Dysregulated Expression of the T Cell Cytokine *Eta-1* in CD4$^-$8$^-$ Lymphocytes during the Development of Murine Autoimmune Disease

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Summary

The development of autoimmune disease in the MRL/MpJ-*lpr* inbred mouse strain depends upon the maturation of a subset of T lymphocytes that may cause sustained activation of immunological effector cells such as B cells and macrophages. We tested the hypothesis that abnormal effector cell activation reflects constitutive overexpression of a T cell cytokine. We found that a newly defined T cell cytokine, *Eta-1*, is expressed at very high levels in T cells from MRL/l mice but not normal mouse strains and in a CD4$^-$8$^-$ 45R$^+$ T cell clone. The *Eta-1* gene encodes a secreted protein that binds specifically to macrophages, possibly via a cell adhesion receptor, resulting in alterations in the mobility and activation state of this cell type (Patarca, R., G. J. Freeman, R. P. Singh, et al. 1989. *J. Exp. Med.* 170:145; Singh, R. P., R. Patarca, J. Schwartz, P. Singh, and H. Cantor. 1990. *J. Exp. Med.* 171:1931). In addition, recent studies have indicated that *Eta-1* can enhance secretion of IgM and IgG by mixtures of macrophages and B cells (Patarca, R., M. A. Lampe, M. V. Iregai, and H. Cantor, manuscript in preparation). Dysregulation of *Eta-1* expression begins at the onset of autoimmune disease and continues throughout the course of this disorder. Maximal levels of *Eta-1* expression and the development of severe autoimmune disease reflect the combined contribution of the *lpr* gene and MRL background genes.

The MRL/MpJ-*lpr* (MRL/l) inbred mouse strain spontaneously develops a systemic autoimmune disease with histopathological features of human SLE and rheumatoid arthritis (1). The development of this syndrome is associated with abnormal expansion of a subset of T cells that co-express CD1 (Thy-1), TCR-α/β, and the B cell marker CD45R (B220 isoform of leukocyte common antigen) and lack both CD4 and CD8 surface antigens (CD1$^+$CD45R$^+$CD4$^-$CD8$^-$) (2). The developmental pathway of this subset of T cells, termed double-negative (DN)[1] T cells, is poorly understood, although recent studies suggest that it may arise relatively late in ontogeny from CD4$^+$ precursors (3). Although the development of autoimmune disease is heralded by the appearance of large numbers of DN cells in peripheral lymphoid tissues, there is little information on the potential contribution of these cells to the sustained activation of immunological effector cells that may mediate the autoimmune process.

In the present studies we determined the level of cytokine genes expressed by a clone of DN T cells in vitro. We found that these cells selectively and constitutively expressed high levels of the recently defined T cell cytokine *Eta-1* (for early T lymphocyte activation 1). This prompted an analysis of the expression of this gene during the development of autoimmune disease in the MRL/l model. We found that expression of this cytokine is substantially increased at the onset of the disease process and remains elevated throughout the course of this disorder. The relative contributions of MRL background genes and the *lpr* mutation to dysregulated expression of *Eta-1* are defined, and the implications of these findings are discussed in the context of our current understanding of the immunological activities of the eta-1 protein.

Materials and Methods

Mice. MRL/l and MRL/MpJ+ (MRL/n) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and from Dr. C. Reinisch (Tufts University School of Veterinary Medicine, Boston, MA). Lymphoid tissues from C3H/HeJ-*lpr* (C3H/l) and C57BL/6-*lpr* (B6/l) mice were kindly provided by Dr. H. C. Morse III (National Institutes of Health, Bethesda, MD). Age-matched C3H/HeJ (C3H/n) and C57BL/6J (B6/n) inbred mouse strains were obtained from The Jackson Laboratory.

[1] *Abbreviations used in this paper:* DN, double-negative; Eta-1, early T lymphocyte activation 1.

Cell Lines and Immunofluorescence. Ar5 is a CD4+ TH1 murine clone specific for arsonylated proteins (4) and Ar5v is a variant of this clone first detected because of its enhanced growth in vitro (5). Cell surface immunofluorescence was performed by flow cytometric analysis using a FACS. Ar5 and Ar5v cells (>90% viability) were incubated with either anti-CD1 (anti-Thy-1, AMT-13, 1:100), anti-CD4 (1:25), anti-CD8 (1:100) (Becton Dickinson & Co., Mountain View, CA), or anti-CD45R (anti-B220, RA3-2C2/1, 1:20, from American Type Culture Collection, Rockville, MD) mAbs at 4°C for 40 min. Cells were then washed thrice with PBS and incubated with FITC-conjugated goat anti-rat IgG [$F(ab')_2$ heavy and light chains, 1:300; Cappel Laboratories, Durham, NC) for an additional 30 min before additional washing in PBS and analysis.

Slot Blot Analysis. Cellular RNA was extracted using the guanidium isothiocyanate method with cesium chloride gradient modification (6). The amount of RNA in each sample was estimated by determining its absorbance at 260 nm (OD_{260}). 20-μg aliquots of RNA from each cellular sample (unless otherwise indicated) were blotted onto nitrocellulose filters using a Minifold II slot blotter (Schleicher & Schuell, Inc., Keene, NH) and hybridized to ^{32}P-labeled cDNA corresponding to *Eta-1* (a 1.6-kb HaeIII fragment from pcD-Eta-1) (7), *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase, a housekeeping gene) (8), IL-2 (9), IL-3 (10), and IFN-γ (5). Northern blot analysis showed that the cDNA probes for *Eta-1* and *GAPDH* hybridized to a single species of RNA and the levels of RNA quantitated by densitometric measurement of Northern blots were equivalent to those obtained by slot blot analysis. Quantitation was performed as described previously (7) and detailed below. After preflashing, Kodak X-OMAT films were exposed and the intensity of radioactivity of the autoradiograms was quantitated using an Ultroscan II laser densitometer (LKB Instruments, Inc., Gaithersburg, MD), adjusting exposure times so that the intensity of autoradiographic signals corresponded to the linear range of densitometric detection. To ensure that comparisons of *Eta-1* RNA levels in different cellular samples were based upon the same amount of RNA in each sample, the area under the densitometric peak for *Eta-1* for each cellular RNA sample was divided by the area under the *GAPDH* densitometric peak for the same cellular RNA sample. The ratios of *Eta-1* RNA to *GAPDH* RNA for each cellular sample are referred to as relative densitometric units (RDU).

Results

Expression of Eta-1 by a DN B220+ T Cell Clone. We examined a DN T cell clone that arose during the in vitro growth of a CD4+ TH1 clone, Ar5 (4, 5). The variant, termed Ar5v, retained expression of CD1 but expressed neither CD4 nor CD8. In addition, the variant clone but not the parent expressed CD45R according to immunofluorescence and thus had acquired the typical surface phenotype of DN cells (Fig. 1 *A*). Analysis of cDNA libraries derived from Ar5 and Ar5v also showed that the variant retained expression of the Ar5 TCR-α/β (not shown).

We asked whether transition from the $CD4^+8^-CD45R^-$ phenotype of Ar5 to the $CD4^-8^-CD45R^+$ DN phenotype of Ar5v was accompanied by a change in the expression of genes encoding T cell cytokines. Resting Ar5 cells did not express detectable levels of cytokine genes, including *IL-2*, *IL-3*, *IL-4*, and *IFN-γ* or a newly defined T cell cytokine, *Eta-1* (7, 11) (Fig. 1 *B*). The *Eta-1* gene encodes a secreted protein that binds specifically to macrophages, possibly via a cell adhesion receptor, resulting in alterations in the mobility and activation state of this cell type (7, 11). In addition, recent studies have indicated that *Eta-1* can enhance secretion of IgM and IgG by mixtures of macrophages and B cells (Patarca, R., M. A. Lampe, M. V. Iregai, and H. Cantor, manuscript in preparation). The DN variant Ar5v cells constitutively expressed high levels of *Eta-1* RNA and showed a less pronounced but significant elevation in expression of *IFN-γ* RNA (Fig. 1 *B*). Enhanced constitutive expression of other TH1 cytokines, including *IL-2* and *IL-3*, was not detected, nor was there evidence for aberrant expression of the TH2-associated cytokine, *IL-4* (Fig. 1 *B*).

Expression of Eta-1 by DN T Cells In Vivo. We asked whether the expanded population of DN cells in peripheral lymphoid tissues of MRL/l mice also showed evidence of dysregulated *Eta-1* expression. Lymphadenopathy in MRL/l mice secondary to expansion of DN cells begins at ~3 mo of age and reaches maximum levels by 5–6 mo of age (12). Elevated levels of *Eta-1* RNA were detected in thymus, lymph nodes (LN), and spleen from the MRL/l mouse strain at 5.5 mo of age but not in lymphoid tissue of age-matched MRL/n controls, which lack the *lpr* mutation and do not exhibit signs of autoimmune disease at this time (Fig. 2). The *Eta-1* RNA expressed by LN cells from MRL/l mice, and by Ar5v cells, was indistinguishable from that found in activated T cells from normal mice, as judged from Northern blot and S1 nuclease protection analysis (not shown). We determined the time course of elevated *Eta-1* expression in the thymus and LN of MRL/l and MRL/n mice to assess its relationship to the development of autoimmune disease. *Eta-1* RNA levels increased in thymus slightly before LN at 2.5–3 mo of age and reached maximum levels by 4.5–5 mo of age (Fig. 3 *A*). In contrast, *Eta-1* RNA levels were not significantly increased in LN of control MRL/n mice during this time (Fig. 3 *A*). These kinetics of enhanced *Eta-1* expression paralleled the development of overt autoimmune disease, which begins at ~3.5 mo of age and results in 50% mortality by 5–5.5 mo of age (1, 12).

The increase in *Eta-1* expression compared with levels of a housekeeping gene, *GAPDH*, over the period from 2.5 to 4.5 mo after birth, was ~25-fold (Fig. 3 *A*). However, the absolute increase in *Eta-1* expression in LN tissues was substantially higher because of the development of severe lymphadenopathy during this time: total levels of *Eta-1* RNA in peripheral LN of MRL/l mice increased by ~4 orders of magnitude (Fig. 3 *B*).

$CD1^+$ DN cells comprise >90% of the cells of LN from MRL/l mice (2) and therefore were likely to account for the bulk of elevated *Eta-1* RNA levels. To define the contribution of this subset to enhanced *Eta-1* expression, RNA was extracted from separated DN cells and from $CD4^+$ or $CD8^+$ T cells in LN. The DN population contained almost all of the *Eta-1* RNA expressed by unseparated lymph node cells (Fig. 4).

Comparison of Eta-1 Gene Expression to Expression of Other T Cell Cytokines. Analysis of the DN clone Ar5v indicated high constitutive levels of *Eta-1* expression and a modest but

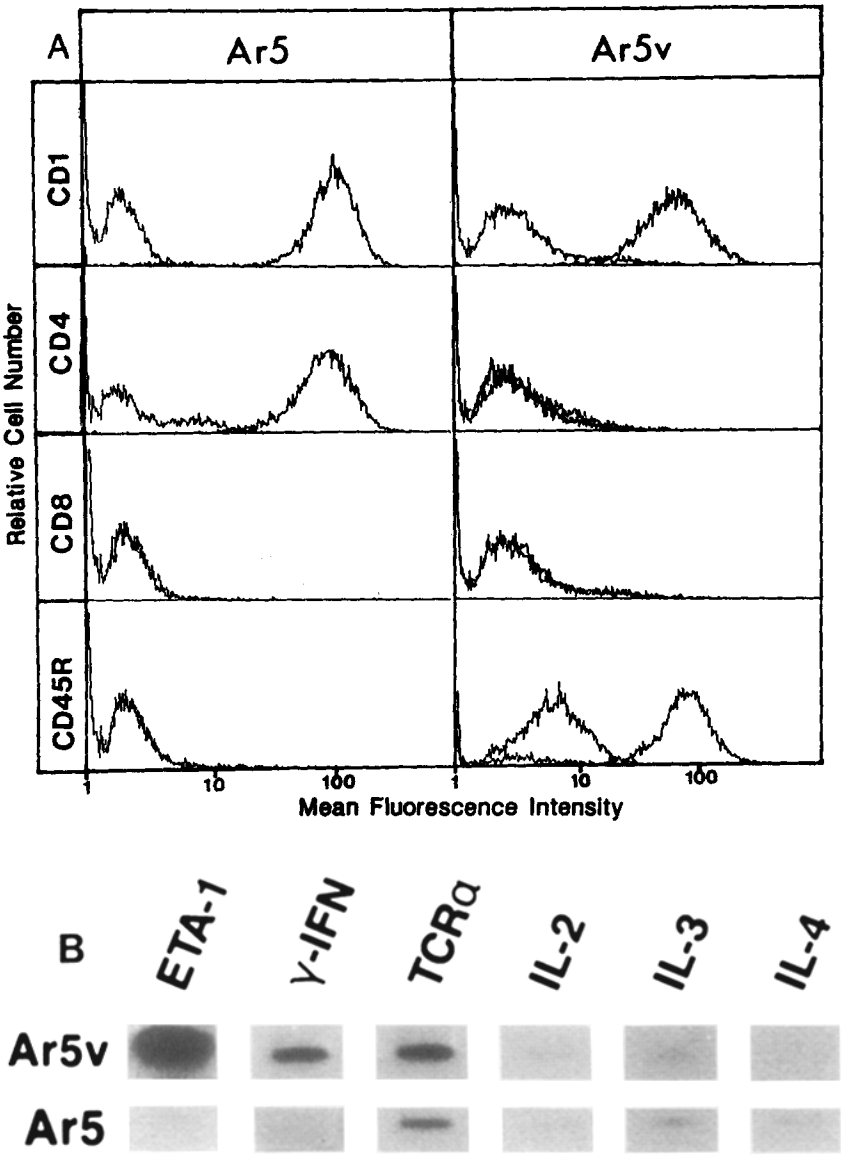

Figure 1. (*A*) Surface phenotype of the T cell clones Ar5 and Ar5v. The $CD4^-8^-$ surface phenotype of Ar5v cells, but not the parent $CD4^+8^-$ Ar5 cells, corresponds to that of DN T cells that are abnormally expanded in MRL/l mice, including the expression of the leukocyte common antigen (LCA) isoform CD45R (B220) characteristic of B cells. Ar5v is a variant clone that arose from the arsonate-reactive inducer T cell clone Ar5 (4, 5). The FACS profiles obtained for each antibody are shown and the intensity of fluorescence is presented on a $\log_{10}$ scale. Background fluorescence profiles due to nonspecific binding of the second antibody were obtained using PBS instead of the relevant mAbs in the first incubation. (*B*) Cytokine RNA expression for clones Ar5 and Ar5v. Northern blot analysis showed that the probes used for the *Eta-1* gene hybridized to a single species of RNA and that the size of the *Eta-1* RNA transcript in Ar5v cells was the same as that in Ar5 cells. S1 nuclease protection analysis using the 5'-labeled 1 kb BstXI-SspI fragment from the *Eta-1* cDNA insert in pcD-Eta1 (7) confirmed the identity of both transcripts at the nucleotide sequence level (not shown).

Tissue	MRL strain	Age (mos)	ETA-1 Expression
Thymus	n	6	
	l	5	
Lymph node	n	6	
	l	5	
Spleen	n	6	
	l	5	

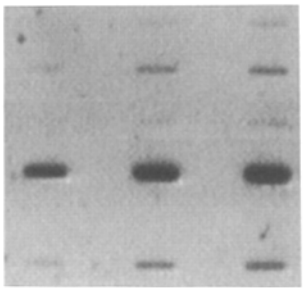

Figure 2. Eta-1 gene expression in lymphoid tissues of MRL/l and MRL/n mice. Thymus, spleen, and mesenteric LN were obtained from 5-mo-old MRL/l mice (which display marked lymphoid hyperplasia and activated macrophages) and 6-mo-old congenic MRL/n mice that had been housed in the same colony after their arrival from The Jackson Laboratory. 1, 3.5, and 7 μg of total RNA were subjected to slot blot analysis using a ^{32}P-labeled murine *Eta-1* cDNA probe as described in Materials and Methods.

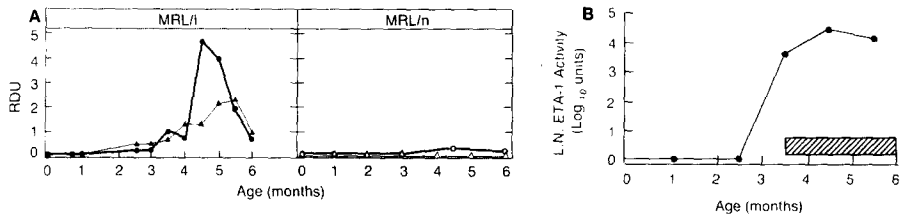

Figure 3. (*A*) Time course of Eta-1 RNA expression in LN and thymus cells of MRL/l and MRL/n mice. Cellular RNA was extracted from LN (●) and thymus (▲) from cohorts of MRL/l and MRL/n mice at the ages indicated on the abscissa. The ratio of *Eta-1* RNA levels to *GAPDH* levels of RNA from each cellular sample, as measured by densitometric analysis, is shown on the ordinate and expressed as relative densitometric units (RDU) (see Materials and Methods for details). The points indicated in the graph correspond to the mean of at least three different samples; standard deviation of each mean value is <20%. The intensity of the autoradiographic signals obtained after blotting RNA from heart, muscle, or adrenal gland with this *Eta-1* cDNA probe did not exceed the background signal of the film. (*B*) Total *Eta-1* activity in LN from MRL/l mice. The levels of *Eta-1* RNA expressed as relative densitometric units (*Eta-1/GAPDH*) as described for *A* are multiplied by the ratio of LN weight at the age indicated to LN weight at 1 mo of age. LN *Eta-1* activity is depicted on a $\log_{10}$ scale. The hatched horizontal bar indicates the interval from the initial signs of autoimmune disease to the age corresponding to 50% mortality from the disease process.

significant elevation of *IFN-γ* (Fig. 1). We asked whether this was also the case for the expanded population of DN cells in the LN of MRL/l mice, or whether these cells exhibited a more generalized dysregulation of lymphokine gene expression. We found that the levels of *IL-2*, *IL-3*, and *IL-4* RNA were not significantly elevated in MRL/l mice compared with age-matched MRL/n controls, in contrast to the striking elevation of *Eta-1* RNA levels (Fig. 5; Table 1). As was the case for the DN Ar5v clone, *IFN-γ* RNA levels were significantly elevated in LN from MRL/l mice. Increased expression of this cytokine was transient and was not evident until relatively late in the disease process (4.5–5.5 mo). Moreover, the degree of elevation at this time was considerably less than noted for *Eta-1* (Fig. 5; Table 1).

We studied expression of *Eta-1* in nonlymphoid tissues of MRL/l mice. *Eta-1* was not significantly elevated in muscle, heart or adrenal tissue (not shown). However, substantial levels of *Eta-1* RNA were found in lung, consistent with the development of peribronchial lymphocytic infiltration and pneumonitis associated with MRL/l disease (1). Analysis of TCR-α levels in pulmonary tissue as a genetic marker of infiltrating T cells showed that increased levels of TCR-α RNA correlated closely with elevation in *Eta-1* expression in lung tissue (Fig. 6).

Genetic Control of Eta-1 Expression in MRL/l Mice. The above comparisons of *Eta-1* RNA expression in MRL/l and MRL/n mice suggest that the *lpr* gene is necessary for elevated expression of this gene in peripheral lymphoid cells. However, they did not distinguish between the following possibilities: (*a*) the *lpr* gene is necessary and sufficient for elevated *Eta-1* expression or (*b*) the *lpr* gene and the associated MRL background gene(s) are both necessary for maximal expression of *Eta-1*. The *lpr* mutation has been bred onto several normal inbred strains, including C57BL/6J (B6/n) and C3H/HeJ (C3H/n) (2). This results in the development of lymphadenopathy and formation of high levels of IgG autoantibodies. However, the C3H/l and B6/l strains do not develop the severe manifestations of autoimmune disease that characterize MRL/l mice (2). Comparison of *Eta-1* RNA levels in enlarged LN from B6/l, C3H/l, and MRL/l mice showed that although the *lpr* gene was sufficient for elevation of *Eta-1* RNA levels, MRL background genes (derived from five inbred strains: C57BL/6J, C3H/HeJ, LG, AKR, A/J) were required for maximal levels of expression (Fig. 7). The *lpr* mutation is not likely to map to the *Eta-1* locus since its presence does not alter the restriction fragment length polymorphic patterns of the *Eta-1* gene in a panel of inbred mouse strains (data not shown).

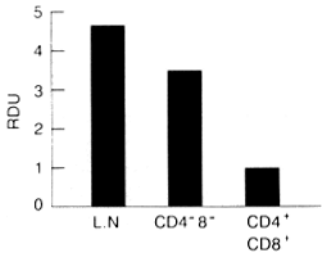

Figure 4. *Eta-1* RNA levels in LN cells and in DN subpopulations of T cells. RNA was extracted from either total lymph node cell suspension or from the adherent Ig^+ ($CD4^+8^+$) and nonadherent Ig^- ($CD4^-8^-$) subpopulations obtained after panning LN cells with anti-CD4 and anti-CD8 antibody as described previously (24). Immunofluorescent analysis of the separated subpopulations indicated that the nonadherent cells contained <1% $CD4^+$, $CD8^+$, or Ig^+ cells while contamination of the $CD4^+8^+$ cells with DN cells was 10–15%.

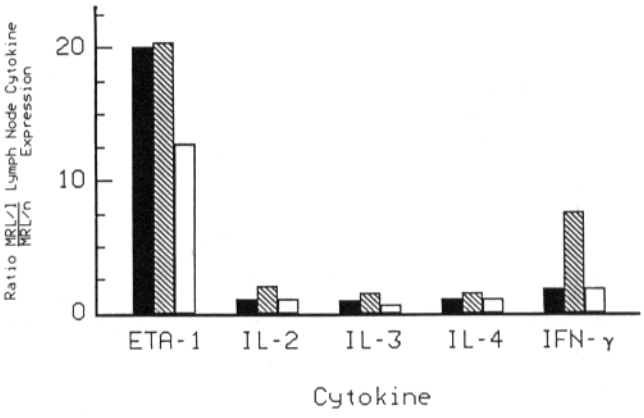

Figure 5. Comparison of in situ cytokine gene expression in MRL/l and MRL/n mice. The ratios of cytokine expression in MRL/l and MRL/n mice (RDU-MRL-l / RDU-MRL-n) at 3.5 mo (■), 4.5 mo (▨), and 6.5 mo (□) are shown.

Table 1. *Ontogeny of In Vivo Lymphokine/Cytokine Expression in MRL/1 and Congenic MRL/n mice*

		RNA expression at different months of age														
		ETA-1			IL-2			IL-3			IL-4			IFN-γ		
Mice	Age:	3.5	4.5	6.5	3.5	4.5	6.5	3.5	4.5	6.5	3.5	4.5	6.5	3.5	4.5	6.5
								RDU								
MRL/l		1.0	4.7	1.9	0.1	0.2	0.1	0.2	0.3	1.9	0.05	0.13	0.3	0.4	1.4	0.43
MRL/n		0.05	0.23	0.15	0.1	0.1	0.1	0.23	0.23	3.9	0.05	0.09	0.3	0.23	0.18	0.26

RNA samples from LN of MRL/l and MRL/n were analyzed by slot blots, and levels of gene expression for each cytokine are expressed as relative densitometric units (RDU) as described in Materials and Methods. Exposure times for autoradiograms were adjusted so that the intensity of the autoradiographic signals associated with *GAPDH* was equivalent for MRL/l and MRL/n RNA samples.

Discussion

The data presented in this report shed new light on the role of abnormal T cell development in autoimmune disease in MRL/l mice. DN cells that are abnormally expanded in these mice constitutively express extremely high levels of *Eta-1* and the time course of enhanced expression is closely associated with the onset and development of autoimmune disease. *Eta-1* expression is also elevated in association with abnormal expansion of DN cells in the C3H/l and B6/l mouse strains. However, increased *Eta-1* expression in these two strains is intermediate between normal inbred strains and the MRL/l strain. This finding is consistent with the milder form of autoimmune disease displayed by B6/l and C3H/l, which develop anti-DNA antibodies and rheumatoid factor but do not develop severe immune complex glomerulonephritis (1, 2).

Several explanations have been put forward to explain the role of abnormal T cell differentiation in the development of autoimmune disease in the MRL/l model. One involves a differentiative defect that results in failure to delete autoreactive T cell clones when they arise in the thymus. However, several recent studies have failed to detect such abnormalities (13, 14). A second holds that the DN subset constitutively expresses elevated levels of cytokines, resulting in chronic activation of immunologic effector cells such as macrophages and B cells (15). The studies reported here are consistent with the latter explanation. DN cells that accumulate during this disease do not divide rapidly in situ and by this criterion are not in an activated state. However, DN cells may express an alternative activation program marked by elevated *Eta-1* gene expression as shown here, as well as constitutive phosphorylation of CD3 (16) and expression of the proto-oncogene *c-myb* (17). The availability of the DN Ar5v cell line may allow analysis in vitro of the molecular basis of this potential alternative activation pathway.

Elevated levels of *IFN-γ* in MRL/l mice have been reported previously, although the degree of this elevation and its temporal pattern were not determined (18, 19). We find that the degree of elevation is relatively small compared with *Eta-1* and occurs late and transiently in the course of disease. We did not detect enhanced levels of *IL-2*, *IL-3*, or *IL-4* RNA, which represent cytokines normally associated with T cell inducer activity. Both *Eta-1* and *IFN-γ* can bind to and acti-

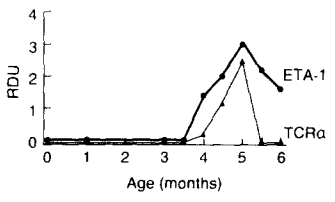

Figure 6. Expression of *Eta-1* and TCR-α in lung tissue of MRL/l mice. RNA was extracted from the lungs of MRL/l mice at the ages indicated in the figure and subjected to slot blot analysis. Levels of *Eta-1* and TCR-α RNA are expressed as relative densitometric units as described in Materials and Methods.

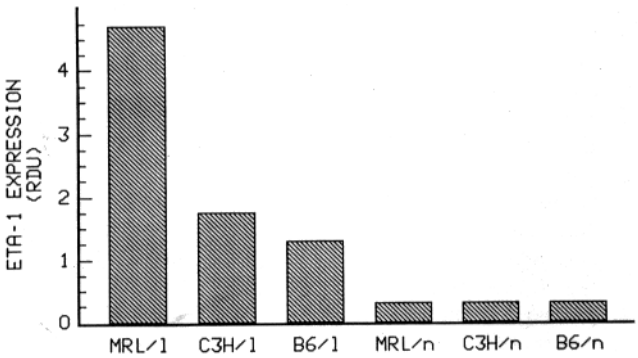

Figure 7. Maximum levels of *Eta-1* in LN cells from MRL/l, C3H/l, and B6/l. Peripheral (axillary, brachial, femoral) and mesenteric lymph nodes were obtained from the indicated mouse strains at 3-mo intervals. Maximum levels of *Eta-1* expression shown in the graph were observed at 4.5 mo for MRL/l mice, and at 7 mo for C3H/l and B6/l mice. Cellular RNA was extracted and analyzed as described in Materials and Methods. The height of the bars corresponds to mean relative densitometric units. Standard errors were <20% of mean.

vate macrophages (7, 11), consistent with evidence that MRL/l mice harbor activated macrophages that may mediate tissue destruction (20) and indirectly contribute to enhanced Ig production by B cells. Although the findings reported here do not directly implicate *Eta-1* in the pathogenesis of this murine model of lupus, they indicate that elevated *Eta-1* expression may represent a highly specific genetic marker for the T cell developmental defect associated with this type of autoimmune disease. The appearance of elevated levels of *Eta-1* gene expression in the thymus before the appearance of substantial levels of DN T cells in the periphery and the dramatic and relatively selective increase of *Eta-1* gene expression in DN T cells during the disease process suggest that this cytokine may also contribute to the pathogenesis of this syndrome. The relationship of *Eta-1* to the B cell differentiation factor produced by T cells from MRL/l mice is currently unknown (21), although recent experiments indicate that eta-1 can enhance secretion of IgM and IgG by mixtures of macrophages and B cells (Patarca, R., et al., manuscript in preparation). The eta-1 protein differs in biochemical properties from the B cell maturation factor found in spontaneously autoimmune moth-eaten mice (22) and in sequence from the B cell differentiation factor associated with autoimmunity in patients with cardiac myxoma or uterine carcinoma (23). Further studies of the biological effects of *Eta-1* on macrophages and on B cell secretion of IgG isotypes should help clarify the role of this cytokine in the MRL/l murine model of autoimmunity.

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