Brief Definitive Report

Double-negative T Cells from MRL-lpr/lpr Mice Mediate Cytolytic Activity when Triggered through Adhesion Molecules and Constitutively Express Perforin Gene

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Summary

The lpr gene induces in mice, accumulation of large numbers of $CD4^-CD8^-$ (double negative [DN]) T lymphocytes which bear adhesion molecules not characteristic of normal resting T cells. These cells fail to acquire interleukin 2 (IL-2) receptors, produce IL-2, and proliferate when activated with mitogens or monoclonal antibodies (mAbs) against the T cell receptor (TCR). Because of these poor functions in vitro, the nature and significance of DN T cells in the autoimmune disease process is not clear. In the current study, we describe a surprising finding that mAbs against CD3-TCR-α/β complex can strongly trigger the lytic activity of the DN T cells to induce redirected lysis of Fc receptor–positive targets. Similar redirected lysis was also inducible using mAbs against CD44 and $gp90^{MEL-14}$, molecules involved in the binding of lymphocytes to endothelial cells. The spontaneous cytotoxic potential of the DN T cells was further corroborated by demonstrating that the lpr DN T cells constitutively transcribed perforin gene but failed to express granzyme A. The current study suggests that DN T cells are capable of mediating lysis of autologous cells bearing the specific ligands for adhesion molecules involved in the signaling of cytotoxicity. These findings provide a novel insight into the functional significance of DN T cells in lpr mice and their potential role in the pathogenesis of autoimmune disease.

MRL-lpr/lpr (lpr) mice develop massive lymphadenopathy, hypergammaglobulinemia, and autoantibody production leading to premature death (1). Lymphadenopathy is characterized by an age-related, accumulation of $CD4^-CD8^-$ (double negative [DN]) T cells that are $CD3^+$ and TCR-α/β^+ and bear an unusual phenotype by expressing high densities of many markers that are not expressed by normal resting T cells such as J11d, Ly6C, CD45R, and CD44 (for a review see reference 2). The lpr phenotype may result from defects in Fas gene (3).

Despite extensive research, the nature and significance of lpr DN T cells are not clear. It has been shown that lpr DN T cells are refractory to stimulation with mitogens or Abs against the CD3-TCR complex as measured by their inability to proliferate, acquire IL-2 receptors, and produce IL-2 (4–8). Such studies have suggested that lpr DN T cells are inactive or anergic T cells whose role in autoimmune disease remains unknown.

Recently we demonstrated that CTL can be activated independent of the TCR, via adhesion molecules such as CD44 and $gp90^{MEL-14}$, to mediate redirected lysis (9). This, combined with our earlier studies that DN T cells from lpr mice exhibit spontaneous NK-like cytotoxicity (7), prompted us to undertake studies to investigate whether lpr DN T cells would exhibit cytotoxic potential when activated via a variety of adhesion molecules. We report a surprising finding that mAbs against the CD3-TCR-α/β complex, as well as against certain other adhesion molecules such as CD44 and $gp90^{MEL-14}$, can activate the lytic potential of lpr DN T cells. Furthermore, unstimulated DN T cells constitutively expressed transcripts for perforin gene. Our studies demonstrate that lpr DN T cells may represent activated cytotoxic T cells in vivo and that such a functional characteristic may contribute to the pathogenesis of autoimmune disease.

Materials and Methods

Mice. MRL-lpr/lpr (lpr) and MRL +/+ mice were bred in our animal facility (10).

Antibodies. The mAbs used were in culture supernatants and were from the following hybridomas: 9F3 directed against CD44; MEL-14 against lymphocyte homing receptor for endothelium ($gp90^{MEL-14}$); 2.4G2 against FcR; H57-597 against TCR-α/β; 145.2C11 against CD3; 6B2 against CD45R; M17/4 against LFA-1;

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/93/12/2225/06 $2.00
Volume 178 December 1993 2225–2230

and 53.6.72 against CD8. All hybridomas were procured and mAbs were purified as described elsewhere (7–10).

DN T Cells. The DN T cells were purified as described elsewhere (10), by treating LN cells twice with anti-CD4 and anti-CD8 Abs followed by complement. Next, the viable cells were isolated by density gradient centrifugation over histopaque (Sigma Chemical Co., St. Louis, MO). The purity of the DN T cells was >95% as determined by flow cytometry as described (10).

CTL Clone. In some experiments, a $CD8^+$ TCR-α/β^+ CTL clone, designated PE-9, was used to compare cytotoxicity with DN lpr T cells. This clone was isolated from C57Bl/6 mice rejecting a syngeneic tumor, LSA (9). This clone is cytotoxic to LSA tumor cells but not to any other allogeneic or syngeneic targets. Also, the clone is also $CD44^+$ and $gp90^{MEL-14+}$ and mediates lysis when activated through these adhesion molecules (9). The clone is maintained in culture by activating the cells using rIL-2 (50 U/ml, generously provided by Hoffmann-La Roche, Nutley, NJ) plus irradiated LSA as described (9).

Cytotoxicity Assay. Redirected cytotoxicity was determined by the capacity of DN T cells to lyse $Fc\gamma R^+$ tumor target cells in the presence of mAbs directed against adhesion molecules expressed by the DN T cells. The cytotoxicity was studied by using a ^{51}Cr-release assay as described (9). Tumor targets or hybridoma cells were labeled with ^{51}Cr and seeded in 96-well plates at 5×10^3 cells/well, along with varying numbers of effector cells and mAb supernatants. The plates were incubated at 37°C for 4 h. The amount of ^{51}Cr released by target cells was measured with a gamma counter (TM Analytic, Elk Grove Village, IL). Percent cytotoxicity was calculated from the ^{51}Cr release as follows: 100 × [(Experimental release − control release)/(total release-control release)].

Detection of N-α-benzyloxycarbonyl-L-lysine Thiobenzyl Esterase (BLTE or Granzyme A) in DN T Cells. The total cellular content of BLTE in T cells was determined as described by Lancki et al. (11). Briefly, varying numbers of purified DN T cells were lysed with 1% Triton X-100. Of this lysate, 20 μl were added to microtiter wells containing 180 μl of assay solution consisting of PBS, pH 7.2 with 2.2×10^{-4} M 5,5'-dithio-bis(2-nitro)-benzoic acid (Calbiochem-Novabiochem Corp., La Jolla, CA) and 2×10^{-4} M BLT (Sigma Chemical Co.). After a 30 min incubation at room temperature, the absorbance was read in an ELISA reader at λ = 410 nm. In assays involving activation of DN T cells through adhesion molecules, DN T cells were added to Ab-coated plates and incubated for 4–6 h at 37°C before the BLTE assay.

PCR Analysis of IL-2 and Perforin Gene Expression in DN T Cells. PCR method was employed to study whether the lpr DN T cells spontaneously expressed perforin gene as described by others (12) and modified as follows. Purified DN T cells or other cells were lysed using a buffer containing 0.14 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris chloride, pH 8, 0.5% NP-40, and 1 mM 2-ME. The total RNA was isolated by digesting the cell lysate at 37°C for 35 min with proteinase K (50 μg/ml) in a digestion buffer containing 0.2 M Tris chloride, pH 7.6, 25 mM EDTA pH 8, 0.3 M NaCl, and 2% SDS. Next, proteins were extracted in a phenol/chloroform 1:1 mixture. The nucleic acid from aqueous phase was precipitated in ethanol at −80°C overnight. The concentration of recovered RNA was measured by UV absorption spectrophotometry, and the RNA was reverse transcribed into cDNA as described in the Perkin-Elmer Cetus (Norwalk, CT) protocol. Reverse transcription was performed at 42°C for 45 min followed by denaturation of reverse transcriptase at 99°C for 5 min. The resulting cDNA samples were subjected to PCR amplification using synthetic oligonucleotide primers. The primers for β-actin, IL-2, and perforin cDNA were selected using the Genetic Computing Group program assisted search from GenBank sequences. The PCR was run at 94°C for 2 min followed by 60 cycles of denaturing at 94°C for 1 min, annealing at 50°C for 2 min, and extension at 72°C for 2 min. The primer sequences were as follows: For β-actin 5'-TATCCTGACCCTGAACTACCCCATT and 3'-AGCACAGCTTCTCTTTGATGTCACG; for IL-2, 5'-ATGTACAGCATGCAGCTCGCATC and 3'-GGCTTGTTGAGATGATGCTTTGACA; and perforin was 5'-GGTCAGAATGCAAGCAGAAGCACAA and 3'-TTGAAGTCAAGGTGGAGTGGAGGTT. 5 μl of the PCR product was electrophoresed on a 1.5% agarose gel stained with ethidium bromide. The demonstration of a single 502-, 464-, or 499-bp band was considered to be indicative of the expression of IL-2, β-actin, and perforin genes, respectively.

Results and Discussion

We investigated whether DN T cells would mediate lysis of target cells when activated via the CD3-TCR-α/β complex. Inasmuch as the ligand for the DN TCR is unknown, we used "redirected" lysis to address this, by employing mAbs against the CD3-TCR complex and P815 target cells because these cells were $Fc\gamma R^+$ and were resistant to direct lysis by the DN T cells (7, 9). The data presented in Fig. 1 suggested that purified DN T cells from lpr mice lysed YAC-1 targets but not P815 target cells as demonstrated before (7). Interestingly, in the presence of mAbs against CD3 or TCR-α/β, the DN T cells mediated efficient lysis of P815. In contrast, mAbs against TCR-γ/δ failed to evoke cytotoxicity. These data demonstrated that cells mediating cytotoxicity were TCR-α/β^+ T cells. Furthermore, depletion of DN T cells by using mAbs against CD44, CD45R, and J11d plus complement, virtually abolished the redirected cytotoxicity of P815 cells in the presence of anti-CD3 or anti-α/β Abs (data not shown), thereby further confirming that the cytotoxicity was mediated by the DN T cells.

We had demonstrated earlier that naive normal T cells would not mediate spontaneous lysis in a redirected assay (9). However, further studies were carried out to exclude the possibility that the cytotoxicity seen with lpr DN T cells may have resulted from contaminating $CD4^+$ or $CD8^+$ T cells. In this experiment, we used LN cells from MRL-+/+ mice

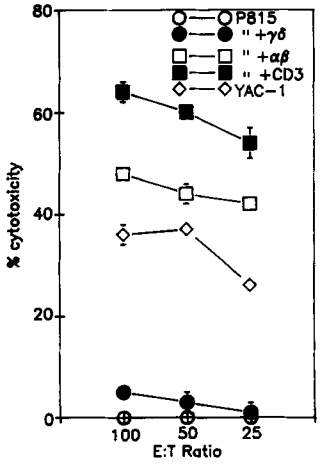

Figure 1. Activation of DN T cells from lpr mice through the TCR leads to efficient induction of cytotoxicity. Freshly isolated and purified DN T cells from lpr mice were tested for spontaneous cytotoxicity against YAC-1 or P815 tumor targets using ^{51}Cr-release assay. The cytotoxicity against P815 targets was performed in the absence or presence of mAbs against CD3,α/β and γ/δ TCR. The data is expressed as mean percent cytotoxicity ± SEM at E/T cell ratios.

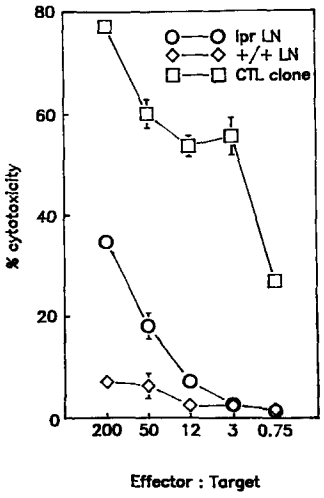

Figure 2. Lytic activity of LN cells from MRL-+/+ or MRL-lpr/lpr mice. Freshly isolated LN cells from 4-mo-old MRL-+/+ or MRL-lpr/lpr mice were tested for spontaneous cytotoxicity against P815 tumor targets in the presence of anti-CD3 mAbs as described in Fig. 1. The CTL clone, PE-9, was used as a positive control.

as a negative control and an TCR-α/β^+ CTL clone designated PE-9 (9) as a positive control. The LN cells from 4-mo-old MRL-+/+ mice failed to mediate significant redirected lysis of P815 target cells in the presence of anti-CD3 mAbs (Fig. 2). In contrast, MRL-lpr/lpr LN cells exhibited marked lysis of P815 targets in the presence of anti-CD3 mAbs. The fact that MRL-lpr/lpr but not MRL-+/+ LN cells mediated lysis of P815 cells in the presence of anti-CD3 mAbs suggested that the cytotoxicity can be attributed to the unique DN T cells found in lpr mice and not to the normal $CD4^+$ or $CD8^+$ T cells.

CD44 is a broadly expressed glycoprotein implicated in leukocyte-endothelial cell binding and may direct lymphocyte homing to certain peripheral lymphoid microenvironments (13). Several recent studies (13) have demonstrated that after activation naive T cells express high density CD44 which plays an important role in T cell activation. In addition, $gp90^{MEL-14}$, a selectin which is structurally distinct from CD44, also recognizes high endothelial venules and is involved in organ-specific homing of lymphocytes. Recently, we demonstrated that activated CTL that expressed CD44 and $gp90^{MEL-14}$, could mediate lysis of target cells when activated via these adhesion molecules, independent of the TCR (9).

To study whether the DN T cells could also be activated to mediate lysis via the TCR-independent alternate pathway, DN T cells were incubated with a variety of $FC\gamma R^+$ tumor targets in the presence of Abs against adhesion molecules (Fig.

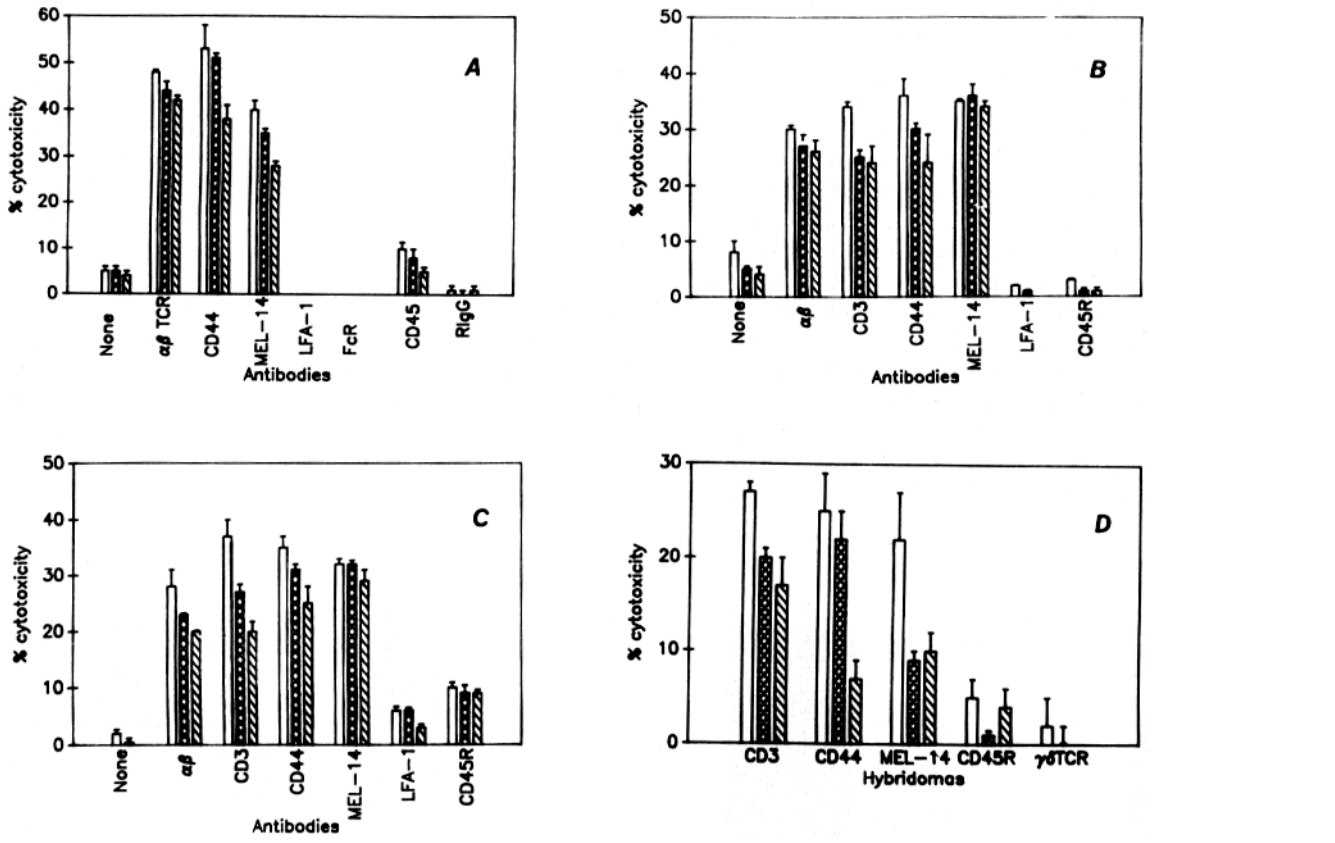

Figure 3. Role of adhesion molecules in the activation of lytic activity by lpr DN T cells. Freshly isolated and purified lpr DN T cells were tested for cytotoxicity against ^{51}Cr-labeled P815 (*A*), LSA (*B*), or EL-4 (*C*) target cells in the presence or absence of mAbs against a variety of adhesion molecules expressed by the DN T cells. In Fig. 2 (*D*), purified DN T cells were tested for cytotoxicity against ^{51}Cr-labeled hybridoma cells. The cytotoxicity was studied as described in Fig. 1. (*Open*, *hatched*, and *diagonal bars*) Cytotoxicity obtained at E/T ratios of 100, 50, and 25, respectively, as described in Fig. 1.

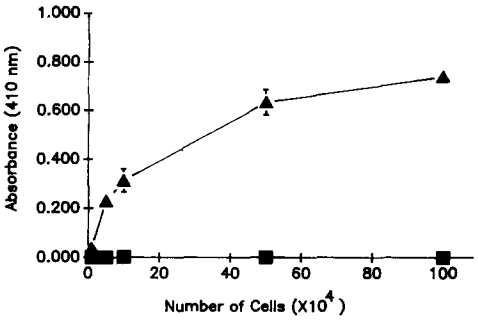

Figure 4. BLTE (granzyme A) activity in lpr DN T cells. Freshly isolated and purified lpr DN T cells (□) or activated CTL clone, PE-9 (△) used as a positive control, were lysed and BLTE activity was measured as described in Materials and Methods.

3 *A*). Interestingly, mAbs against CD44 and gp90^{MEL-14} induced strong lysis of P815 target cells, whereas mAbs against LFA-1, FcγR, and CD45R failed to trigger significant lysis. Similar observations were also made using other allogeneic FcγR$^+$ tumor cells such as LSA and EL-4 (Fig. 3, *B* and *C*). This fact was further corroborated by demonstrating that DN T cells could also mediate lysis of hybridomas secreting mAbs against CD3, CD44, and gp90^{MEL-14} (Fig. 3 *D*). In this experiment, DN T cells failed to mediate significant lysis of hybridomas secreting mAbs against CD45R and TCR-γ/δ, which also served as appropriate negative controls for other hybridomas. Together these studies demonstrated that lpr DN T cells can mediate efficient lysis of target cells when activated through the CD3-α/β TCR complex, as well as other adhesion molecules such as CD44 and gp90^{MEL-14}.

The cytoplasmic granules of CTL and NK cells have been shown to contain a number of proteins, known as perforin, and a family of serine esterases (14, 15). In this study, we addressed whether the DN T cells from lpr mice would exhibit perforin and serine esterases such as granzyme A either spontaneously or after activation via the CD3-TCR complex or through adhesion molecules. To this effect, we lysed purified DN T cells freshly isolated from lpr mice and measured the level of BLTE. As a positive control, we used the in vitro activated CTL clone, PE-9 (9). The data shown in Fig. 4 suggested that whereas the CTL clone exhibited dose-dependent BLTE activity, the DN T cells failed to demonstrate spontaneous BLTE activity. Furthermore, activation of the DN T cells through the CD3-TCR complex or through adhesion molecules such as CD44, gp90^{MEL-14}, CD45R, etc., also failed to trigger BLTE activity (data not shown), even at a concentration of 10^6 cells. These data together demonstrated that DN T cells from lpr mice fail to demonstrate detectable levels of granzyme A.

Naive CTL do not express perforin. However, after activation, they synthesize perforin (15). We therefore investigated whether lpr DN T cells expressed perforin either constitutively or after activation. The presence of perforin in lpr DN T cells was detected by studying the expression of perforin gene in unstimulated cells using PCR. In addition, we also tested whether the lpr DN T cells would express IL-2 gene spontaneously. In this assay, we used primers for β-actin as an internal standard to allow comparison of various samples. The data shown in Fig. 5 indicated that unstimulated lpr DN T cells expressed the perforin but not IL-2 gene. The DN T cells failed to express IL-2 gene despite stimulation through the TCR (data not shown), consistent with previous studies that lpr DN T cells fail to produce IL-2 when activated (1).

It is possible that the perforin band in the PCR using DN T cells may have been contributed by contaminating NK cells. To exclude this possibility, we isolated total RNA from MRL-lpr/lpr or MRL-+/+ LN cells and from the control CTL clone, PE-9. Next, 0.5 or 1 μg of RNA was processed for PCR analysis, and the product was electrophoresed as described earlier. The data shown in Fig. 6 suggested that when 0.5 μg of RNA was used in PCR analysis, no visible perforin band was seen using MRL-+/+ or PE-9 cells. However, a demonstrable perforin band was seen using MRL-lpr/lpr LN cells. In this experiment, the expression of β-actin served as an internal control. Furthermore, when PCR was initiated with 1 μg of RNA for all samples, the expression of perforin was seen in all three cell types. However, the expression of perforin band was stronger using MRL-lpr/lpr cells when compared with MRL-+/+ cells. These data suggested that perforin expression seen using lpr LN can be attributed to lpr DN T cells.

This study has two important implications on the nature

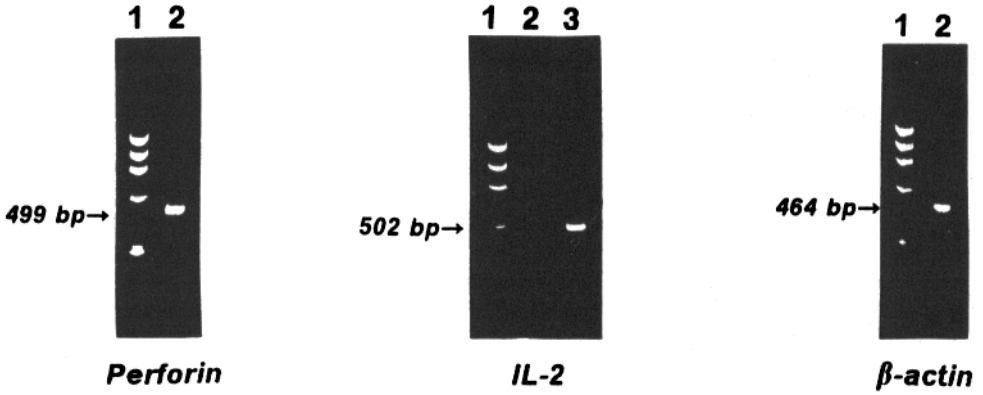

Figure 5. Spontaneous expression of perforin gene by DN T cells from lpr mice. Total RNA was extracted from cells, reverse transcribed, and cDNA samples were subjected to PCR amplification using synthetic oligonucleotide primers for perforin, IL-2 and β-actin. The PCR products were electrophoresed through a 1.5% agarose gel containing ethidium bromide. Lane *1* is a molecular standard, lane *2* depicts DN T cells, and lane *3* for IL-2 represents normal splenic T cells stimulated with anti-CD3 mAbs.

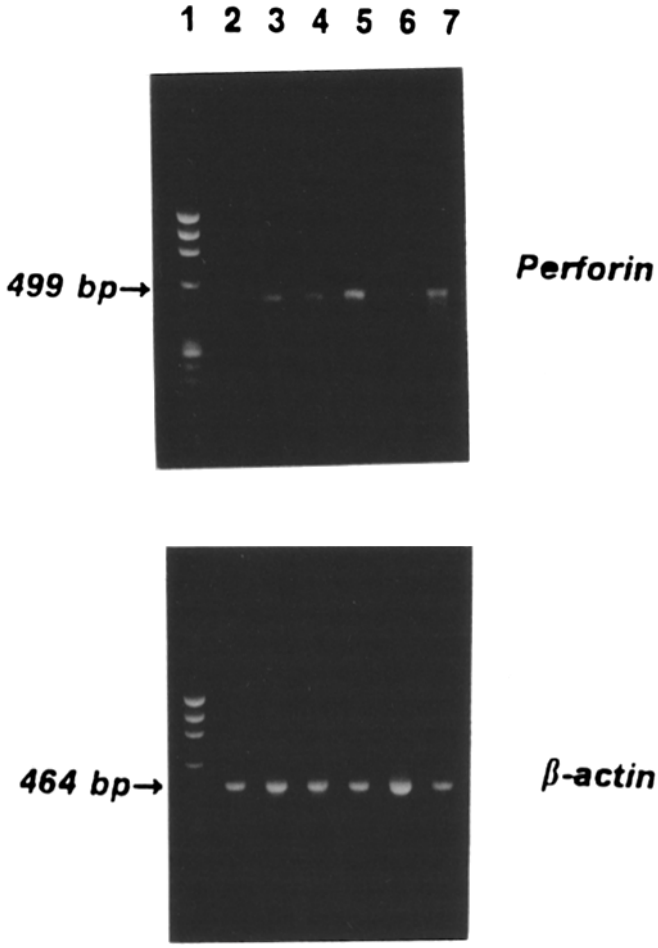

Figure 6. Comparison of expression of perforin gene by LN cells from MRL-lpr/lpr and MRL-+/+ mice. Perforin gene expression at mRNA level was studied as described in Fig. 5, using varying concentrations of RNA in the PCR analysis as follows: lane *1* is a molecular standard; lane *2*, 0.5 μg of RNA from MRL-+/+ cells; lane *3*, 1.0 μg of RNA from MRL-+/+ cells; lane *4*, 0.5 μg of RNA from MRL-lpr/lpr cells; lane *5*, 1 μg of RNA from MRL-lpr/lpr cells; lane *6*, 0.5 μg of RNA from CTL clone, PE-9; and lane 7, 1 μg of RNA from CTL clone, PE-9.

of lpr DN T cells. First, lpr DN T cells, although incapable of producing IL-2 and dividing when activated through the TCR, and therefore believed to be unresponsive, are fully functional as cytotoxic cells. The ligand for the DN TCR is not known. If the ligand is a self-antigen expressed on lpr cells, the DN T cells may kill such cells, thereby contributing to the autoimmune disease process. However, it should be noted that lpr DN T cells have been shown to have undergone negative selection and that their TCR is polyclonal in nature (for a review see reference 2). Thus, it is less likely that they would cause damage to autologous cells after activation through the TCR. However, our findings that DN T cells can be activated via other adhesion molecules such as cD44 and $gp90^{MEL-14}$, to mediate cytotoxicity, further suggests that the DN T cells may be cytotoxic to autologous cells that bear ligands for CD44 and $gp90^{MEL-14}$. Such a mechanism may explain the observation that lpr LN contain cells capable of spontaneous cytotoxicity against autologous cells (16). Second, CD44 and $gp90^{MEL-14}$ have been implicated in lymphocyte adhesion to endothelial cells (13). Thus, it is possible that the interaction between DN T cells and endothelial cells can lead to activation of the lytic properties of the DN T cells and consequently damage endothelial cells, resulting in vascular disease seen in lpr mice (17). Our data support earlier observations that depletion of DN T cells reduces the immunopathology (18) and that the DN T cells may mediate their effect by their capacity to spontaneously transcribe IFN-γ and TNF-α genes (19).

Based on this study, we suggest that DN T cells are not inert or anergic T cells but may represent activated cytotoxic cells constitutively expressing certain cytokines. This, combined with the fact that DN T cells can be activated via a variety of adhesion molecules to mediate cytotoxicity suggests that they play an important role in the induction of autoimmune disease.

The authors thank Ms. Jackie Hamblin and Ms. Sue Rasmussen form excellent secretarial assistance.

This work was partially supported by National Institutes of Health grant CA-45009, grants-in-aid of research from Sigma Xi, and the Horsley Cancer Research Fund.

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Received for publication 6 April 1993 and in revised form 11 August 1993.

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