Endogenous Myelin Basic Protein Inactivates the High Avidity T Cell Repertoire

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Summary

To study the contribution of endogenous myelin basic protein (MBP) to the positive and/or negative selection of the MBP-specific T cell repertoire, we studied the T cell response to MBP in MBP-deficient shiverer and MBP-expressing congenic C3H mice. Immunization with MBP induced a vigorous T cell response in shiverer mice directed against a single I-A<sup>k</sup>-restricted immunodominant determinant, the core of which is peptide MBP:79-87 (DENPV-VHFF). Injection of this peptide induced a high avidity T cell repertoire in shiverer mice that primarily consisted of clones capable of recognizing the native MBP protein in addition to the peptide itself. These data show that endogenous MBP is not required for the positive selection of an MBP-specific T cell repertoire. C3H mice, in contrast, were selectively unresponsive to the MBP protein and injection of MBP:79-87 peptide induced a low avidity repertoire that could be stimulated only by the peptide, not by the protein. Therefore, endogenous MBP induced profound inactivation of high avidity clones specific for the immunodominant determinant making that determinant appear cryptic.

Key words: cryptic determinant • T cell tolerance • MBP-specific repertoire • T cell affinity • determinant hierarchy

There have been three mechanisms implicated in the development of self tolerance: clonal deletion, anergy, and ignorance. At present, it is unclear which of these mechanisms underlies the naturally developing self tolerance to myelin basic protein (MBP)

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, an abundant central nervous system protein and the best-studied autoantigen. For decades it was thought that MBP is sequestered behind the blood–brain barrier, but more recently MBP transcripts have been detected in the thymus and in other lymphoid organs (1–3), and evidence that MBP is presented in the thymus has emerged (4). However, the extent to which endogenous MBP contributes to the positive and/or negative selection of the MBP-reactive T cell repertoire remained unclear. Studies using three independently generated TCR transgenic mice showed that MBP:Ac1-11-specific T cells are positively selected in the thymi of MBP-expressing H-2<sup>k</sup> mice and, in the absence of cross-reactive environmental stimulation, persist as naive T cells in their secondary lymphoid tissues (5–7). Although they do not yield conclusions about the role of endogenous MBP in positive selection, these results seemed to imply that the dominant determinant of MBP does not cause clonal deletion in H-2<sup>k</sup> mice and that ignorance is the primary tolerance mechanism to MBP. Only when activated by immunization or environmental antigens would these MBP-reactive T cells induce the inflammatory central nervous system (CNS) disease, experimental allergic encephalomyelitis (EAE), an animal model of human multiple sclerosis (8). In support of this data, MBP-reactive T cells are present in unmanipulated, healthy mice, but these T cells do not cause EAE without immunization with MBP and treatment with pertussis toxin to open up the blood brain barrier (9). Do such T cells in MBP-expressing hosts represent the full MBP-reactive repertoire or are they low avidity remnants that slipped through the negative selection process? How fully do the existing TCR-transgenic models reflect the mechanisms of self tolerance to MBP?

We studied the contribution of endogenous MBP to positive and negative selection by comparing the MBP-specific T cell repertoires in MBP-deficient Shiverer mice (H-2<sup>b</sup>) and in the congenic, MBP-expressing C3H strain. Shiverer mice have a deletion in the last five exons of the MBP gene; the first two MBP exons are present but their transcripts are unstable (10, 11). We report here, first, that, shiverer mice can generate a vigorous high avidity T cell response to MBP and its immunodominant determinant suggesting that endogenous MBP is not required for the positive selection of MBP-reactive T cells. Second, we re-

1 Abbreviations used in this paper: CNS, central nervous system; EAE, experimental allergic encephalomyelitis; HEL, hen egg white lysozyme; MBP, myelin basic protein; gpM BP, guinea pig MBP; mMBP, mouse MBP.
port that the MBP-expressing C3H mice are selectively unresponsive to this protein and its immunodominant peptide, but retain low avidity MBP-reactive T cells. Therefore, endogenous MBP induces profound clonal inactivation of the high avidity MBP-reactive T cell repertoire.

When H-2<sup>2</sup> mice (which express MBP) are immunized with MBP, Ac1-11 is the peptide that elicits the strongest immune response and, hence, has been defined as the immunodominant determinant for this haplotype (12, 13). When the inflammatory CNS lesions develop, additional MBP peptides are targeted by T cells; these include the peptide 121-141, which has been designated as cryptic (14, 15). This assignment of determinant hierarchy has defined the immunotherapeutic approach to the EAE model. It assumes that determinants hierarchy within the T cell response to MBP follows the rules of T cell responses to foreign antigens, where the most abundantly presented peptide is likely to be the immunodominant one (16, 17). However, if endogenous MBP induces tolerance, its dominant determinant(s) might be the one that inactivates T cells most efficiently, as was observed after tolerogenic injection of hen egg lysozyme (18) and the transgenic expression of that protein (19). If the MBP-specific T cell repertoire were shaped in a similar fashion by endogenous MBP, one might expect the determinant that is immunodominant in the MBP-deficient shiverer mouse to be a cryptic determinant in the MBP-expressing congenic strain. Here we report evidence for this apparent reversal of determinant hierarchy.

Materials and Methods

Mice, Antigens, and Immunizations. Shiverer C3H/eB/FeJ-Mbp<sup>III</sup> mice, congenic C3H/eB/FeJ mice, and B10.A(2R) and B10.A(4R) mice were purchased from The Jackson Laboratories (Bar Harbor, ME) and bred at CWRU facilities under specific pathogen-free conditions. Hen egg lysozyme (HEL) and ovalbumin (OVA) were purchased from Sigma Chemical Co. (St. Louis, MO). Guinea pig MBP (gpMBP) and mouse MBP (mMBP) were prepared as described (20). MBP:79-87 peptide (DENPVHFF) was purchased from Princeton Biomolecules (Columbus, OH). IFA was purchased from GIBCO BRL (Gaithersburg, MD) at 2 mg/ml to IFA. Individually, MBP:79-87 peptide (DENPVHFF) was purchased from Princeton Biomolecules (Columbus, OH). IFA was purchased from GIBCO BRL (Gaithersburg, MD) at 2 mg/ml to IFA. Individually, antigens or peptides were mixed with the adjuvant to yield CFA and C3H mice were generated using a standard protocol (21). In brief, 9 d after immunization with 50 µg of MBP peptide 79-87 in CFA, subcutaneously, draining LN cells were cultured in vitro with 50 µg/ml MBP:79-87 as specified for proliferation assays. After 78 h, 10 ng/ml of recombinant human IL-2 (a gift from Sandoz Ltd., Basel, Switzerland) was added overnight and the cells were fused with BW1100 T lymphoma cells (provided by Dr. C. Harding, Case Western Reserve University, Cleveland, OH). The cells were plated under limiting dilution conditions. The resulting hybrids were tested for MBP:79-87 specificity in CTLL assays and positive hybridomas were cloned under conditions of limiting dilution. Restriction of the hybridomas was mapped using recombinant mouse strains and the I-A<sup>g</sup>-transfected cell line C3F6 (22), the H-2 allele expression of which is shown in Fig. 1B.

Results

Mice Respond to MBP, but MBP<sup>+/+</sup> Mice Do Not. We immunized C3H (MBP<sup>+/+</sup>) and congenic shiverer (MBP<sup>-/-</sup>) mice with two control "foreign" antigens,
OVA and HEL, and tested the antigen-specific recall response in draining LN. We performed cytokine ELISA spot assays to measure the frequency of antigen-specific T cells in freshly primed cell populations. The magnitude of the IFN-γ recall response to these control antigens was comparable in C3H and shiverer mice (Table 1). The immunizations with OVA and HEL also induced similar frequencies of memory cells that produced IL-2 but did not induce detectable production of IL-4 or IL-5 (data not shown). These data show that C3H and shiverer mice mount type-1 responses of comparable magnitudes to two prototypic “foreign antigens”; despite their neurologic defect, shiverer mice are fully immunocompetent.

When immunized with either mM BP or gpMBP (the two proteins differ at 15 residues scattered over the 168-amino acid sequence), shiverer mice developed a vigorous response that could be recalled with either protein (Table 1). As with the aforementioned control antigens, IL-2 was detected in addition to IFN-γ, a result confirmed in proliferation assays, whereas neither IL-4 or IL-5 was detected (data not shown). In two experiments performed using Shiverer mice (six mice per group), the frequency of the M BP-specific IFN-γ-producing memory cells was three to eight times higher in M BP-immunized shiverer mice than the frequency of memory T cells specific for HEL or OVA in mice immunized with the respective antigen (Table 1). Because C3H mice are classic high responders for HEL and OVA, these data show that mice of the C3H (H-2k) background are high-responders to MBP when MBP is a “foreign” antigen.

Unlike the case in shiverer mice, mM BP did not induce an IFN-γ recall response in C3H mice (Table 1), nor was M BP-specific proliferation or the production of IL-2, IL-4, or IL-5 detected in immunized mice (data not shown). Immunization with gpMBP resulted in a response that was not recalled with mM BP (Table 1). Despite the extensive sequence similarities between the two proteins, the anti-gpMBP response did not cross-react with mM BP but seemed to be directed against the species-foreign sequences of the protein. In light of the fact that MBP−/− mice respond strongly to mM BP, the complete absence of spontaneous mM BP-reactivity in the unimmunized MBP−/+ mice suggests that endogenous MBP did not prime a type-2 immune response, that is, the spontaneous development of tolerance to MBP did not involve immune deviation. Instead, the failure of C3H mice to respond to mM BP even after immunization with gpMBP suggests that their endogenous MBP had inactivated the majority of MBP-reactive precursor T cells.

IFN-γ responses to MBP (Table 1) were not detected in C3H or shiverer mice immunized with control antigens, nor was production of IL-2, IL-4, or IL-5 or a proliferative response seen (data not shown). Exposure to potentially cross-reactive environmental antigens did not, therefore, result in significant spontaneous priming of MBP-reactive T cells.

MBP Peptide 79–87 Is the Core Immunodominant Determinant in Shiverer Mice. We immunized shiverer mice with mM BP and mapped the fine specificity of the response using an overlapping nonamer peptide series. Fig. 1 A shows that, from the entire series, only three peptides induced a recall-response, 78–86, 79–87, and 80–88, with the response to peptide 79–87 by far the strongest. No reactions were detected to other peptides, including peptide Ac1-11, which has been defined as an I-Ak–restricted determinant (23). This result identifies peptide 79–87 on the MBP molecule as the core immunodominant determinant in shiverer mice. Further characterization showed that this MBP:79–87-specific recall response could be blocked with anti-CD4 mAb (data not shown) and that this peptide is I-Ak–restricted (Fig. 1 B).

Immunization of C3H mice with mM BP (or with gpMBP) did not result in a recall response to any of the MBP peptides (Fig. 1 A); therefore, the MBP:79–87 peptide that was immunodominant in the MBP−/− mouse was non-stimulatory in the congenic, MBP+/+ strain after the injection of the MBP protein.

Table 1. Immunogenicity of MBP in Shiverer vs. C3H Mice

| Immunization* | Antigen-Induced† IFN-γ spots‡ |
|---------------|-----------------------------|
| Mice          | mM BP | gpMBP | OVA | HEL |
| Shiverer      | <3    | 81 ± 7| 181 ± 40 | <3    | <3    |
| (MBP−/−)      | <3    | 83 ± 20| 227 ± 95| <3    | <3    |
| OVA           | <3    | <3    | <3    | 31 ± 13| <3    |
| HEL           | <3    | <3    | <3    | 12 ± 1| <3    |
| C3H           | <3    | <3    | <3    | <3    | <3    |
| (MBP+/+)      | <3    | 23 ± 5| <3    | <3    | <3    |
| OVA           | <3    | <3    | <3    | 21 ± 4| <3    |
| HEL           | <3    | <3    | <3    | 11 ± 4| <3    |

*50 μg/mouse in CFA, subcutaneously.
†50 μg/ml
‡Number of spots/million draining lymph node cells tested on day 9 by IFN-γ ELISA spot (mean ± SE for three mice per group tested in two experiments). SD for triplicate wells for individual mice was <10%.

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In C3H mice, on the contrary, the 50 µg immunization dose primed 19 IFN-γ-producing cells per million LN cells when tested at the same 10⁻² M MBP:79-87 recall concentration, the highest that was not toxic to the cells. No cells producing IL-4 or IL-5 were detected. The dose–response curve in C3H mice did not approximate a plateau and, compared with that of shiverer mice, was shifted 4 Log units to the right. Immunizations with the 0.5 µg M BP:79-87 peptide dose still primed a vigorous response in shiverer mice but failed to induce a significant response in the C3H strain; the 5 µg immunizing dose gave intermediate results.
Conclusions about the functional avidity of the MBP:79-87 peptide-specific repertoires in shiverer and C3H mice can be drawn from the data in Fig. 2 A (along with the peptide dose required for immunization). T cell activation (including the induction of cytokine production) occurs when a critical number of MHC-peptide ligands is available for the TCR binding (25), a number that is dependent on the TCR's intrinsic affinity for this MHC-peptide complex (26) and that can be modulated by variations in TCR and/or coreceptor densities (27). Following the classic definition of avidity for polyvalent ligand-receptor interactions, functional T cell avidity can be defined as the concentration of peptide that leads to half the maximal activation of T cells in a given population for a constant number of APCs. According to this definition, the functional avidity of the T cell response in shiverer mice was at least three orders of magnitude greater than that in C3H mice. MBP:79-87-reactive T cells yielded identical dose response curves when activated by APCs derived from C3H and shiverer mice (data not shown); this shows that the differences in the responses do not reflect differences in the APC compartment but in the avidity of the T cells themselves. These data strongly argue for the functional inactivation of the high avidity MBP:79-87-reactive T cell repertoire in the MBP-bearing mice.

Using a third independent approach to assess and compare the avidity of MBP-reactive T cells in shiverer and C3H mice, we studied the dose response characteristics of MBP:79-87-reactive T cell hybridomas generated from both mouse strains. Fusing T cells from mice immunized with 50 µg MBP:79-87, we obtained 346 hybrid clones from shiverer mice and seven hybrid clones from C3H mice (a number that reflects the frequency of peptide-reactive T cells detected in the freshly isolated draining LN, Fig. 2 A). All seven T cell hybridomas from C3H mice required concentrations of MBP:79-87 in excess of 10^{-5} M to be activated (Fig. 2 B). In contrast, the 25 shiverer mouse T cell hybridomas that we randomly selected for closer characterization fell into three categories. Nine of these clones were triggered by peptide concentrations as low as 10^{-10} M, as exemplified in Fig 2 B by clone 2G9.4. Seven clones responded only to peptide concentrations in excess of 10^{-6} M (exemplified in Fig. 2 B by clone 1B2.5). The third type of clone (9 of 25) showed intermediate results similar to those seen with the clone 3D8.5 shown in Fig. 2 B. According to Tonegawa's algorithm (26), which stipulates that the peptide concentrations at which T cell activation occurs is inversely proportional to the hybridoma's TCR affinity, the differences in the concentrations required to activate the clones reflect differences in the affinity of the clones, while the plateau, because of the limitations in this assay, reflects the maximum CTLL proliferation rather than the maximum production of IL-2. Importantly, the T cell hybrids from all three categories and from both mouse strains responded comparably to anti-CD3 stimulation. Because of the similarity of these responses to antibody-mediated TCR ligation, and because these cells were grown under the same conditions, in long-
term culture and in the absence of MBP, which could have affected their function, the differences in their MBP:79-87 response characteristics could not have resulted from differences in the functional states of the cells, but must reflect differences in TCR-affinities for this peptide. The fact that the repertoire of T cell hybridomas obtained from shiverer mice consists of clones with high, intermediate, and low avidities is consistent with the notion that this repertoire has not undergone negative selection. In contrast, the fact that only low avidity T cell hybrids were obtained from C3H mice points towards a repertoire that has been depleted of precursor cells that have high and intermediate avidity for MBP:79-87.

MBP:79-87 is a Cryptic Determinant in the MBP+/+ Mice. We tested freshly isolated MBP:79-87 peptide-primed T cells and the T cell hybridomas obtained after immunization with this peptide to see if they could be stimulated by the native MBP protein. In shiverer mice, the peptide-primed primary cells gave a vigorous recall response when cultured with MBP the magnitude of which was comparable to the response recalled by the peptide itself (Fig. 3 A). Similarly, 12 of the 25 T cell hybridomas obtained from these mice responded to the protein as well as the peptide, as exemplified in Fig. 3 B by clone 2G9.4. The remaining thirteen shiverer-derived clones were stimulated only by the peptide, as was the clone 3D8.5 in this figure. In C3H mice, the MBP:79-87 peptide-induced response was not recalled by mMBP when freshly isolated cells were tested (Fig. 3 A) and none of the seven hybridomas obtained from such immunization was stimulated by the native antigen (Fig. 3 B). Because the MBP:79-87-specific repertoire in shiverer mice consisted of clones that recognized type-A and type-B conformations (28) of the MBP:79-87 determinant, these data also suggest that the repertoire of these mice had not been negatively selected. (The type-B conformation is assumed only when the peptide directly binds to MHC molecules on the cell surface; type-A conformation is assumed after natural processing of protein.) In contrast, the MBP:79-87-reactive repertoire in C3H mice was heavily biased towards the recognition of the type B determinant.

Discussion

The extent to which homologous self peptides contribute to the positive selection of T cells has been controversial. Early experiments suggested that the selecting peptide for a given T cell might need to be related in sequence to the antigenic peptide (29-31). Recently, it was demonstrated that a CD4+ T cell repertoire selected in the thymus for a single peptide could react with an array of different peptides (32). Similar observations were made in mice whose thymic peptide diversity was extremely limited by the prevalence of the CLIP peptide (33), which also suggests that the peptide that triggers an immune response can be of a sequence unrelated to that of the peptide(s) that select(s) the clone. Our data strongly support the latter notion. The MBP-deficient shiverer mice responded vigorously to MBP (Table 1), showing that positive selection by endogenous MBP is not required to generate an MBP-specific repertoire. On the contrary, with all shiverer mice tested, the response to MBP involved a three- to eightfold greater clonal size than did the response to the "foreign" antigens HEL and OVA (Table 1) to both of which C3H mice are high responders. It would seem that, if endogenous lysozyme or albumin were to contribute to the shaping of responses specific for HEL and OVA, it would be by negative rather than positive selection of the preimmune repertoires.

Although antigens like HEL and MBP include several sequences that can bind a given class II allelic product, frequently only one of these potential determinants actually induces a response after immunization with the protein (16). This immunodominance of a single determinant is a poorly understood feature of T cell responses. It could be the result of unique aspects of antigen presentation, e.g., MHC-guided processing (34-36), or it could be the result of repertoire limitations imposed by the homologous autoantigen. For example, sequences shared by the foreign (e.g., HEL) and self (e.g., mouse lysozyme) antigens could contain several immunodominant determinants, but because these determinants would negatively select the repertoire, only a fraction of the potential determinants, those that are species-foreign, would be available to elicit a response. The fact that MBP:79-87-shiverer mice recognize a single determinant (Fig. 1 A) strongly argues against negative selection of the repertoire being a major defining factor for the single-determinant dominance of this response.

On the other hand, the fact that the immunodominant determinant in the MBP+/− mice behaved as a cryptic determinant in the MBP+/+ mice suggests that the repertoire selected by the endogenous antigens contributes to the defining of the determinant hierarchy. Unlike the situation with foreign antigens, where peptides with the highest affinity for MHC are likely to be the most immunogenic (17), with self antigens, such peptides are prone to induce tolerance most efficiently (18, 19), thus making them appear cryptic.

Our data suggest that the determinant that is actually immunodominant on MBP induces profound clonal inactivation in mice that express MBP. Although immunization with peptide MBP:79-87 triggered a response in C3H mice, the clones induced were of low avidity (Fig. 2) and recognized only the peptide (Fig. 3). Apparently, the vast majority of high avidity cells capable of recognizing the endogenous protein did encounter it before the immunization and the outcome was functional inactivation. This tolerance might have resulted from clonal deletion or from the induction of an anergic state, or both, possibly affecting T cells with different avidities for MBP differently. TCR-transgenic models using TCRs with different avidities for MBP might be required to clearly distinguish between these possibilities. Because MBP messenger RNA is expressed in the thymus (1-3) and because it has recently been demonstrated that there is sufficient antigen presentation of MBP in this organ to stimulate MBP-reactive T cell
MBP would need to be high. Of these T cells, the concentration of endogenous free peptide available for direct cell surface binding to APC, and, because of the apparent low avidity to be extracellular peptide available for direct cell surface binding to APC, the transgenic T cells are likely to be ignorant of the autoantigen as well.

MBP:79-87 qualifies as cryptic determinant in C3H mice because it does not recall a response after the immunization with native MBP (Fig. 1 A) but is immunogenic when injected as a peptide (Figs. 2 A and 3 A). Because all seven hybrids that we obtained from C3H mice recognized the MBP:79-87 peptide, but not the native protein, the MBP:79-87-reactive T cell repertoire in these mice seemed to be biased towards recognition of the type B determinant (28). It is unclear whether such T cells are capable of mediating autoimmune pathology. For type B determinants to constitute a target in the CNS there would have to be extracellular peptide available for direct cell surface binding to APC, and, because of the apparent low avidity of these T cells, the concentration of endogenous free peptide would need to be high.

We found C3H mice to be resistant to MBP-induced and MBP:79-87-induced EAE when using protocols that cause severe disease in EAE-susceptible mouse strains. Even grafting lymphoid cells from naive shiverer mice into C3H mice did not render C3H mice susceptible to the induction of active disease. Neither could we induce passive disease by injecting MBP:79-87–primed T cells from shiverer into C3H mice, nor with primary cells or T cell lines. Although this outcome suggests that the shiverer/C3H model might not qualify for studies of EAE, it sends a novel and surprising message about disease susceptibility. Whereas the primary focus of research addressing susceptibility to T cell–mediated autoimmune disease has been on the ability to generate a vigorous type-1 response to the autoantigen (MHC-linked Ir gene effects, T cell repertoires, type-1/type-2 biases), these data suggest that even the most vigorous, unipolarly type 1-biased T cell responses to MBP do not cause clinical disease if they occur in a host that lacks the genetic susceptibility to develop downstream inflammatory pathology. In such mice, in addition to central tolerance, powerful peripheral tolerance mechanisms seem to prevent T cell–mediated immunopathology of the central nervous system.

Because of the EAE-resistance of C3H mice, we could not address the issue of whether the low avidity T cells that escape negative selection can be pathogenic. Previous studies performed in the H-2u model might provide clues to this question showing that peptide, MBP:121-140, a cryptic determinant in MBP+/+ H-2u mice, is codominant with MBP:Ac1-11 in the congenic MBP−/− mice (43). These data can be interpreted as reflecting similar avidity-repertoire selections, as we do here. If this were the case, the fact that MBP:121-140 is encephalitogenic in H-2u mice (15) might be seen as evidence that negative selection for a dominant determinant is incomplete and can cause autoimmune pathology.

The inactivation of T cells that are specific for the dominant MBP determinant is likely to occur in EAE-susceptible mice too, but even if it were as profound in those strains as we observed it to be in C3H mice, for theoretical reasons it is unlikely to be complete. A central question in the understanding of autoimmune disease will be how auto-reactive T cells that escaped negative selection and have the autoantigen presented to them at a subthreshold level can be induced to mediate autoimmune pathology. Like T cell activation itself, negative selection is threshold-dependent (24), that is, T cells will be deleted only if their TCR binds a critical number of MHC–peptide ligands, a number defined by the affinity of the TCR for the peptide (26). The MBP-specific clones that escape negative selection will, therefore, fall into two broad categories: first, T cells with receptors having a low affinity for an abundantly presented peptide (T cell crypticity); second, T cells that have high, low and intermediate avidities for peptides that are presented in low copy numbers on the APC (determinant crypticity). Because the autoantigen is presented at subthreshold levels to both types of clones, they are ignorant of it. This activation threshold seems to be lowered after the naive cell is primed to a memory/effector state. Studies of TCR–transgenic cells showed that the activation of naive T cells requires peptide concentrations up to 50 times higher than those needed by primed/memory T cells (44). If the activation threshold of T cells, however, is not constant for an individual clone but varies as a function of its state of activation, environmental priming by cross-reactive antigens, or by immunization in adjuvant could make the number of endogenous MHC/peptide ligands that was substimulatory to the naive cell stimulatory to the memory cell. The autoantigen that was ignored by the naive cells will be attacked by the memory cells.

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