZW, or B6NWF1 mice were cultured with Con A. B, BALB/c spleen cells were cultured with Con A or LPS. C, Purified T cells from BALB/c were cultured with Con A, anti-CD3 mAb, PHA, or PMA plus ionomycin. Results represent the mean cpm of triplicate assays from one experiment of three and are given as the percentage of the mean cpm of controls without NAD. The proliferative responses of splenocytes to Con A, irrespective of mouse strains, were in the range of 100,000 cpm, whereas thymidine incorporation of nonactivated control cells was in the range of 600 cpm. The proliferative response of BALB/c splenocytes to LPS was in the range of 60,000 cpm, control cells in the range of 300 cpm. The proliferative responses of BALB/c-c-purified T cells to Con A were in the range of 50,000 cpm, to coated CD3 mAbs in the range of 70,000 cpm, to PHA in the range of 40,000 cpm, and to PMA plus ionomycin (IONO) in the range of 90,000 cpm. Whereas proliferation of nonstimulated control cells was under 800 cpm. D, Cells were cultured in the presence of 10 μM NAD, and cell viability was followed for 48 h by staining with annexin V and PI to determine apoptotic and dead cells, as described in Materials and Methods. Assays were performed in triplicate, and results represent mean numbers of viable cells (nonapoptotic and non-PI-stained cells) from one experiment of two, given as the percentage of the mean viable cell numbers in controls without NAD. Vertical bars correspond to SDs.](http://www.jimmunol.org/DownloadedFrom)
annexin V and PI to detect apoptotic and dead cells. A rapid fall of cell viability was observed as early as 1.5 h after incubation of BALB/c and B6NWF1 T cells (Fig. 2D). NZW T lymphocytes were much less affected even after 48 h. An intermediate and slower toxicity of ecto-NAD was observed on C57BL/6 T cells. At 48 h, cell viability was rather similar in all mice except NZW. This was in good agreement with the inhibitory effect of ecto-NAD on T cell proliferation, but suggested a differential mechanism of toxicity between C57BL/6 and BALB/c or B6NWF1, mice.

To test whether CD4 and CD8 subsets were equally sensitive to NAD toxicity, BALB/c spleen cells were incubated with 50 μM NAD for 48 h and were then analyzed by flow cytometry (Fig. 3). In the absence of NAD, the CD4:CD8 ratio was close to 2. With 50 μM NAD, most T cells were eliminated but the CD4:CD8 ratio remained unchanged. This demonstrated that extracellular NAD has a direct toxic effect on both naive CD4+ and CD8+ T lymphocytes in the absence of additional stimulus. The results also confirmed that B220+ B cells were not sensitive to NAD toxicity.

**Rapid induction of T cell apoptosis by NAD in BALB/c and B6NWF1, mice**

The rapid toxic effect of NAD on naive T lymphocytes from BALB/c and B6NWF1 mice could result from necrosis or from the induction of programmed cell death. To address this issue, purified splenic T cells from BALB/c or C57BL/6 mice were incubated for 1.5 h with NAD and then stained with annexin V/PI. As illustrated in Fig. 4A, NAD at 10 μM induced apoptosis of T lymphocytes from BALB/c but not from C57BL/6 mice. T cell apoptosis was dependent on the NAD concentration added to cultures (Fig. 4B) and could be detected as early as 10 min after incubation (data not shown). C57BL/6 mice could thus be considered as resistant to NAD-induced apoptosis as previously suggested (17). Analysis of DNA strand breaks by TUNEL after 6 h of incubation confirmed that BALB/c T cells were sensitive to NAD-induced apoptosis (Fig. 4C). Again, only a small fraction of C57BL/6 T cells were engaged into programmed cell death. Taken together, these observations indicated that ecto-NAD blocks T cell activation in both BALB/c and C57BL/6 mice, but selectively induces rapid apoptosis in BALB/c mice. As expected from their resistance to NAD-mediated inhibition of T cell proliferation, NZW T cells were also resistant to NAD-mediated apoptosis. Interestingly, apoptosis was induced in B6NWF1 T cells, with similar kinetics as in BALB/c, although both their C57BL/6 and NZW parents were resistant (Fig. 4). This demonstrated that at least two genetic factors are involved in NAD-induced apoptosis.

**Apoptosis is mediated by NAD and involves GPI-anchored cell surface proteins**

The observation that NAD-induced apoptosis in BALB/c and in B6NWF1, T cells raised the question of the molecular mechanism underlying this effect. Extracellular NAD cannot penetrate into cells but can be degraded by several enzymes, including NAD-glycohydrolases such as CD38 (29) and phosphodiesterases such as PC-1 (30). These enzymes can transform NAD into metabolites that could deliver signals of programmed cell death (31). These metabolites include adenosine, ADP, AMP, nicotinamide, ADP-ribose (ADPR), and cyclic ADP-ribose (cADPR). To test the possibility that the toxic effect of NAD was mediated by one of these metabolites rather than by NAD itself, purified T lymphocytes from BALB/c mice were incubated for 30 min with NAD metabolites, and the relative number of apoptotic cells was evaluated. As illustrated in Fig. 5A, apoptosis was induced by incubation with NAD but not by any other NAD metabolites or derivatives tested. This demonstrated that the apoptotic signal was dependent on a surface protein acting as a direct NAD acceptor. The role of P1 purine receptors, which bind adenosine, and of P2 purine receptors, which bind ATP, ADP, UTP, and to a lesser extent NADP (32, 33), was ruled out. Results also excluded the possible intervention of CD38, which is only expressed on a small fraction of murine T lymphocytes (34), because no effect was observed with ADPR or cADPR. As also shown in Fig. 6, double staining of B6NWF1 T cells with CD38 and annexin V after incubation with increasing concentrations of NAD revealed that T cells engaged into apoptosis belonged to the CD38+ population and that CD38- cells were resistant.

ARTs expressed on the cell membrane are GPI-anchored proteins, which directly bind NAD and transfer the ADP-ribose moiety from NAD onto other cell surface proteins. It is thus conceivable that they are involved in NAD-mediated apoptosis. Direct evidence for the involvement of GPI-anchored proteins was brought by treatment of cells with bacterial PI-PLC. Previous studies have shown that this treatment almost completely dislodges ART activity from the membrane of CTL cell lines (15). If GPI-anchored ARTs were involved in the NAD-mediated apoptotic effect, PI-PLC treatment should result in resistance to NAD-induced apoptosis. Results in Fig. 5B support this prediction by showing
that removal of GPI-anchored proteins leads to concomitant loss of sensitivity to extracellular NAD.

Direct involvement of ART activity in the apoptotic process

We took advantage of a recently developed ART2.2-specific mAb and of ART2-specific antisera to evaluate the functional role of ART2 and ADP-ribosylation in NAD-mediated T cell apoptosis. In a first series of experiments, we used Nika-102, a mAb that binds ART2.2 (22) but does not inhibit its enzymatic activity (data not shown), to follow cells undergoing apoptosis in the presence of NAD. Nylon-purified T cells were incubated with different concentrations of NAD for 90 min before annexin V/PI staining. As illustrated in Fig. 7, 66% of T cells from B6NWF1 mice were ART2.2⁺. With increasing concentrations of NAD, the number of cells undergoing programmed cell death increased concomitantly with a diminution of the ART2.2-expressing population. Furthermore, annexin V⁺ cells were largely restricted to the Nika-102⁺ population. By contrast, the fraction and number of ART2.2⁻ cells, which were not labeled by annexin V, remained unchanged. Similar results were obtained with BALB/c T cells (not shown). These observations demonstrated that the cells committed to apoptosis by NAD were only those expressing ART2.2 on their surface.

We then tested the effect of polyclonal rabbit and rat Abs raised against ART2.2 on apoptosis. BALB/c or B6NWF1 T lymphocytes were incubated with serial dilutions of K12760 or R8 serum and NAD. Whereas preimmune sera did not prevent apoptosis, ART2-specific Abs from R8 (not shown) and K12760 selectively blocked cell death (Fig. 8). As illustrated below (Fig. 9), this was consistent with the ability of these Abs to inhibit ART2-transferase activity. Together, this demonstrated that NAD-mediated apoptosis requires ART2-mediated ADP-ribosylation of membrane proteins.

**FIGURE 4.** Induction of apoptosis by NAD. A, Nylon-purified T lymphocytes from BALB/c, C57BL/6, NZW, and B6NWF1 mice were incubated in the presence or absence of 10 μM NAD for 90 min before annexin V/PI staining. B, Cells were incubated as in A with different concentrations of NAD. Apoptosis is expressed as the mean percentage of annexin V⁺/PI⁻ cells from three independent experiments upon subtraction of the respective percentage in controls. C, Cells were incubated for 6 h with different concentrations of NAD before determination of apoptosis by TUNEL assay. Results correspond to the mean percentage of apoptotic cells from three independent tests. Vertical bars represent SD.

**FIGURE 5.** Induction of apoptosis by different nucleotide metabolites and effect of PI-PLC treatment on NAD-induced apoptosis. A, Purified T cells from BALB/c mice were cultured with the indicated compounds at 37°C for 30 min before annexin V/PI staining. Results are expressed as the mean percentage of apoptotic cells from three independent assays. B, Purified T cells were treated with PI-PLC as described in Materials and Methods and incubated for 1 h with NAD before quantification of apoptotic cells by annexin V/PI staining. Each point corresponds to the mean of three independent experiments. Vertical bars represent SD.

**FIGURE 6.** CD38⁺ cells are less sensitive to NAD-mediated apoptosis than CD38⁻ cells. Nylon-purified T lymphocytes from B6NWF1 mice were incubated in the presence of NAD for 30 min before annexin V/CD38 staining. CD3/CD38 and B220/CD38 control staining revealed the presence of 15–17% CD38⁺ T cells and 8–10% contaminant CD38⁻ B cells (data not shown). Viable cells were gated based on forward and side scatter and analyzed by flow cytometry. The percentage of gated cells in each quadrant is given. Data are from one of two independent experiments.
Membrane protein ADP-ribosylation on cells expressing ART2.1, ART2.2, or both.

The presence of ART(s) on naive T cells predicts that incubation with radioactive NAD should result in labeling of cell surface proteins. To test this prediction, nylon-purified T lymphocytes isolated either from natural knockout mice for \textit{Art2a} (C57BL/6) or \textit{Art2b} (NZW) or from mice expressing \textit{Art2a} and \textit{Art2b} (BALB/c and B6NWF1) were incubated with [32P]NAD for 1 h. Crude cell lysates were then prepared, and labeled proteins were analyzed by autoradiography after SDS-PAGE. As illustrated in Fig. 9A (lane 2), strong ADP-ribosylation of several proteins was routinely observed with cells from C57BL/6 mice, consistent with their high level of ART2.2 expression (22). The pattern of proteins labeled was very similar to that already reported by Okamoto et al. in these mice (17). A similar pattern of bands was observed also in cells from BALB/c and B6NWF1 animals (Fig. 9A, lanes 1 and 4), although the intensity of labeling was lower than in T cells from C57BL/6 mice. In marked contrast, little if any ADP-ribosylated cell surface proteins were detected on cells from ART2.2-deficient NZW mice (Fig. 9A, lane 3). Preincubation of T cells with ART2-specific antisera K12760 (Fig. 9, B and C) or R8 (data not shown) reduced labeling of cell surface proteins to background levels in all strains tested. These findings confirmed that most if not all of the ART activity on resting murine T lymphocytes can be attributed to the ART2 ADP ribosyltransferase.

Taken together, these results further suggest that resistance of NZW T cells to NAD-mediated apoptosis can be attributed to the virtual absence of cell surface ADP-ribosyltransferase activity on these cells. Similarly, T cells with strong cell surface ART activity, e.g., those of BALB/c and B6NWF1, can be rendered resistant to NAD-mediated apoptosis by inhibiting ART activity with ART2-specific Abs. In contrast, resistance of C57BL/6 T cells to NAD-mediated apoptosis cannot be attributed to lack of ADP-ribosyltransferase activity. Instead, it seems most likely that these cells carry a defect in a downstream effector of ART activity.
**Discussion**

Increasing evidence suggests that extracellular NAD can regulate T cell functions via cell surface GPI-anchored ADP ribosyltransferases (6). Our results demonstrate that extracellular NAD strongly inhibits the proliferation of normal splenic T cells from BALB/c, C57BL/6, or B6NWF1 mice, at doses ranging from 1 to 10 μM (Fig. 2). This is consistent with previous studies reporting an inhibitory effect of NAD on the proliferation of CTL lines and of naive peripheral T cells (15–17). Yet, more importantly, we demonstrate here that extracellular NAD has a direct toxic effect on T cells from BALB/c mice and induces programmed cell death in the absence of any additional cosignal (Fig. 4). This contrasts with the situation in ART2.1-deficient C57BL/6 mice, in which NAD blocks T cell activation but does not induce apoptosis (17), and in ART2.2-deficient NZW mice, which are refractory to both NAD-mediated apoptosis and inhibition of T cell proliferation.

It has previously been shown that the suppressive effects of ecto-NAD on T cells of C57BL/6 mice depend on GPI-anchored ART(s) (15). Similarly, we demonstrate that removal of GPI-anchored cell surface proteins by treatment of BALB/c T cells with bacterial PI-PLC strongly reduces their susceptibility to NAD-induced apoptosis (Fig. 5B). Implication of ART(s) rather than other NAD-metabolizing ectoenzymes such as CD38 or PC-1 (29, 30) is also attested by the observation that none of the NAD metabolites tested can mediate apoptosis (Fig. 5A), that cells undergoing apoptosis are ART2.2+ (Fig. 7), and that apoptosis can be inhibited by polyclonal Abs to ART2.2 (Fig. 8).

In mice, at least six distinct GPI-anchored ARTs, namely ART1, ART2.1, ART2.2, ART3, ART4, and ART5, have been identified that could be involved in the apoptotic process (3, 4, 18, 19). ART1 has been cloned by different groups from mouse lymphoma cells (Yac-1), from a BALB/c skeletal muscle cDNA library, or from mouse genomic DNA. Northern blot and RT-PCR analyses previously reported indicate that Art1 is prominently expressed in cardiac and skeletal muscles and at much lower level in lymphoid tissues (12, 20, 21). Although ART1 has been detected in T cell lymphomas and hybridomas, direct evidence was still lacking for its expression on normal peripheral T lymphocytes. Our attempt to detect ART1 by RT-PCR on naive splenic T cells was negative even by using different pairs of specific primers and irrespective of the mouse strain tested (Fig. 1). It can thus be concluded that Art1 is not expressed on normal T lymphocytes. Weak signals detected by others for Art1 expression in lymphoid organs may be due to its expression on other cell types (35). Alternatively, Art1 expression may be developmentally regulated and limited to fully differentiated T cells like activated CTL or to transformed T cell lymphomas (20). A rather similar situation has been reported for ART5, which was isolated from Yac-1 lymphoma cells but, again, is not expressed on normal lymphoid spleen cells (18). In contrast, our results confirm previous observations (9, 22, 24) that Art2a and Art2b transcripts are found in resting BALB/c T cells (Fig. 1). In C57BL/6 mice, a low level of Art2a transcripts is detected, but this mRNA contains a stop codon that prevents the expression of a functional enzyme (23). Moreover, our finding that ART2-specific antisera abolish labeling of cell surface proteins following incubation with [32P]NAD (Fig. 9) supports the conclusion that the ART2s represent the predominant ARTs expressed on normal peripheral T cells. It further implies that ART2.2, and presumably also ART2.1, can ADP-ribosylate a number of different cell surface proteins.

The very high sensitivity of T cells from B6NWF1 animals to NAD-induced apoptosis, whereas T cells of either parental animal are resistant (Fig. 4), is important because it indicates that complementation of (at least) two genetic factors is operative in cells of the F1 animals in NAD-mediated apoptosis. We propose that one of these is ART2 cell surface ADP-ribosyltransferase activity. This hypothesis is supported by our findings that ART2-specific antisera block both ADP-ribosylation of cell surface proteins (Fig. 9) and NAD-mediated apoptosis of B6NWF1 cells (Fig. 8). It is further supported by our finding that cells from ART2.1/ART2.2 double-knockout mice are completely resistant to NAD-mediated apoptosis (our unpublished observations). The resistance of NZW T cells to NAD-induced apoptosis (Fig. 4), then, could be attributed to the virtual absence of ART activity on these cells (Fig. 9). Consistent with this interpretation, we find that treatment of NZW T cells with DTT, which is known to stimulate the activity of ART2.1 (13), enhances both NAD-mediated ADP-ribosylation of cell surface proteins and apoptosis (our unpublished observations).

We propose that the second genetic factor governing sensitivity to rapid NAD-mediated apoptosis of resting T cells in B6NWF1 animals concerns a downstream effector of ADP-ribosylation. The observation that C57BL/6 T cells are resistant to NAD-mediated apoptosis (Fig. 4) despite a high level of cell surface ART activity (Fig. 9) implies that ART activity alone is not sufficient for mediating apoptosis. We hypothesize that C57BL/6 mice are resistant to NAD-mediated apoptosis due to a defect in a downstream effector of ADP-ribosylation and that this defect is complemented in B6NWF1 animals by the corresponding factor from the NZW parent. Further analyses are required to determine whether this factor is itself a target protein for ADP-ribosylation or a signaling component activated by ART activity. In this context, it may be interesting to note that lysates of BALB/c and B6NWF1 T cells contain a prominent 35-kDa protein that is labeled weakly in lysates of C57BL/6 mice (arrows in Fig. 9A).

The molecular mechanisms underlying rapid NAD-mediated apoptosis of naive T lymphocytes are still unknown but several hypotheses can be put forward. Activation of the caspase pathway is a well-established mechanism for inducing apoptosis of T cells. However, preliminary evidence using caspase inhibitors indicate that NAD does not activate this pathway in resting T cells. Signaling through GPI-anchored proteins has been reported in diverse settings (36). One could thus postulate that ART2.2 itself, as a GPI-anchored protein, would deliver the apoptotic signal to naive T cells. This possibility is supported by the observation that RT6 is associated with tyrosine kinases and could thereby, potentially, generate intracellular signaling (37). Alternatively, a membrane protein distinct from ART might be involved in apoptotic signal delivery. For example, the apoptotic signal could be delivered via ADP-ribosylation of important costimulatory signaling cell surface molecules. Indeed, CD45, CD44, CD43, or CD27 have been shown to be ADP-ribosylated on NAD-treated T cells (17). Conceivably, this could result in inappropriate weak stimulation signals that in turn would cause programmed cell death. It is indeed established that costimulatory molecules are associated with protein kinases involved in signal transduction (38). Such weak stimulatory signals have been shown to induce apoptosis at different stages during lymphocyte differentiation (39). However, our results indicate that strong stimulation of cells with mitogenic activators fails to rescue cells from death (Fig. 2). Recently, an additional mechanism involving CD38 has been proposed to mediate apoptosis of activated T cells (40). Indeed, ADP-ribosylation of CD38, by blocking its cyclase activity and cADPR production, was shown to induce apoptosis in T cells recently activated by soluble CD3 mAb (40). However, our results indicate that resting T cells lacking CD38 are sensitive to NAD-induced apoptosis whereas CD38-expressing cells are resistant (Fig. 6). Conceivably,
the apoptotic mechanisms induced by NAD may differ in activated and nonactivated T cells.

Next to the remarkable rapid induction of apoptosis of resting T cells described here, NAD evidently influences T cell functions in other ways. This is supported by the finding that C57BL/6 mice are resistant to rapid induction of apoptosis (Fig. 4), but are sensitive to NAD-mediated inhibition of proliferation (Fig. 2). Given that several different proteins are ADP-ribosylated following treatment of cells with ecto-NAD (Fig. 9), it is not surprising that ecto-NAD affects T cell functions in more than one way. As shown by Denner and coworkers, ADP-ribosylation of various membrane proteins affects T cell signaling and functions via ADP-ribosylation of important cell surface molecules including CD8 coreceptors and important cell surface molecules including CD8 coreceptors and effects of nicotinamide adenine dinucleotide on their function. J. Immunol. 160:4190.


Under physiological conditions, the concentration of NAD in extracellular body fluids is low, but it can increase in injured tissues due to the liberation of their intracellular content by dead cells. On the basis of our findings, it is tempting to speculate that NAD would then kill naive T cells present in these tissues or block their recruitment by nonprofessional APC. In contrast, recently activated, i.e., Ag-specific T cells, that have shed ART2 (14) would be resistant to the apoptotic effects of ecto-NAD. NAD-mediated apoptosis could thereby play a role in restricting the proliferation of bystander lymphocytes. This provides a plausible scheme for NAD to participate in the control of autoimmunity as already suggested by ART deficiency in mice and rats prone to autoimmune diseases (8, 24, 25).

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References


