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High Incidence of Spontaneous Disease in an HLA-DR15 and TCR Transgenic Multiple Sclerosis Model

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Multiple sclerosis (MS) is thought to involve CD4 T cell recognition of self myelin, many studies focusing on a pathogenic role for anti-myelin, HLA-DR15-restricted T cells. In experimental allergic encephalomyelitis, it is known which epitopes trigger disease and that disease is associated with determinant spread of T cell reactivity. Characterization of these events in human MS is critical for the development of peptide immunotherapies, but it has been difficult to define the role of determinant spread or define which epitopes might be involved. In this study, we report humanized transgenic mice, strongly expressing HLA-DR15 with an MS-derived TCR; even on a RAG-2 wild-type background, mice spontaneously develop paralysis. Disease, involving demyelination and axonal degeneration, correlates with inter- and intramolecular spread of the T cell response to HLA-DR15-restricted epitopes of myelin basic protein, myelin oligodendrocyte glycoprotein, and αβ-crystallin. Spread is reproducible and progressive, with two of the epitopes commonly described in responses of HLA-DR15 patients. The fact that this pattern is reiterated as a consequence of CNS tissue damage in mice demonstrates the value of the transgenic model in supplying an in vivo disease context for the human responses. This model, encompassing pathologically relevant, spontaneous disease with the presentation of myelin epitopes in the context of HLA-DR15, should offer new insights and predictions about T cell responses during MS as well as a more stringent test bed for immunotherapies. The Journal of Immunology, 2005, 174: 1938–1946.

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1 This work was funded by grants from the Multiple Sclerosis Society of Great Britain and Northern Ireland and the Biotechnology and Biological Sciences Research Council.

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Received for publication September 14, 2004. Accepted for publication November 11, 2004.

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showed a high incidence of spontaneous disease that was dependent to some extent on the environment in which the mice were housed (16). The immunogenetics of human MS have previously been modeled by expressing HLA class II genes, or HLA-DR with a matched TCR, in transgenic mice (17–20). Disease in HLA-DR/TCR transgenics has been achieved by injection of myelin peptides under EAE-inducing conditions or by crossing onto RAG-2 knockouts, eliminating T regulatory cells, although a high incidence of spontaneous disease in humanized models has rarely been observed. In this study, we describe a unique model in which there is a very high incidence of spontaneous disease, allowing the analysis of CNS pathology that is faithful to clinical MS and accompanied by epitope spread on a RAG-sufficient background and in the context of human HLA molecules. The model thus offers clear advantages over existing ones for testing immunotherapies.

**Materials and Methods**

**Line 7 mice**

(C57BL/6 × CBA)F1 oocytes were microinjected with an HLA-DRA1*0101 cosmid, as previously described (21), and clone 11, a full-length DRB1*1501 cosmid encompassing extensive, flanking 5' and 3' regulatory sequence. Founder 24 was mated for more than six generations with C57BL/6 Aβ2 mice to yield DR15 Aβ2 mice. To generate mice expressing an anti-MBP 85–99/DR15 TCR, rearranged Var3.1Jαα 40 and Vβ2.1Jβ2.1 segments amplified from genomic DNA of the Ob1A12 T cell clone were subcloned into pTOCass and pTβJcass (12, 22). A number of TCR-positive founders were generated, of which one, termed line 7, is described in this work. A subline was generated by two generations of backcrossing to C57BL/6 RAG-2/–/– mice.

**Flow cytometry**

The phenotype of lymphocyte populations was assessed by flow cytometry on a BD Biosciences FACSCalibur running CellQuest software and using fluorochrome conjugated Abs against CD4, CD8, B220 (BD Biosciences), HLA-DR, and human VJ2 (Serotec). All staining reactions were performed on 5 × 10^6 cells using standard procedures. Briefly, the cells were incubated with the Abs at 4°C for 30 min, washed three times, and resuspended in FACS buffer before assessment.

**T cell peptide responses**

T cell responses were analyzed in HL-1 medium (Cambrex) to MBP, MOG, or αβ-crystallin peptides, added to triplicate wells at a final concentration of 50 μg/ml, unless indicated otherwise. In some cases, T cells were prepared by centrifugation of disrupted CNS cells over a 30–70% Percoll density gradient. For CNS cell assays, 2 × 10^6 infiltrate cells/well were used with 3 × 10^3 irradiated (2000 R) splenocytes as APC and peptide added at a final concentration of 50 μg/ml. Peptide panels representing the sequences of human MBP, MOG, and αβ-crystallin were made by Biosynth International. Tissue culture supernatant was removed from each well after 48 h for ELISA measurement of IFN-γ and IL-4 responses (R&D Systems).

In some cases, long-term T cell lines were generated against myelin peptides by four or more cycles of restimulation at 14-day intervals using irradiated HLA-DR15 transgenic spleen cells as APC to present peptide added at 50 μg/ml and cultured between restimulations in medium containing 20 U/ml rIL-2. To investigate adoptive transfer of disease, T cell lines were propagated by repeated restimulation with spread epitopes. Cells were generated by i.v. injection of 5 × 10^6 cells to HLA-DR15 transgenic recipients, given 24 h after irradiation of mice with 400 R. At the time of cell transfer and again 24 h later, mice received an i.v. injection of 200 ng of pertussis toxin (Valent Pharmaceuticals).

To analyze responses of purified populations, CD4^+ and CD8^+ cells were negatively purified from line 7 spleen using rat anti-mouse anti-CD4/CD8 Abs and anti-rat IgG Dynal beads (Dynal Biotech). Anti-DR Abs were also used to eliminate any DR^+ cells. CD4^+ or CD8^+ cells were added at 3 × 10^5 cells/well in 96-well plates supplemented with 3 × 10^5 DR15^+ APC and MBP 85–99 at concentrations from 0 to 25 μg/ml. IL-2 at a 1 U/ml was added to the wells containing the CD8^+ cells. To test for killing by selected populations of line 7 cells, splenocytes were stimulated with 1 μg/ml MBP 85–99 for 48 h, and CD4^+ and CD8^+ cells were negatively selected, as described above. PGF HLA-DR15^+ human B lymphoblastoid cells were labeled with 100 μCi of chromium^{51} and pulsed with 1 μg/ml MBP 85–99 for 2 h at 37°C. PGF target cells were added at 5 × 10^4 per well with CD4^+ or CD8^+ effector cells. At 4 h, supernatants were removed from each well for counting in an automated gamma counter. Specific lysis was calculated according to the formula: (mean sample cpm – mean spontaneous release)/(mean total cpm – mean spontaneous release) × 100.

**Paralysis scores**

Mice were scored for spontaneous disease, as follows: 0; normal; 1, limp tail; 2, impaired righting reflex or waddling gait; 3, partial hind limb paralysis; 4, total hind limb paralysis; 5, total limb paralysis; 6, moribund.

**Histopathology**

Mice were perfused with 1% paraformaldehyde/1% gluteraldehyde/1% dextran/PIPES buffer. Tissues were postfixed in osmium tetroxide and embedded in Durcupan resin. Semithin sections were stained with thionin and acridine orange, and ultrathin sections with uranyl acetate and lead citrate. Alternatively, 5-μm wax sections were prepared and stained with H&E or luxol fast blue/cresyl fast violet.

**Results**

**Characterization of TCR/HLA expression and function**

A number of TCR and HLA-DR15/Aβ2 transgenic lines was generated, of which one, termed line 7, is described in this work. Flow cytometric analysis of thymocytes showed that 97% of CD4 single-positive (SP) thymocytes carry the human Vβ2 TCR (Fig. 1), and the receptor is also present on 79% of CD8 SP cells. In the spleen, 97% of CD4 SP cells express the transgenic TCR (Fig. 2a). Analysis of MHC class II expression in the mice shows strong HLA-DR15 expression, with no expression of mouse/human class II mixed pairs (Fig. 2b).

Naïve splenocytes from TCR transgenics show a strong proliferative (Fig. 3a) and IFN-γ response (Fig. 3b) to low doses of MBP 85–99 peptide. CD4 and CD8 cells can recognize MBP 85–99...
transgenic lines expressing the Ob1A12 receptor: one line showed a very low incidence of disease, unless injected with MBP peptide or crossed onto RAG-2 knockouts, while a line reported by us showed poverty of movement, but only rare paralysis (19, 20). Approximately 60% of line 7 mice develop paralysis by 6 mo. This phenotype is greatly exacerbated by crossing onto a RAG-2-null background, suggesting a possible role of regulatory cells.

**Histopathological changes in line 7 mice**

Paralysis in the RAG-sufficient line was associated with profound histopathological changes. Several mice of various ages were analyzed, with representative findings shown in Fig. 5. CNS and peripheral nerves of wild-type control, age-matched mice showed no histological abnormalities (data not shown). In transgenics, inflammation is associated with myelin loss in the spinal cord and cerebellum. Analysis of mice aged under 4 mo shows demyelination, for example in the lumbar cord, with extensive infiltrates and perivascular cuffing (Fig. 5, a and b). Mice with chronic, overt paralysis show widespread axonal degeneration (Fig. 5, c, e, and f), associated with dense areas of lymphocytic infiltration, predominantly in the spinal cord, most marked distally and in the brain (Fig. 5d). This involves extensive infiltration of the pia arachnoid and perivascular cuffing of blood vessels in cord and brain (Fig. 5d). Lesions are widespread in the spinal cord, but particularly evident in the dorsal and lateral columns. In the brain, lesions are found in the cerebellum, brain stem, and origin of the fifth nerve. In younger animals, many of the lesions associated with these infiltrates are demyelinating. In older animals, axonal degeneration is more frequently found. Collections of axonal organelles are found in some demyelinated and remyelinated axons in spinal cord lesions (Fig. 5f), indicating cessation of axonal flow. Infiltrating lymphocytes are often closely associated with large, unmyelinated axons (Fig. 5e), suggesting active demyelination. Lesions in the peroneal nerves and dorsal root ganglia are more focal, but follow the same pattern. In the peripheral nervous system (PNS), both sensory (dorsal root ganglion) and motor nerves contained lesions.

**Inter- and intramolecular determinant spread during spontaneous disease**

As a preliminary to analysis of the relationship between disease progression and epitope spread, we quantified ingress of T cells into the CNS during the preclinical phase of disease (Fig. 6). That is, we needed some estimate of the immunopathological events in the CNS that may lead to epitope spread before paralysis is detectable. Indeed, we found that T cells progressively accumulate in the CNS of line 7 mice throughout adult life. Eighteen-week mice were then separated by spontaneous disease score into groups with score 0, 1–2, or >3, and each group was analyzed for epitope spread; a representative experiment is shown in Fig. 7a. Three percent of CD4 SP cells do not express the transgenic TCR, and dual TCRα cells can further widen the range of Ags recognized. Mouse showing no overt signs of clinical disease showed epitope spread, both within MBP, to MBP 38–59, but also to an αB-crystallin epitope, 161–175. These mice responded to eight of the epitopes tested. The development of overt disease (score 1–2) was associated with more extensive epitope spread though the C terminus of MBP, again also including MBP 38–59 and αB-crystallin. These mice responded to eight of the epitopes tested. Among the MBP epitopes specifically recognized in the context of disease were MBP 101–120 and MBP 142–161, echoing findings in terms of spread responses following actively induced disease (20). Mice with a disease score >3 were in generally poor health with small, lymphopenic spleens. They showed a refocused T cell response, recognizing four of the tested epitopes. At this stage in the disease,
histopathology shows very advanced degeneration, and CNS presentation of myelin peptides may be limited. In addition to MBP 38–59 and β-crystallin 161–175, identified in the studies above as HLA-DR15-presented disease-associated epitopes, preliminary studies identified an additional spread epitope, MOG 82–96. These three epitopes were used for more detailed analysis in groups of mice at various stages of disease. A clear and reproducible pattern of progressive spread was identified (Fig. 7b). The response initially spreads to MBP 38–59, and then, correlating with the period during which more T cells infiltrate the CNS and mice progress to full-blown paralysis, to MOG 82–96, and to β-crystallin 161–175. This model thus echoes the type of hierarchy of spread seen in EAE, except that it is in this study demonstrated in spontaneous disease for HLA-DR-restricted self epitopes with direct relevance to MS. Although the data shown in Fig. 7b clearly demonstrate a hierarchy of spread for these epitopes correlating with age, there is...
no clear distinction by disease score. However, clinical hind-limb paralysis is an extreme measure of CNS damage, and it should not surprise us that, with greater than 10^5 myelin-reactive Th1 cells in the brain, it is possible to get local tissue breakdown and priming of responses to spread epitopes before the appearance of full-blown paralysis. Responses to these spread epitopes are inhibited by Ab to HLA-DR and not class I (Fig. 8). T cell responses to epitopes in the region of MBP 38–59 and MOG 82–96 have previously been described in the responses of HLA-DR15 MS patients (15, 24). It is likely that the responses to spread epitopes are due to peptide recognition by dual TCR^+/H9251 CD4^+/H11001 T cells, as T cell lines selected against the spread epitopes show altered TCR V^+/H11001 usage compared with the starting population (data not shown). Long-term T cell lines cultured from diseased line 7 mice against MOG 82–96 transfer disease to naive HLA-DR15 recipients, indicating that the responses to spread epitopes can contribute to pathogenesis (Table I). When passive disease was induced in HLA-DR15 recipients by transfer of anti-MBP-85–99 T cell lines, the pattern of spread observed for spontaneous disease was reiterated, with the development of responses to the MOG and ^+/H11001 B-crystallin spread epitopes (data not shown).

Responses of CNS-infiltrating T cells

We then purified lymphocytic infiltrates from the brain and spinal cord of paralyzed mice for more detailed analysis (Fig. 9). The majority of cells were TCR^+/CD4^+, but there were also TCR^+/CD8^+ cells, as well as a population of CD4^−/8^+ TCR^+ cells, not detected in the peripheral population (Fig. 9a). An extrathymic CD4^+8^+ double-positive population has been described as having a highly activated phenotype in other models (25). CNS-derived T cells proliferate in response to MBP 80–99, MBP 38–59, and ^+/H11001 B-crystallin 161–175 peptide.
When these proliferative responses are compared with matched cultures containing the same number of splenic T cells, the responses of CNS cells are reduced and the response to MOG 82–96, which can be detected in splenocytes, is not readily detectable in the infiltrating cells. However, when cytokine production of CNS-infiltrating cells and spleen cells was compared, CNS T cells made a more potent IFN-γ response, particularly to MBP 80–99 and to a lesser extent to MBP 38–59 and αB-crystallin 161–175.

Discussion

EAE studies have yielded substantial progress in understanding the pathogenesis of MS; yet, of many treatments developed for EAE, few have had major impact on clinical practice. If therapies based on a single antagonist peptide are to be applied (10), it is vital to understand whether the response involves epitope spread, and, if so, whether the antagonist treatment inhibits the spread response (3). This new model encompasses pathologically relevant, spontaneous disease with the presentation of myelin epitopes in the context of an immune system, in which the only class II molecule

### Table 1. Long-term T cell lines against both MBP 85–99 and the spread epitope, MOG 82–96, can transfer disease to HLA-DR15 transgenic recipients

<table>
<thead>
<tr>
<th>T Cell Line Cells</th>
<th>Number Affected</th>
<th>Disease Scores (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBP 85–99</td>
<td>3/4</td>
<td>5, 4, 4, 0 (3.25)</td>
</tr>
<tr>
<td>MOG 82–96</td>
<td>3/4</td>
<td>1, 1, 1, 0 (0.75)</td>
</tr>
</tbody>
</table>
for Ag presentation is the MS-associated HLA-DR15 heterodimer. The localization of disease lesions may be to some extent dictated by specificity of the TCR transgene (26), and thus, the approach is helpful for delineating the in vivo pathogenicity of human T cell clones. However, severity of the phenotype may be variable: we described a sister line, expressing the same human TCR, albeit at a lower level and limited to the CD4 lineage, showing a mild phenotype with rare paralysis, but general poverty of movement (20). As might be expected, the pattern of epitope spread in the context of milder neuropathology was different from the pattern seen in the line 7 mice described in this work. Thus, with differential expression of the same TCR, we described different disease severity, albeit with similar CNS localization and different patterns of associated epitope spread. The latter point is not surprising, because one would expect the pattern of epitope spread to depend largely on the extent of tissue damage rather than the initiating TCR sequence.

Epitope spread in this model was very reproducibly found to MBP 38–59, MOG 82–96, and αB-crystallin 161–175. Some of these responses correlated with age rather than paralysis score (presumably though correlating with the accumulation of subclinical tissue damage), while responses to some epitopes such as MBP 101–120 and 142–161 were more specifically correlated with paralysis score. The response is hierarchical and progressive: as mice age and become sicker, responses to more epitopes are found. The MOG 82–96 and MBP 38–59 spread responses echo the finding that these are key HLA-DR15-restricted epitopes in MS (14, 15, 24, 27). However, from the studies with human PBL, it is uncertain how and whether the responses are associated with pathogenesis. What our mouse/human hybrid model adds to this is the answer that responses to these epitopes are indeed generated as part of epitope spread, activated through pathogenic CNS tissue damage. Of these epitopes, the αB-crystallin epitope is conserved between human and mouse, as is the 9-aa core of MOG 82–96 and the MBP epitope, with the exception of a substitution from glycine to serine at residue 46. The αB-crystallin 161–175 epitope was not identified in a small study of T cell responses by HLA-DR15 MS patients, although we have preliminary data that patient T cell lines can indeed be cultured in response to this epitope (28) (D. Price and R. Hewitt, unpublished observation). Responses to αB-crystallin are believed to reflect its appearance within perivascular macrophages in MS lesions (29). In rodent models, tolerance is more robust and T cell responses are often poor (30). From a danger hypothesis perspective, circumvention of tolerance, whether in patients or transgenics, will depend on αB-crystallin expression in an inflammatory context. We have previously shown that in the case of another stress protein, heat shock protein 60, autoreactivity can develop in the face of strong thymic expression that would normally be associated with central tolerance (31).

This model offers new and unique features in comparison with previously published human TCR transgenics for MS research (19). In an earlier example, mice were described that expressed the same TCR, had a low frequency of the transgenic TCR-positive cells, and could be actively induced to develop EAE after an aggressive regime of high dose peptide with pertussis toxin. The new model has little overlap with the earlier work, describing a highly penetrant, spontaneous disease phenotype in mice, even when not crossed to RAG knockouts. This makes this a unique and valuable humanized model for the analysis of mechanisms and the testing of therapeutics in a more stringent and relevant model than EAE induction. For example, as commented upon the subject of promising drug trials in EAE models, “none of these three mouse models of EAE develops spontaneously; instead, they are induced by

FIGURE 9. Responses by CNS-infiltrating T cells. a, Brain- and spinal cord-infiltrating cells from three mice, score 3–4, were purified and analyzed by flow cytometry. The lower FACS plot indicates TCR+ cells within the marked gate of CNS-infiltrating CD4+CD8+ cells. b, T cell proliferation, IFN-γ release, and IL-4 release in response to spread epitopes in pooled cells from spleen (■) or CNS (□) of mice (three per group) with a disease score of 2–4.
aggressive immunization protocols. So, it is hard to use the results of testing potential drugs in these models to predict what will happen in human multiple sclerosis. (32). Our new model for the first time fills this niche, and in the context of a humanized class II; so, observations with respect to T cell specificities are closely applicable to human patient studies. Furthermore, the evidence that there is a hierarchy of HLA-DR15-presented intermolecular epitope spread encompassing MBP, MOG, and αβ-crystallin, relating to age and disease progression, is novel in the field and has not been touched upon by other studies. This allows prediction of HLA-DR15-presented, patient myelin epitopes. This is a unique and valuable feature of the new model: for the first time, one can predict epitopes relevant to and testable in human MS patients, but with the knowledge that the epitopes have the credentials of being identified through CNS damage in an immunogenetically humanized, severe, MS-like in vivo model. That is, they are HLA-restricted myelin epitopes selected by autoimmune CNS damage, not by the peptide panel synthesizer, as is necessarily the case in most studies. Although more work will have to be done to delineate the contribution of these HLA-DR15-restricted epitopes to disease, they offer a bridge between analysis of the in vitro responses of humans and paralysis in mice.

The spread epitopes are most likely recognized through dual TCRα expression, endogenous murine α-chains paired with the human β-chain. Spread in the context of paralysis is associated with presence in CNS infiltrates of T cells expressing various murine Vα sequences, including a nested murine Vα17 receptor (A. Al Anizi, S. Ellmerich, and D. M. Altmann, manuscript in preparation). These responses, depending on dual TCRα cells expressing mouse/human hybrid receptors, clearly have meaning in their ability to mimic the normal repertoire in view of the similarity of the epitopes singled out by this disease process with those identified in vitro in patients’ cells. Although this in vivo model moves us no nearer to reconciling the conflicting views from the mouse models as to whether epitope spread is a critical step for EAE progression or indeed in MS (4–7), it offers an important platform, linking the mouse disease models with the human epitopes so that the pathological context of the specific human responses may be better understood. Armed with a flow chart of the hierarchy of spread through disease progression in an HLA-DR15 mouse model, one can design experiments to test whether a similar sequence is followed in the development of disease in HLA-DR15 patients. Evidence for determinant spread in the pathogenesis of this model derives partly from the responses to spread epitopes by CNS-infiltrating T cells. The proliferative response of CNS T cells is lower than that of peripheral cells, yet the IFN-γ response is much greater. CNS IFN-γ release has previously been highlighted as a marker of disease severity (33). Another possibility is that cytotoxic killing has a role in this model (34), although this will require further study. A key difference between these mice and the less severely affected line 8 mice we have previously described is that the TCR is expressed in this work both by CD4 and CD8 cells, both able to lyse MBP 85–99-pulsed targets.

This model is pertinent to analysis of T cell determinant spread to HLA-DR15-restricted epitopes, and also shows a relevant spectrum of histopathological damage, including progression from an inflammatory demyelination phase to a phase of neurodegeneration. Despite being driven initially by a response to MBP, disease progresses to incorporate demyelination in the PNS, reiterating a pattern observed in MS and EAE (35, 36). In summary, we have presented a new MS model, which, encompassing spontaneous paralysis and determinant spread to a series of HLA-DR15-restricted epitopes, will serve well, both for the prediction of epitopes involved in patient responses and for investigation of immunotherapies.

Acknowledgments

We thank Drs. D. Douek and N. Karandikar for use of their myelin peptide panels.

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