Brief Definitive Report

ABERRANT PRODUCTION OF LEUKOTRIENE C_4 BY MACROPHAGES FROM AUTOIMMUNE-PRONE MICE

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Eicosanoids have been implicated in the pathogenesis of autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). The evidence linking eicosanoids and rheumatic diseases is primarily based upon the concept that several eicosanoids that are potent mediators of inflammation can also modify immune function and that SLE and RA are inflammatory diseases associated with altered immune responses. The specific contribution any individual lipoxygenase or cyclooxygenase product has in predisposing to lupus remains to be established. Although prostaglandins (PGs) may promote inflammation by virtue of their capacity to induce edema, erythema, and hyperalgesia (1), certain prostanoids appear to protect against the development of autoimmune disease. Parenteral administration of prostaglandin E_1 (PGE_1) prevents the expression of lupus in autoimmune-prone New Zealand (2) and MRL-*lpr/lpr* (3) mice. In contrast, leukotrienes (LT) may have a permissive effect on the development of autoimmunity (reviewed in reference 4). Oxygenation of arachidonic acid by a 5-lipoxygenase yields 5-hydroperoxyeicosatetraenoic acid, which can be further metabolized to leukotriene A_4 (LTA_4). In murine peritoneal macrophages (Mø), stimulation with zymosan preferentially converts LTA_4 into the sulfidopeptide LTC_4 (5). Peptidolysis of LTC_4 sequentially produces LTD_4 and LTE_4, which collectively comprise the slow reacting substance of anaphylaxis (SRS-A). The SRS-A are bronchoconstrictors, stimulate mucous production in the trachea, increase vascular permeability, and induce the release of lysosomal enzymes (reviewed in reference 6). More recently, LTC_4, and/or its metabolites, have been reported to exert potent effects on the murine system, inducing the synthesis of IFN-γ (7) and inhibiting the proliferation of T cells (8).

Leukotriene synthesis in the setting of autoimmunity has not been previously investigated and is the focus of this study. Mice that spontaneously manifest lupus-like illnesses were chosen as the experimental model (reviewed in references 9, 10).

Materials and Methods

Mice. Mice were purchased from the Jackson Laboratory, Bar Harbor, ME. MRL-*lpr/lpr*, MRL-*+/+*, and C57BL/6-*lpr/lpr* mice were bred in the Animal Research Facility at the Denver

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Veterans Administration Medical Center. MRL-*lpr/lpr* mice exhibit an accelerated and severe disease that has features of both SLE and RA (10). Congenic MRL- +/+ mice, which lack the *lpr* gene but possess an autoimmune background (9, 10), and C57BL/6-*lpr/lpr* mice, which possess the *lpr* gene on a nonautoimmune background, both develop an indolent type of lupus without an arthritic component.

In Vitro Culture. 2×10^5 resident peritoneal Mø were dispensed in 96-well flat-bottomed plates (Costar, Cambridge, MA) in Eagle's Basal Medium (BME) (Gibco Laboratories Chagrin Falls, OH) plus 10% FCS and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 2 h. The cells were then washed and cultured for 2–24 h in BME with 0–150 μg/ml zymosan (Sigma Chemical Co., St. Louis, MO). The supernatants were harvested and analyzed freshly for eicosanoid activity using enzyme immunoassays (11) with tracers purchased from AIA, Inc. (Aurora, CO) or dialyzed and examined for IL-1 activity, as previously described (12). After adherence, 80–92% of cells were Mø as evaluated by nonspecific esterase staining. There were no significant differences in the percentage of Mø obtained from autoimmune vs. normal mice. In experiments using HPLC, 10^6 Mø were dispensed in 35-mm petri dishes in 2 ml BME and cultured for 2 h with 150 μg/ml of zymosan at 37°C in 5% CO_2.

HPLC Analyses. Supernatants from freshly prepared samples were spiked with [^{3}H]LTC_4 (New England Nuclear, Boston, MA) (16,000 dpm, 120 pg) and [^{3}H]LTD_4 (15,000 dpm, 90 pg) to determine recovery and with PGB_2 (Cayman Chemicals, Ann Arbor, MI) (500 ng) to provide a reference point for chromatographic analysis. HPLC analysis was performed using a liquid chromatograph (model 1090; Hewlett-Packard Co., Palo Alto, CA). The gradient was run at a flowrate of 1 ml/min with the initial mobile phase being methanol/water/phosphoric acid (30:70:0.02, vol/vol, pH 5.7, with ammonium hydroxide) for 6 min, followed by a linear gradient to 100% methanol over 44 min. The overall recovery of radioactivity was 81 ± 3% for [^{3}H]LTC_4 and 74 ± 3% for [^{3}H]LTD_4 in these samples.

Results

The profile of lipoxygenase metabolites produced by zymosan-stimulated peritoneal Mø from 16-wk-old autoimmune-prone and immunologically normal mice was initially investigated using reverse-phase HPLC and a gradient-mobile phase. The chromatogram obtained in zymosan-induced Mø from normal C57BL/6 +/+ mice (Fig. 1 *A*) shows a peak that elutes with a retention time identical to that of authentic LTC_4 (Fig. 1, peak 1) and with a UV spectrum (Fig. 1, *insert*) characteristic of a leukotriene. Further analysis of this peak by enzyme immunoassay (EIA) (Fig. 1 *A* histogram) confirmed the presence of LTC_4. The second peak represents the 11-trans isomer of LTC_4 (Fig. 1 *A*). PGB_2, the internal standard (peak 3) elutes at 30.3 min. LTB_4 was not detectable in the Mø supernatants. Similar profiles were observed in zymosan-activated Mø from autoimmune C57BL/6-*lpr/lpr*, MRL- +/+ , and MRL-*lpr/lpr* mice (Fig. 1, *B-D*, respectively) and from immunologically normal C3H/HeN mice (data not shown). Thus, Mø obtained from autoimmune and normal mice demonstrate qualitatively comparable lipoxygenase products on stimulation with zymosan, and in all cases the predominant leukotriene synthesized is LTC_4.

The capacity of Mø from autoimmune-prone MRL mice and immunologically normal C3H/HeN mice of various ages to produce LTC_4 was next investigated by directly measuring eicosanoid activity in the supernatants of zymosan-induced cultures using EIA. An age-associated increase in the ability of MRL-*lpr/lpr* Mø to produce LTC_4 in response to zymosan was observed (Fig. 2). In Mø from MRL- +/+ mice, no such enhancement was seen (Fig. 2), and, in response to zymosan, Mø from the latter strain produced levels of LTC_4 that were comparable with those from C3H/HeN mice (not shown). Zymosan stimulation of Mø from 6- and 20-wk-old

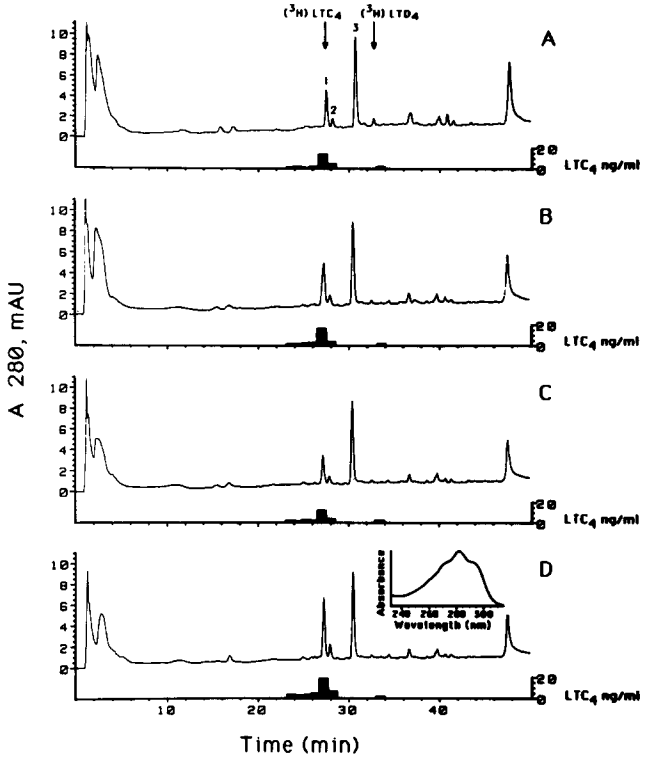

FIGURE 1. Lipoxygenase metabolites of zymosan-stimulated Mø from autoimmune and normal mice. Reverse-phase HPLC of supernatants derived from zymosan-stimulated Mø of 16-wk-old mice. The retention times of authentic [^{3}H]LTC_4 and [^{3}H]LTD_4 are indicated by arrows in *A*. Peak 2 represents the trans-isomer of LTC_4. The peak (3) at 30.3 min is the PGB_2 internal standard. Solid bars indicate LTC_4 immunoreactivity in fractions assessed by EIA. The insert represents the UV absorbance spectrum of the eluent with a retention time (27.1 min) identical to that of authentic LTC_4. (*A*) C57BL/6- *+/+* ; (*B*) C57BL/6-*lpr/lpr*; (*C*) MRL- *+/+* ; (*D*)MRL-*lpr/lpr*.

autoimmune C57BL/6-*lpr/lpr* mice produced levels of LTC_4 that were equivalent to those generated by Mø from age-matched control C57BL/6- *+/+* and BALB/c mice (data not shown).

It remained possible that increased LTC_4 production by Mø from MRL-*lpr/lpr* mice was the consequence of enhanced responsiveness to zymosan. This was investigated by optimally stimulating Mø (10^6/ml) from young (3–5 wk) and old (12–20 wk) MRL mice with zymosan (150 μg/ml) for 24 h, then testing the dialyzed

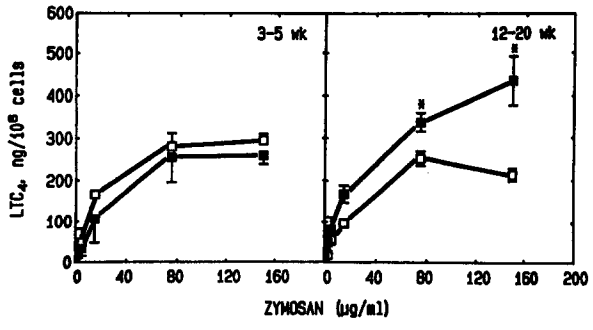

FIGURE 2. Age-associated changes in zymosan-stimulated LTC_4 production. Peritoneal Mø from young (3–5 wk) and old (12–20 wk) MRL- *+/+* (*open squares*), MRL-*lpr/lpr* (*closed squares*), and C3H/HeN (not shown) mice were stimulated with up to 150 μg/ml of zymosan. Supernatants were harvested after a 2-h culture and assayed for LTC_4 by EIA. Values represent the mean ± SE of three experiments. (*) $p < 0.05$ using a nonpaired *t* test. The dose response curves for Mø from young and old C3H/HeN mice were equivalent to those of the MRL- *+/+* .

supernatants for IL-1 activity. Comparable levels of IL-1 were produced by Mφ from MRL-*+/+* mice (74 ± 5 U/ml) and MRL-*lpr/lpr* mice (80 ± 6 U/ml) at all ages tested. Similar results were obtained when both the total time of culture and the dose of zymosan were varied (data not shown).

In contrast to those from normal mice, a significant increase in spontaneous LTC_4 production was observed in Mφ from MRL-*lpr/lpr* mice. A systematic survey of various autoimmune-prone strains revealed high spontaneous production of LTC_4 to be a common feature of Mφ from mice that manifested lupus-like illnesses (Fig. 3). The augmented spontaneous LTC_4 activity was found to increase further with age and was unrelated to either gender (not shown) or to MHC haplotype. Spontaneous LTC_4 release was most marked in Mφ from MRL-*lpr/lpr* mice (Fig. 3). Measurements of PGE_2 in Mφ cultures from 4- (not shown) and 16- (Fig. 3) wk-old autoimmune mice for up to 24 h revealed no differences in the spontaneous release of prostanoids relative to normal mice.

Discussion

The results presented herein demonstrate that Mφ from autoimmune-prone mice exhibit a novel aberration in arachidonic acid metabolism, producing levels of LTC_4 that were up to 10 times greater than those from age-, sex-, and MHC-matched immunologically normal mice in the absence of deliberate addition of exogenous stimulants. Levels of LTC_4 comparable with those spontaneously released by Mφ from MRL-*lpr/lpr* mice 8 wk of age or older ($\sim 10^{-8}$ M) have been shown in vitro to (*a*) stimulate DNA synthesis in human epidermal keratinocytes (13); (*b*) augment the proliferation of human cultured fibroblasts (14); (*c*) induce the mitogenesis of human glomerular epithelial cells (15); and (*d*) replace the helper cell requirement for immune IFN production by murine T cells (7). The age-associated increase in spontaneous LTC_4 production was independent of sex and was shared by Mφ from MHC disparate autoimmune mice with distinct patterns of disease. The data suggest that augmented spontaneous LTC_4 release may be a common feature of mu-

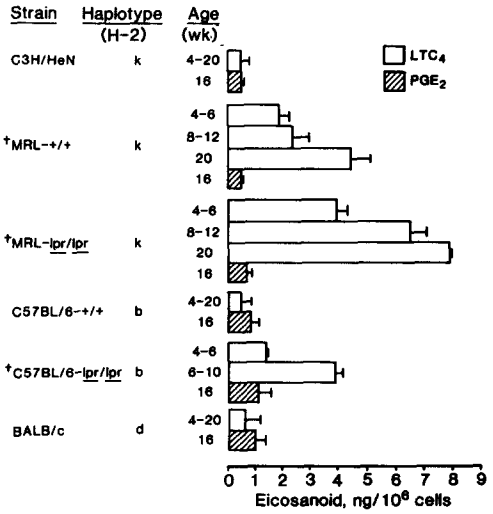

FIGURE 3. Spontaneous eicosanoid production by Mφ from normal and autoimmune (+) murine strains at different ages. Supernatants from peritoneal Mφ were cultured for 2 h or 24 h and assayed freshly for LTC_4 (*open bars*) and PGE_2 (*hatched bars*), respectively, by EIA. Results represent the mean ± SE of 3-5 experiments. Spontaneous LTC_4 release was significantly enhanced ($p < 0.05$) in Mφ from 20-wk-old MRL-*+/+* mice, 8- and 20-wk-old MRL-*lpr/lpr* mice, and 10-wk-old C57BL/6-*lpr/lpr* mice relative to that from the respective control by a non-paired Student's *t* test.

rine lupus. That Mϕ from certain strains (e.g., MRL-+/+) display enhanced spontaneous production of LTC_4 at a time when no overt manifestations of autoimmunity are present indicates that this aberration may be of etiopathogenetic significance. Mϕ from MRL-*lpr/lpr* mice, which manifest the most aggressive lupus-like illness of all strains tested, possessed the greatest capacity to produce LTC_4 both spontaneously and in response to zymosan stimulation. The relationship between disease severity and augmented LTC_4 production further indicates that the two phenomena may be pathogenetically linked. The mechanism by which spontaneous production of LTC_4 may predispose to the development of autoimmune disease is a matter of speculation. However, by increasing vascular permeability and inducing the release of lysosomal enzymes (4, 5), LTC_4 could contribute to the inflammation and tissue destruction characteristically seen in lupus.

Summary

Eicosanoids have been implicated in the pathogenesis of autoimmune diseases. In this study, peritoneal macrophages from autoimmune-prone mice were examined for their capacity to produce proinflammatory 5-lipoxygenase metabolites. The results indicate that enhanced production of leukotriene C_4 is a common feature of murine autoimmunity and suggest further that aberrations in 5-lipoxygenase activity may play a role in the development of lupus.

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