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.
Cell Lines and Immunofluorescence. Ar5 is a CD4+ TH1 murine clone specific for arsionylated 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, RA3-2C5/1, 1:20, from American Type Culture Collection, Rockville, MD) mAbs at 4°C for 40 min. They 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 (OD260). 20-μg aliquots of RNA from each cellular sample (unless otherwise indicated) were blotted onto nitrocellulose filters using a Minifold II slot bloter (Schleicher & Schuell, Inc., Keene, NH) and hybridized to 32P-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 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+ CD8+ CD45R- phenotype of Ar5 to the CD4+ CD8- 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 Viva. 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 sorted 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/MpJ 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 arsenate-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 log10 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-Etal (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         |                 |
| Lymph node | n          | 6         |                 |
| Spleen     | n          | 6         |                 |

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 32P-labeled murine Eta-1 cDNA probe as described in Materials and Methods.
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 IL2, IL3, and IL4 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/1 and B6/1 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).
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/1 and B6/1 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 B6/1 and C3H/1, 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 IL2, IL3, or IL4 RNA, which represent cytokines normally associated with T cell inducer activity. Both Eta-1 and IFN-γ can bind to and acti-

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**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 | 1.0   | 0.0  | 0.1  | 0.2  | 0.3   | 1.9  | 0.05 | 0.13 | 0.3 | 0.4 | 1.4 | 0.43 |
| MRL/l | 0.05 | 0.23 | 0.15 | 0.1  | 0.1  | 0.23 | 0.23 | 3.9  | 0.05 | 0.09 | 0.3 | 0.23 | 0.18 | 0.26 |
| MRL/n |

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.

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**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 mouse-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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