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Diverse Repertoire of the MHC Class II-Peptide Complexes Is Required for Presentation of Viral Superantigens

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Among other features, peptides affect MHC class II molecules, causing changes in the binding of bacterial superantigens (b-Sag). Whether peptides can alter binding of viral superantigens (v-Sag) to MHC class II was not known. Here we addressed the question of whether mutations limiting the diversity of peptides bound by the MHC class II molecules influenced the presentation of v-Sag and, subsequently, the life cycle of the mouse mammary tumor virus (MMTV). T cells reactive to v-Sag were found in mice lacking DM molecules as well as in A\textsuperscript{b Ep}-transgenic mice in which MHC class II binding grooves were predominantly occupied by an invariant chain fragment or E\textsubscript{a}, peptide, respectively. APCs from the mutant mice failed to present v-Sag, as determined by the lack of Sag-specific T cell activation, Sag-induced T cell deletion, and by the aborted MMTV infection. In contrast, mice that express I-A\textsuperscript{b} with a variety of bound peptides presented v-Sag and were susceptible to MMTV infection. Comparison of v-Sag and b-Sag presentation by the same mutant cells suggested that presentation of v-Sag had requirements similar to that for presentation of toxic shock syndrome toxin-1. Thus, MHC class II peptide repertoire is critical for recognition of v-Sag by the T cells and affects the outcome of infection with a retrovirus. The Journal of Immunology, 2001, 166: 2244–2250.

Diverse Repertoire of the MHC Class II-Peptide Complexes Is Required for Presentation of Viral Superantigens

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Peptides constitute an integral part of the MHC proteins without which these molecules aggregate and get degraded. The tertiary complex between peptide and \(\alpha\)- and \(\beta\)-chains of the MHC is seen by the TCR as a single entity. The nature of a peptide modifies the complexes in such a way that the overall conformation of the MHC molecule is changed. The changes in conformation could be estimated by determination of SDS stability and hydrophobicity (1–5) and by mAb binding (6–9).

MHC class II molecules are also critical for presentation of superantigens (Sag), which are protein products of bacterial (b-Sag) or viral (v-Sag) origin. Sag are able to activate large numbers of T cells bearing TCR \(\beta\)-chains that interact with Sag-MHC class II complexes. For exogenous mouse mammary tumor virus (MMTV) the Sag presentation is an absolutely critical step in its life cycle (10, 11). Cells of the immune system are the first targets of this virus (12). The infected B cells present v-Sag in the context of MHC class II molecules to T cells, leading to stimulation and consequent proliferation of specific V\(\beta\)-bearing T cells (13). These events result in viral amplification and transport to the mammary glands. Without recognition of v-Sag by cognate T cells, there is no T cell activation, no infection of mammary epithelium, and no viral transmission to the progeny via milk (10, 11). Sags that are present in the germline cause deletion of the Sag-reactive T cell subsets during formation of the immune repertoire (14). The MHC class II isotype and allotype matters for v-Sag presentation: I-E molecules are more efficient than I-A (14) and among I-A allotypes there is a hierarchy of v-Sag presentation (14, 15). As a result, mice lacking Sag-cognate T cells (10, 11) or animals with inappropriate MHC alleles (16) are either completely protected from, or are relatively resistant to, MMTV infection.

Whether v-Sag presentation by a given MHC molecule depends on the nature of a bound peptide was unknown. Targeted disruption of genes involved in MHC class II Ag presentation (DM, Refs. 17–19 and invariant chain (II), Ref. 20), as well as generation of mice expressing single MHC-peptide complexes (21), allowed the studies of the importance of the peptide repertoire limitation for T cell selection. Here, taking advantage of the array of animals with altered peptide presentation we address a specific question whether qualitative differences between MHC class II complexes affect presentation of v-Sag.

Materials and Methods

Mice and approved symbols

CB/A\(\ddagger\), CBA/Ca\(\ddagger\), C57BL/6\(\ddagger\) (B6), 129/\(\ddagger\) (B6 \(\times\) 129/\(\ddagger\)) \(F_{1}\), BALB/c\(\ddagger\), LP/J, D1.LP/J (D1.LP-H\(\beta\)\(2\) H2-T18\(\ddagger\)/Sn), and DM knockout (KO) (B6, 129\(\ddagger\)-H2-M\(\alpha\)\(\ddagger\)Mm1Luc\(\ddagger\); Ref. 18) mice were obtained from The Jackson Laboratory; Ii KO mice (20) were backcrossed to B6 mice for more than six generations at The Jackson Laboratory. To generate Mtv\(7\)-positive mutant mice, D1.LP/J (H-2\(b\), Mtv\(7\)\(\ddagger\)) females were crossed to Mtv\(7\)-negative DM KO (H-2\(b\), Mtv\(7\)\(\ddagger\)) males, and resulting F\(1\) females were backcrossed to DM KO males. N\(2\) generation animals were screened by Mtv\(7\)-specific PCR (22) and by FACS analysis to identify Mtv\(7\)+/+, Mtv\(7\)-/+, DM+/+ and DM\(-/-\) offspring. Mtv\(7\)-/+ Ii KO mice expressing single E\(\alpha\)E\(\delta\) peptide-MHC class II complexes (A\(\text{Ep}\)) (21) were provided by Dr. Philippa Marrack (National Jewish Center, Denver, CO). To generate Mtv\(7\)-/+ A\(\text{Ep}\)-transgenic mice, original Mtv\(7\)-/+ A\(\text{Ep}\)-transgenic females were crossed to MHC class II KO, Ii KO, Mtv\(7\)-/+ males, and resulting F\(1\) females (Mtv\(7\)-/+) were backcrossed to MHC class II KO, Ii KO, Mtv\(7\)-/+ males. N\(2\) generation animals were screened by PCR to identify Mtv\(7\)+/+ and
**Mtv7−/−** mice, and by FACS analysis to identify Aαp- transgenic and nontransgenic mice.

**Superantigens**

b-Sag were purchased from Toxin Technology (Sarasota, FL). A mixture of three exogenous MMTVs (BALB2, BALB/A, and BALB14; Ref. 23) was a gift from Dr. Isabele Piazzon (Instituto de Investigaciones Hematologicas, Buenos Aires, Argentina). These viruses were passed on BALB/J mice (called BALB/cLA throughout).

RNase T1 protection assays were performed as previously described (24), using BALB/A(Mtv7)-specific probe (22). RNA was isolated from milk of mice after the second pregnancy by the method of Chirgwin et al. (25), and 5 µg was used per assay. The same probe was used to test expression of Mtv7 in spleens. Forty micrograms of total RNA isolated from spleens was used for this analysis. X-ray film exposure was standard (12 h) in all experiments.

**T cell activation**

CD4+ T cells were purified from lymph nodes by treatment with mAbs against MHC class II (Y3JP) (26) and 25-9-17 (27), and anti-CD3 (53-6.72) (28) for 45 min at 4°C (105 cells/ml of culture supernatant) followed by negative selection with the mixture of magnetic beads (anti-mouse IgG, anti-mouse IgM, anti-rat IgG) from PerSeptive Biosystems (Framingham, MA) according to manufacturer’s protocol. Sag-presenting cells were 2000 rad irradiated, T cell-depleted spleen cells (for b-Sag), or nonfractionated splenocytes (for v-Sag). For T cell depletion a mixture of anti-CD8 and anti-CD4 (GK1.5) (29) mAbs was used, followed by treatment with anti-rat Ig magnetic beads.

No response to v-Sag could be elicited in DM KO mice upon MMTV infection. Four groups (1–4) of animals differing in Mtv7 and DM expression were analyzed for the presence of Vβ6+ cells among peripheral CD4+ T cells. Data shown as mean percentage ± SE. n, number of mice used per group. **Bottom**, Expression of endogenous Mtv7 by splenocytes from the four groups of analyzed mice detected by RNase T1 protection assay with the Mtv7-specific probe. B6, No response to v-Sag could be elicited in DM KO mice upon MTV infection. Four days after footpad injection of MTV-containing or MTV-free milk, cells derived from popliteal lymph nodes were double-stained with anti-CD4 PE and anti-Vβ6 FITC-labeled mAbs and analyzed by FACS.

**Results and Discussion**

**Lack of v-Sag presentation by DM KO cells**

In DM KO mice most of class II molecules are occupied by class II binding II peptide. This complex alters recognition of Aα by allogeneic T cells (17, 19) and affects T cell selection (31, 32). In the following experiments we tested whether APCs of DM KO are capable of presenting the endogenous and exogenous v-Sag. To do that we first crossed Mtv7−/− DM KO mice to Mtv7+/+ D1.LP/J (H-2b) mice and then backcrossed hybrid F1 females (Mtv7+−/−) to DM KO males. TCR Vβ profile was analyzed in the N2 generation of mice with or without Mtv7 and DM molecules. Deletion of T cells expressing cognate β-chains (Vβ6) was used as a read-out. Should v-Sag be presented normally, the cognate T cells should be deleted. As expected all DM-sufficient Mtv7+ N2 mice have deleted Vβ6+ T cells (Fig. 1A). In contrast, none of DM-negative Mtv7+ mice showed deletion of Sag cognate T cells, even though expression of this provirus could be easily detected in the spleens of these mice by Mtv7-specific RNase T1 protection assay (Fig. 1A).
We also tested the anti-v-Sag-specific response of DM KO mice to a mixture of exogenous MMTVs. Mice were injected with partially purified virus derived from BALB/cLA milk. BALB/cLA mice produce a complex of three viruses with Vβ2-, Vβ14- and Vβ6-specific Sag, respectively (22, 23). BALB/cLA virus encodes a Sag with the same Vβ specificity as Mtv7, capable of interacting with both I-E and I-A MHC class II molecules (22, 30). A significant increase in the percentage of CD4+ Vβ6+ T cells was detected in the regional lymph nodes of DM-sufficient mice injected into foot pads with virus-containing milk compared with control mice injected with MMTV-free milk (Fig. 1B). In contrast, no T cell-specific stimulation was observed in DM KO mice injected with virus-containing milk (Fig. 1B). These results indicated that APCs from DM KO mice failed to present exogenous and endogenous v-Sag.

T cells from DM KO mice are fully capable of recognizing a v-Sag

The lack of T cell deletion (Fig. 1A) or proliferation (Fig. 1B) in response to v-Sag could be explained by a lack of the proper thymic positive selection in DM KO mice. Thus, we tested the ability of T cells from DM KO mice to react to v-Sag presented by DM-sufficient APC. To determine whether CD4+ T cells from DM KO mice were able to recognize v-Sag, we tested their ability to increase the proportion of cells with appropriate TCR Vβ-chains in response to an endogenous v-Sag in vitro. Purified CD4+ T cells from DM KO and control B6 mice were cultured with irradiated splenocytes from Mtv7-positive D1.LP/J (H-2b) mice. T cells expressing several Vβs were expected to expand in reaction to Mtv7, including Vβ6+ T cells. After 3 days in culture the cells were stained with anti-Vβ mAbs. In two independent experiments, responding T cells have shown a increase in the percentage of Vβ6+ T cells after stimulation with Mtv7-presenting cells (Fig. 1C). At the same time the numbers of noncognate Vβ14+ cells were not increased. In contrast, activation of the same cells with Mtv7-negative splenocytes from LP/J mice did not cause any special increase in Vβ6+ T cell subsets. The relatively small increase in CD4+ Vβ6+ T cells from DM KO mice is most likely due to vigorous proliferation of T cells in response to MHC class II Aβ molecules. This is because Aβ-reactive cells are not deleted in DM KO mice (18, 31). Thus, DM KO mice have T cells that can respond to the endogenous v-Sag.

Lack of v-Sag presentation by Aβ-E-transgenic APC

Aβ-E-transgenic mice express a single Aβ-peptide complex (21). This complex is SDS stable and is recognized by Aβ-specific Ab YAe (33, 34). Furthermore, it is not recognized by an anti-Aβ mAb 25-9-17 (8), suggesting a special conformation of MHC class II complexes imposed by the peptide. We sought to determine whether APC from Aβ-E-transgenic mice are capable of presenting endogenous v-Sag. The Ii KO mice were used as controls for these experiments because Aβ-E-transgenic mice lacked Ii molecule (21). Both Aβ-E-transgenic mice and Ii KO mice have reduced MHC class II expression (20); however, Ii KO mice express a diverse peptide repertoire (34). Aβ-E-transgenic mice were found to inherit and express Mtv7 (data not shown) contributed by one of the embryonic stem cell donors during generation of these mice (21). To determine whether Aβ-E-transgenic mice delete CD4+ Vβ6+ T cells when Mtv7 is present we generated Aβ-E-transgenic mice without Mtv7 (see Materials and Methods). None of the Aβ-E-transgenic Mtv7+/+ mice have deleted their CD4+ Vβ6+ T cells even though expression of the provirus could be easily detected in the spleens (Fig. 2A) and in lymph nodes (data not shown) of these mice. Thus, like DM KO mice with limited MHC peptide repertoire, the Aβ-E-transgenic animals expressing the single MHC class II-peptide complex were unable to present endogenous v-Sag to the T cells.

T cells from Aβ-Ep mice are fully capable of recognizing a v-Sag

Next we sought to determine whether Aβ-Ep-transgenic mice were able to generate CD4+ T cells capable of v-Sag recognition. Purified CD4+ from Aβ-Ep-transgenic and control Ii KO mice were stimulated with Mtv7+/+ cells from D1.LP/J mice (Fig. 2B). CD4+ T cells from both Aβ-Ep-transgenic and Ii KO mice recognized v-Sag as determined by their ability to increase the percent of Vβ6+ T cells after stimulation in vitro (Fig. 2B). In contrast, activation with Mtv7-/- LP/J cells did not show any increase in Vβ6+ subsets (Fig. 2B). Although this increase was consistent in several experiments (p = 0.0015), it was very small. That relatively small increase was explained by a strong reactivity to Aβ molecules with various peptides (21), which was obviously involving TCRs with diverse Vβ-chain repertoire. To circumvent this problem we used a pair of allogeneic H-2a strains CBA/J and CBA/CaJ, which are positive and negative for Mtv7, respectively. We expected that the frequency of cells responding to MHC class II H-2b molecules would be lower and that we would be able to detect the anti-v-Sag response. In fact, in this case CD4+ T cells isolated from Aβ-Ep-transgenic mice responded to Mtv7 v-Sag by selective proliferation of the Vβ6+ cells, whereas proliferation to H-2b molecules was nonselective in terms of Vβ usage (Fig. 2C). It is unlikely that the Mtv7 v-Sag in Aβ-Ep mice elicits stronger response when complexed with the MHC class H-2d compared with MHC class II H-2b molecules because the response of control B6 T cells to the Mtv7 v-Sag presented by H-2b and H-2d MHC class II molecules did not differ (Figs. 1C and 2C). It could have been a problem for another v-Sag, but it is very unlikely for such a strong activator as Mtv7 v-Sag. Thus, CD4+ T cells in Aβ-Ep-transgenic mice are capable of reacting to v-Sag that is processed and presented by normal APC with a diverse MHC-peptide repertoire.

Exogenous MMTV infection depends on diversity of peptides presented by MHC class II

MMTV life cycle is dependent on the v-Sag T cell activation, and viruses that lack Sag sequences cannot propagate in vivo (35). Having established that peptides bound to MHC class II are important for presentation of exogenous MMTVs, we sought to determine whether exogenous MMTVs could infect MHC class II presentation mutants. Thus, we allowed DM KO and (B6 x 129/J)F1 control females to ingest infected milk from BALB/cLA females and later analyzed their own milk for the presence of MMTVs. All three viruses could be readily detected in all milk samples of (B6 x 129/J)F1 females but not in DM KO mice (Fig. 3A, data for the most abundant LA virus is shown).

We then applied the same strategy for infection of Ii KO and Aβ-Ep-transgenic mice. Ii KO and Aβ-Ep animals were infected with LA viruses by foster-nursing on BALB/cLA females. The control Ii KO mice became infected and produced virus into the milk (Fig. 3B). In contrast, there was no detectable virus in the milk of Aβ-Ep-transgenic mice exposed to MMTV (Fig. 3B). Thus, even though T cells from mutant mice respond to v-Sag in vitro (Figs. 1C and 2, B and C) they fail to do so in vivo due to a failure of Sag presentation leading to an aborted MMTV infection.

The failure to present v-Sag by mutant APC could not be attributed to the differences in the amount of MHC class II expressed on the cell surface. The total class II expression in the wild-type and DM KO mice is very similar (17–19). The level of MHC class II in Ii KO and Aβ-Ep-transgenic mice is below the levels of expression of MHC class II in the wild-type mice, but similar to each
other (21). In addition, the low total number of CD4$^+$ T cells in those mutants could not be at fault, because Ii KO mice have similar CD4$^+$ T cell numbers as DM KO mice (31). However, the viral life cycle is complete in Ii KO mice but not in DM KO mice (Fig. 3). Furthermore, CD4-negative mice that have very few functional non-CD8$^+$ T cells are susceptible to MMTV infection (36). Moreover, mice that had over 90% of v-Sag-responding T cells deleted due to transgenic expression of a v-Sag (10) were producing detectable amounts of MMTV after two or more pregnancies (37). Thus, even practically invisible populations of v-Sag-reactive T cells may promote MMTV infection. In our experiments, all MMTV-infected mice were analyzed after the second pregnancy, and none of the mutant mice became MMTV infected. Thus, qualitative differences between MHC class II complexes must be responsible for the observed differences in the v-Sag presentation.

**Variable presentation of b-Sag by MHC class II-positive cells from mutant mice**

The mode of v-Sag presentation by MHC class II molecules could be better understood if compared with the presentation of b-Sag by MHC class II-positive cells from wild-type and mutant mice. b-Sag vary in the manner they bind MHC class II molecules as demonstrated by analyses of Sag mutants and crystal structures of Sag-MHC complexes. For instance, staphylococcal enterotoxin (SE)A binds MHC class II at two sites (one on $\alpha$- and one on $\beta$-chain) (38, 39), and binding to His81$\beta$ involves a Zn$^{2+}$ ion. Another b-Sag, SEB also cross-links MHC class II molecules using Zn$^{2+}$ (40). In contrast, SEB has a single MHC binding site and does not use Zn$^{2+}$ (41–44). Toxic shock syndrome toxin-1 (TSST-1) can bind Zn$^{2+}$ but does not require it to elicit T cell responses and uses a single site for binding MHC (45, 46).

Such differences in binding properties of b-Sag would likely be detected in MHC class II presentation mutants. Therefore, we compared b-Sag-dependent activation of normal CD4$^+$ T cells from B6 mice by T cell-depleted normal and mutant splenocytes. We readily observed the differences in proliferation of responding CD4$^+$ T cells (Fig. 4). Some of our observations were in line with the previously published studies of b-Sag binding to MHC class II presentation mutants (31, 47). However, to evaluate quantitatively the ability of mutants to present Sag, all of the mutants had to be compared in the same experiment. Although the range of protein concentrations that elicit maximal T cell activation varies for different b-Sag (nanogram range for TSST-1 and SEA, and 10 $\mu$g for SEB), the hierarchy of presentation of different b-Sag by the mutants can be found. Results of the experiments shown in Fig. 4 revealed the rank of presentation of the three tested b-Sag by wild-type and mutant APCs. It became clear that each Sag has its own pattern (Fig. 5). For example, presentation of TSST-1 is weaker by Ii KO cells, but is much weaker by DM KO cells when compared with the wild-type cells. In contrast, SEA is presented
relatively well by DM KO cells, but poorly by AβEp-transgenic cells. Our data indicates that v-Sag presentation resembles (but is not identical to) the mode of presentation of TSST-1 than of other b-Sag tested, suggesting that these two Sag may favor similar MHC class II-peptide complexes for binding. Both SEA and TSST-1 have been previously shown to block the Mtv7 Sag-peptides binding to MHC class II in vitro. However, the concentrations of blocking b-Sag were 10^3 - 10^4 times higher than required for T cell activation (48). The similar mode of presentation such as of TSST-1 and v-Sag does not necessarily imply that the two types of Sag use exactly the same interaction sites on the MHC class II molecules (49).

How do MHC class II variants affect Sag presentation? The most probable mechanism accounting for the Sag-presenting properties is the change in affinity of interaction between MHC class II molecule and Sag. Such differences have been shown to influence T cell activation quite dramatically; even a slight increase in MHC-Sag interaction could compensate for a huge loss of TCR-Sag interaction (50).

b-Sag bind MHC on the cell surface and do not need any additional processing. Presentation of v-Sag has been more enigmatic since their discovery. MMTV Sag is believed to be a type II protein processed from a longer precursor (51, 52). Recent compelling biochemical evidence suggests that v-Sag expression on the plasma membrane is independent of class II expression and that the Sag-MHC complexes are formed on the cell surface (53, 54) even in solution without any accessory proteins (55). That is important, because different MHC-peptide complexes may have their own peculiarities of intracellular trafficking, which theoretically could affect the rate of v-Sag binding and presentation.

That MHC class II-bound peptides can influence the b-Sag presentation has been well documented (56–58), and MHC-binding residues within a peptide as well as the C terminus of the peptide have the most influence on b-Sag binding (58). Two of MHC class II peptide mutants change the binding properties of b-Sag in a way that is compatible with the data (56, 57). The data suggest that the b-Sag binding to MHC class II is determined by the same MHC binding determinants as used by the TCR for recognition of antigen.

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FIGURE 4. b-Sag are presented differently by MHC class II Ag presentation mutants. Purified CD4^+ T cells from B6 mice were stimulated in vitro by b-Sag presented by T cell-depleted splenocytes from B6 and mutant mice. [3H]Thymidine incorporation was measured 72 h after activation. Vertical axis: SI, calculated as follows: SI = (a - b)/(c - d), where a = counts in response to Sag, c = counts in response to Sag presented by MHC class II-negative splenocytes, and b and d = counts in the same respective cultures but without Sag. Horizontal axis: b-Sag concentration (TSST-1 and SEA, ng/ml; SEB, μg/ml).

FIGURE 5. Patterns of v-Sag and b-Sag presentation by MHC class II Ag presentation mutants. The responses to individual b-Sag and v-Sag were ranked relative to maximal responses to the same stimuli in the wildtype (WT, B6) animals. The presentation of v-Sag mostly (but not completely) resembles the mode of presentation of TSST-1. * No detectable T cell deletion and MMTV infection.
II presentation mutants used in our study have peptide repertoire limited to either a single peptide (AβEp) or to almost completely a single peptide (class II binding peptide in DM KO mice). It is likely that peptides influence Vα-Sag binding indirectly by inducing MHC class II conformations that either do or do not favor Vα-Sag binding. However, the direct involvement of peptide residues in Vα-Sag binding cannot be excluded. Either way, the presentation of Vα-Sag (as well as the outcome of the retroviral infection) appears to be sensitive to the nature of the MHC-peptide complexes.

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