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*J Immunol* 2006; 176:7079-7086; doi: 10.4049/jimmunol.176.11.7079
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The Role of Noradrenergic Nerves in the Development of the Lymphoproliferative Disease in Fas-Deficient, lpr/lpr Mice

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Lpr/lpr mice develop a lymphoproliferative, autoimmune, lupus-like disease. These mice lack functional Fas (CD95) expression and are resistant to Fas ligand (CD178)-mediated apoptosis, a critical mechanism for the maintenance of peripheral tolerance. In this study, we show that noradrenaline (NA), the main sympathetic neurotransmitter, can induce apoptosis of lymphoid cells independently of functional Fas. Based on this finding, we used lpr/lpr mice as model to study the role of noradrenergic nerves in the expression of a lymphoproliferative disease. Early in ontogeny, the concentration of NA was significantly increased in the spleen of lpr/lpr mice, compared with normal littermates. However, splenic sympathetic innervation gradually declined as the disease progressed, and IgM blood levels and splenic NA concentration inversely correlated when the disease was overtly manifested. When the loss of noradrenergic fibers that occurred naturally during adult life in lpr/lpr mice was experimentally advanced by neonatal sympathectomy, the concentration of IgM and IgG2a in blood was markedly higher than that of control lpr/lpr mice, and the appearance of lymphadenopathy was accelerated. Furthermore, although neonatal denervation did not affect the life span of normal animals, it shortened significantly the survival time of lpr/lpr mice. These data show that, in addition to defects in the Fas pathway, an altered sympathetic innervation in lpr/lpr mice also contributes to the pathogenesis of the autoimmune disease, and strongly support the hypothesis that the sympathetic nervous system can modulate the expression of lymphoproliferative diseases. The Journal of Immunology, 2006, 176: 7079–7086.

Lymphoid cells are in close contact with the nerve fibers that innervate lymphoid organs and express receptors for sympathetic neurotransmitters. Furthermore, the evidence available shows that almost all mechanisms involved in an immune response can be affected by noradrenergic neurotransmitters (for review, see Refs. 1–4). Indeed, noradrenaline (NA), the main sympathetic neurotransmitter, can inhibit or stimulate an immune response depending on the dose of agonist applied and on the type of adrenergic receptor stimulated. The effect of NA also depends on the type of stimulus that triggers the immune response, the subset of cells affected, and, most importantly, at which step of the response, lymphoid and/or accessory cells are exposed to neurotransmitters. Among the processes directly or indirectly affected by sympathetic neurotransmitters are Ag presentation and the expression of costimulatory and adhesion molecules, lymphoid cell activation, cytokine production, clonal expansion and deletion, Ig production, and the generation of cytotoxic cells (for review, see Refs. 1–4). The fact that the simultaneous stimulation of adrenergic receptors and the TCR affects common intracellular signaling pathways (5) may explain why adrenergic agonists can affect so many mechanisms involved in an immune response. However, very little is known about the possible relevance of noradrenergic innervation for the development and expression of lymphoproliferative diseases. To approach this question, we have used a model based on lpr/lpr mice, which develop a genetically determined autoimmune lymphoproliferative disease that shares several characteristics with human systemic lupus erythematosus (SLE) (for review, see Refs. 6 and 7).

Mice homozygous for the autosomal recessive lpr gene lack functional Fas (CD95) expression, and therefore apoptosis cannot be triggered via this receptor. The autoimmune disease in lpr/lpr mice is mainly characterized by marked lymphoproliferation and lymphadenopathy, hypergammaglobulinemia due to increased levels of all immunoglobulins classes and subclasses, and production of autoantibodies. Our data show for the first time that NA can induce apoptosis independently of the Fas pathway, and that noradrenergic nerves play an essential role in the control of the onset and course of the lymphoproliferative disease in lpr/lpr mice.

Materials and Methods

Animals

Normal C57BL/6J (B6) and C57BL/6J homozygous for the lpr mutation (lpr/lpr) were bred in our animal facilities from breeding pairs provided by Dr. K. Hartmann (Institute for Experimental Immunology, Marburg, Germany). Animals were permanently housed in temperature-, humidity-, and light (12-h cycles)-controlled rooms and fed ad libitum. The studies were approved by the Institutional Review Board.

Studies during ontogeny

Groups of male and female B6 and lpr/lpr mice were killed by cervical dislocation at the age indicated in the figures. Blood was collected in EDTA-coated tubes, and plasma samples were immediately frozen until used for Ig determinations. The spleen was weighed and divided into two halves. One half was processed for immunohistochemistry. The other half,...
as well as the left kidney, were immediately frozen and used for NA determination.

**Sympathetic denervation**

Groups of B6 and lpr/lpr mice were sympathetically denervated at birth by i.p. injection of 6-hydroxydopamine hydrochloride (6-OH-DA; Sigma-Aldrich; 150 mg/kg dissolved in 0.01% ascorbic acid; 1 injection per day over 5 consecutive days, starting when mice were <24 h old). Controls received the vehicle alone. Essentially the same procedure was followed to denervate adult mice (two injections; 24 h apart) (8).

**Assessment of disease severity**

Lymph node swelling was assessed by palpation twice per week by an observer blinded to treatment groups and classified according to the criteria established by Shirai et al. (9). The first day when lymph nodes could be palpated (degree 1 of Shirai’s scale) was recorded.

**Evaluation of apoptosis**

A total of $2 \times 10^8$ thymic or spleen cells obtained from 8- to 10-wk-old B6 and lpr/lpr mice was cultured for 4 h in a final volume of 0.5 ml with or without addition of i-NA HCl (Fluka) and/or Ab against mouse Fas (BD Pharmingen). The percentage of apoptotic cells was evaluated by assessment of membrane phospholipid asymmetry, staining with FITC-labeled annexin V (Bender MedSystems) and propidium iodide (Sigma-Aldrich), and by determination of inner mitochondrial membrane transmembrane potential ($\Delta \Psi _m$) using 3,3'-dihexyloxycarbocyanine iodide (DiOC$_{6}$; Molecular Probes) (3, 10). Cells were analyzed by flow cytometry on a FACScan (BD Biosciences).

**Ig determinations**

IgM plasma levels were determined by ELISA using a commercially available kit (Bethyl Laboratories). IgG2a levels also were determined by ELISA using coated and biotinylated anti-mouse IgG2a Abs that specifically recognize the IgG1b allotypic variant of the C57BL/6J strain and purified mouse IgG2a allotype as standard (BD Pharmingen), as described previously (10).

**NA concentration**

NA determinations in the spleen and left kidney were performed by HPLC with electrochemical detection as described previously (10), using the supernatant of tissue samples homogenized in 0.4 M HClO$_4$.

**Immunohistochemistry**

Adjacent sections (7 μm thick) were cut from deparaffinized spleen samples embedded in Paraplast Plus (Merck). Ag retrieval to increase the sensitivity of immunodetection was performed by heating at 92–95°C for 15 min in 0.01 M citrate buffer. Nonspecific binding sites were blocked with 5% BSA (Serva) in PBS followed by an avidin-biotin blocking step (Boehringer Ingelheim). Tissue sections were incubated with sheep antityrosine hydroxylase affinity-purified Ab (Chemicon) overnight at 18°C, followed by 2-h incubation at 37°C. After washing, sections were incubated with biotinylated secondary Ab against sheep Ig (Dianova) for 45 min at 37°C, washed, and incubated for 30 min with ABC reagents (Vectorstain Elite ABC kit; Boehringer Ingelheim). Immunoreactions were visualized with 3’3-diaminobenzidine (DAB, Sigma-Aldrich) enhanced by 0.08% ammonium nickel sulfate (Fluka). No binding was detected in the absence of the primary Ab. Sections were analyzed and photographed with an AX70 microscope (Olympus).

**Statistical analysis**

Results are expressed as mean ± SEM. Data were analyzed using one-way ANOVA followed by Fisher’s test for multiple comparisons. Differences were considered significant when $p$ was <0.05.

**Results**

**NA-induced apoptosis is Fas independent**

Since we and others have shown that β-adrenergic stimulation can induce apoptosis of normal lymphoid cells (10–12), we studied whether Fas-deficient lpr/lpr cells are sensitive to the proapoptotic effects of NA. Although, as expected, anti-Fas Abs induced apoptosis only in thymic cells from normal B6 mice, NA induced a comparable degree of apoptosis in cells from both B6 and lpr/lpr mice (Fig. 1). An additive effect of the two substances was observed only on cells from B6 mice. Comparable results were obtained using spleen cells (data not shown).

**Ontogeny of splenic noradrenergic innervation in lpr/lpr mice**

The previous results led us to evaluate NA concentration in the spleen of lpr/lpr mice during ontogeny. IgM levels were determined in plasma of the same animals and used as read-out parameter to evaluate the progression of the disease. Age- and sex-matched B6 mice served as controls.

No significant differences in IgM levels were detected between B6 and lpr/lpr mice during the first 2 wk of life (Fig. 2A). Splenic NA concentration in 1-wk-old B6 mice was reduced relative to the first day of life, confirming our previous results in another strain of normal mice (13). No comparable decrease was observed at this age in lpr/lpr mice, in which splenic NA concentration was even higher than at birth (Fig. 2B). The concentration of the neurotransmitter continued to be significantly elevated in lpr/lpr male mice until they were 4 wk old. The spleen weight of young lpr/lpr mice was comparable to that of normal animals, but the total splenic NA content was elevated in 1-wk-old lpr/lpr females and in 2-wk-old lpr/lpr males (data not shown).

IgM plasma levels were markedly elevated in 18-wk-old lpr/lpr mice and continued to increase during development (Fig. 2C). At 13 wk of age and until the last time point studied (~1 year), a profound reduction in splenic NA concentration was observed in lpr/lpr mice, compared with the typical development of sympathetic innervation in B6 mice (Fig. 2D).

A significant splenomegaly was already observed in 13-wk-old lpr/lpr mice. At 18 wk of age, the spleen of lpr/lpr mice of both
sexes was about three times heavier than that of the normal counterparts, and this difference was maintained or even more marked in older mice (data not shown). However, immunohistochemical studies showed that the decreased splenic NA concentration in lpr/lpr mice was not due to a “dilution factor” caused by spleen enlargement. An example showing nerve fibers stained for tyrosine hydroxylase, the rate-limiting enzyme for NA synthesis, is given in Fig. 3. The immunostaining clearly shows the profound decrease in noradrenergic nerve fibers in the lpr/lpr spleen, which resulted in ~50% lower NA concentration, compared with that of the normal spleen. No comparable differences were observed in NA concentration in the kidney, a nonlymphoid abdominal organ used as control (data not shown).

Correlation between Ig levels and splenic innervation
A positive correlation between IgM plasma levels and splenic NA concentration was observed in B6 and lpr/lpr mice until 2 wk of age (Fig. 4, A and B). In B6 mice, this positive correlation was maintained later in life, between 4 and 18 wk (Fig. 4C). However, an opposite, negative correlation between these two parameters was observed in lpr/lpr mice of the same age range (Fig. 4D). It is worth noting that there was no correlation between IgM and splenic NA concentration before the overt onset of the disease in 4- to 9-wk-old lpr/lpr mice, but these parameters inversely correlated as the disease progressed.

Sympathetic denervation at birth increases IgM and IgG2a levels in lpr/lpr mice
Based on the results described, we explored whether interfering with the early increased splenic sympathetic nerve activity in young lpr/lpr mice and advancing the spontaneous loss of noradrenergic fibers would influence the onset and course of the disease. Noradrenergic nerve fibers were permanently destroyed by injection of the specific neurotoxin 6-OH-DA into lpr/lpr mice at birth. Lpr/lpr mice that received the vehicle alone served as controls. Denervated and vehicle-injected B6 mice were studied in parallel. A moderate decrease in IgM blood levels was observed in denervated B6 mice at all time points studied (Fig. 5, A and B). However, 22-wk-old denervated lpr/lpr female mice had IgM levels that were 3-fold higher than those of the vehicle-injected lpr/lpr mice (Fig. 5D). It is interesting to note that five of eight denervated lpr/lpr male mice died between 4 and 5 wk of age, whereas no death occurred during this period in any of the other seven groups.
whereas 90% of the denervated lymph nodes were palpable in 19% of the control received the vehicle alone were left undisturbed and the day of death

Groups of *lpr/lpr* mice had palpable cervical lymph nodes. One month later, enlarged lymph nodes were palpable in 100% of the control and 90% of the denervated *lpr/lpr* mice had enlarged cervical and inguinal lymph nodes. No lymphadenopathy was detected at any time in control or neonatally denervated B6 mice.

Survival of autoimmune disease in *lpr/lpr* mice

The effect of neonatal denervation on the development of the disease in *lpr/lpr* mice also was assessed by the time at which enlarged lymph nodes could be palpated. Lymph node swelling was first detected in 2 of 16 vehicle-injected *lpr/lpr* mice at 18 wk of age (Fig. 8). At this age, already 50% of the neonatally denervated *lpr/lpr* mice had palpable lymph nodes. One month later, enlarged lymph nodes were palpable in 19% of the control *lpr/lpr* mice, whereas 90% of the denervated *lpr/lpr* mice had enlarged cervical and inguinal lymph nodes. No lymphadenopathy was detected at any time in control or neonatally denervated B6 mice.

Survival of autoimmune *lpr/lpr* mice is affected by neonatal sympathectomy

Groups of *lpr/lpr* mice that were either denervated at birth or received the vehicle alone were left undisturbed and the day of death was recorded. Neonatal denervation clearly affected the survival time: in the vehicle-injected group, 23% of the male and 17% of the female *lpr/lpr* mice died within 1 year, whereas ~70% of the denervated *lpr/lpr* males and females died within this period (Fig. 9). When denervation was delayed until 9 wk of age, no effect in the mortality rate was observed: only 1 of 11 males and 2 of 11 females died within 1 year.

Discussion

The major alterations described in *lpr/lpr* mice, such as lack of Fas-dependent lymphoid cell apoptosis, massive lymphoproliferation, expansion of double-negative B220<sup>+</sup>CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup> cells, and dysfunctions in B cell functioning, the Th1/Th2 balance, and cytokine production, are all defects intrinsic to the immune system (for review, see Refs. 6 and 7). The studies described here show that these mice also have alterations in noradrenergic innervation, a component extrinsic to the immune system, which can modulate the disease.

The results showing that NA-induced apoptosis is Fas independent led us to the hypothesis that the release of this neurotransmitter in lymphoid organs might partially compensate for the lack of Fas-induced cell death in *lpr/lpr* mice. This possibility was further supported by our finding that noradrenergic nerves are necessary for the specific deletion of CD4<sup>+</sup>CD8<sup>+</sup> T cells, and dysfunctions in B cell functioning, the Th1/Th2 balance, and cytokine production, are all defects intrinsic to the immune system (for review, see Refs. 6 and 7). The studies described here show that these mice also have alterations in noradrenergic innervation, a component extrinsic to the immune system, which can modulate the disease.
the MRL strain as background. However, mice of the MRL strain itself (i.e., not carrying the lpr mutation) also develop an autoimmune lymphoproliferative disease although delayed, compared with that of the homozygous MRL lpr/lpr mice (15). Thus, we have chosen animals carrying the lpr gene on a C57BL/6J background, because this allowed comparison with a normal strain.

We have shown previously that a physiological reduction in NA concentration occurs in the spleen of 1-wk-old BALB/c mice when the first T cells are detected in this organ (13). These two events are most likely causally related, because T cell-deficient mice have a hyperinnervated spleen and injection of normal T cells at birth causes a diminution of noradrenergic fibers and a reduced NA content in this organ (13). We show in this study that a decreased splenic NA concentration relative to that of newborn mice also is observed in 1-wk-old B6 mice, indicating that this is a general phenomenon during ontogeny in normal mice. However, the opposite situation was observed in 1-wk-old lpr/lpr mice in which splenic NA concentration and total content were even increased, compared with newborn lpr/lpr mice. Increased NA levels were still noticed in male lpr/lpr mice until 4 wk of age. This early alteration is likely relevant for the control of the expression of the lymphoproliferative disease (see below). Later in ontogeny, splenic sympathetic innervation was progressively lost in male and female lpr/lpr mice, as reflected by the decreased NA concentration in this organ and by the reduced density of noradrenergic nerve fibers, particularly in the lymphoid compartment. The mechanism underlying the loss of sympathetic nerve fibers in the spleen of lpr/lpr mice is not known, but in our view, it is related to increased immune activity. This possibility is supported by the converse evidence showing that reduced immune activity caused either by the lack of mature T cells in athymic nude mice or by reduced antigenic challenge in germ-free rats results in increased sympathetic activity in lymphoid organs (13, 16).

It has been shown that the density of β-adrenergic receptors is increased in lymphocytes of normal mice after surgical or chemical sympathectomy (17, 18). Theoretically, a similar effect could be expected in lpr/lpr lymphoid cells as consequence of the spontaneous decrease in splenic NA levels as the disease progresses.
As expected, a delay in ablating the sympathetic nervous system caused the opposite effect: neonatally denervated B6 mice showed an increase of between 200 and 300% in IgM blood levels, compared with vehicle-injected male mice. These results agree with the report that neonatal sympathectomy results in reduced spontaneous IgM production in normal mice (21). A moderate (~35%) but persistent decrease in IgM blood levels was observed during development in neonatally denervated B6 mice. These results agree with the report that neonatal sympathectomy in rats results in reduced spontaneous IgM production throughout development (22). However, in lpr/lpr mice, this treatment caused the opposite effect: neonatally denervated lpr/lpr female mice showed an increase of between 200 and 300% in IgM blood levels, compared with vehicle-injected lpr/lpr mice, whose IgM levels were already twice those of normal, age-matched B6. As expected, a delay in ablating the sympathetic nervous system until adulthood, before the disease was clearly manifested but shortly before sympathetic innervation was spontaneously lost, did not affect Ig levels.

Denervation at birth resulted in a tendency toward increased blood IgG2a levels in young B6 mice. This finding agrees with the report that B cells exposed in vitro to a β2-receptor agonist produce less IgG2a (27). However, IgG2a levels tended to decrease in denervated B6 animals as they became older, indicating once more the relevance of considering the age of the animals and the time of exposure to neuro-endocrine agents among the variables that can contribute to opposite effects of neurotransmitters on the immune system (for review, see Ref. 4). In any case, our results show that in animals genetically predisposed to develop an autoimmune disease such as lpr/lpr mice, neonatal denervation results in increased IgG2a levels at early and later stages in life.

It has been reported consistently that neonatal administration of 6-OH-DA to mice and rats can affect the activity of the CNS, and there are examples that this pharmacological manipulation can affect some, but not all, hormonal responses. For example, we have reported recently (8) that neither basal corticosterone levels nor the capacity of the hypothalamus-pituitary-adrenal axis to respond with increased glucocorticoid output following stimulation with superantigen is affected in adult normal mice sympathetically denervated at birth. The few reports on endocrine alterations in lpr/lpr mice that we have found involve mainly the hypothalamic-pituitary-adrenal axis. Some reports indicate that young lpr/lpr (MRL background) mice have higher basal corticosterone levels but no differences in corticosterone-binding globulin (28) and disturbances of corticosterone circadian rhythm (29). Other groups have reported decreased hypothalamic corticotropin-releasing hormone and increased arginine vasopressin mRNA expression, increased circulating levels of corticosterone and a trend for levels of corticosterone-binding globulin to be decreased (30, 31). We have neither detected significant differences between B6 and B6 lpr/lpr mice in basal corticosterone levels in the blood of the same 9-, 18-, and 40-wk-old mice used for the studies reported here, nor observed significant differences in blood corticosterone levels in a small group of adult lpr/lpr female mice denervated at birth with 6-OH-DA, compared with aged- and sex-matched, vehicle-injected lpr/lpr mice (data not shown). However, we cannot exclude that other hormonal imbalances in lpr/lpr mice might be exacerbated by neonatal treatment with 6-OH-DA.
Several autoimmune diseases, including multiple sclerosis, type 1 diabetes, and rheumatoid arthritis, are considered the consequence of typical Th1-related autoreactive cellular responses (32, 33), whereas SLE has been mainly associated with a Th2 shift and increased humoral responses (34, 35). NA and several synthetic noradrenergic agents can affect the Th1/Th2 balance, although differentially, depending on whether the effect is local or systemic (for review, see Ref. 4). In intact lpr/lpr mice, all IgG classes and subclasses (36) as well as several cytokines, including INF-γ (typical Th1 cytokine) and IL-4 (typical Th2 cytokine), are altered (37, 38). Because neonatal denervation of lpr/lpr mice resulted in an even further increase in both IgM and IgG2a levels, it seems that both Th1 and Th2 responses were affected, although with different kinetics. Interestingly, an imbalance toward Th1 predominance, as evaluated by increased IgG2a production, has been associated with acceleration of the lupus-like autoimmune syndrome (37), as observed in our studies. The evidence derived from normal animals cannot explain satisfactorily why the change in IgG2a in denervated lpr/lpr mice was observed before the change in IgM levels, especially if we attempt to extrapolate this evidence to a disease in which disregulation of immunoglobulin production is a major defect observed already very early during ontogeny. As mentioned, in contrast with observations in normal mice, IgG2a-producing B cells in lpr/lpr mice are densely clustered in spleen areas with the reactivest noradrenergic innervation (26). One possibility is that, in early life and at least in part due to their particular anatomical localization, lpr/lpr cells producing IgG2a are more susceptible to the effect of NA deprivation than those producing IgM. The fact that denervation tends to decrease IgM levels in normal B6 mice, but that this effect is not observed in young lpr/lpr, and that denervation even results in increased IgM production in older lpr/lpr, indicates that IgM production is differentially affected by NA in disease-prone mice.

The effect of neonatal denervation on the expression of the disease in lpr/lpr mice also was clearly manifested by the accelerated appearance of lymphadenopathy: although enlarged lymph nodes could be detected in 19% of the 22-wk-old control lpr/lpr mice, nearly all of the denervated lpr/lpr mice already showed this symptom at this age. Perhaps the most striking effect of the relevance that an intact sympathetic innervation has for the course of the lymphoproliferative disease derives from the survival rates. When the specific adrenergic neurotoxin was administered close to the time when splenic sympathetic nerves start to be lost spontaneously, the mortality rate was not affected. However, the lethal course of the disease was clearly accelerated by neonatal depletion of endogenous NA. Denervated lpr/lpr male mice died before females. This suggests that the course of the disease was more dependent on endogenous NA in males than in females. We speculate that such dependence may be linked to the more pronounced and prolonged increase in sympathetic activity noticed in lpr/lpr male mice early during ontogeny. Thus, as evaluated by the concentration of IgM and IgG2a in blood, the appearance of lymphadenopathy, and the survival rate, these studies show that noradrenergic nerve fibers can affect the expression of the lupus-like autoimmune disease that lpr/lpr mice develop. In this context, it is interesting to mention that it is known since a long time that treatment with β-blockers is among the causes of drug-induced lupus in humans, although the mechanism underlying this effect is so far unknown (39–41). Also, it has been reported more recently that patients with SLE have a decreased density of β2-adrenergic receptors on peripheral CD19+ cells (42).

In conclusion, the results reported here constitute the first example that noradrenergic nerves contribute to control the expression of lymphoproliferative diseases. When this control system fails, it occurs in lymphoid organs of adult lpr/lpr mice, the onset and lethal course of the disease is markedly accelerated.

Acknowledgments
We thank S. Petzoldt and N. Özen for their excellent technical assistance and Peter J. Hanley for careful review of the manuscript.

Disclosures
The authors have no financial conflict of interest.

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