Brief Definitive Report

Binding Sites for Bacterial and Endogenous Retroviral Superantigens Can Be Dissociated on Major Histocompatibility Complex Class II Molecules

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Summary

Bacterial and retroviral superantigens (SAGs) interact with major histocompatibility complex (MHC) class II molecules and stimulate T cells upon binding to the Vβ portion of the T cell receptor. Whereas both types of molecules exert similar effects on T cells, they have very different primary structures. Amino acids critical for the binding of bacterial toxins to class II molecules have been identified but little is known of the molecular interactions between class II and retroviral SAGs. To determine whether both types of superantigens interact with the same regions of MHC class II molecules, we have generated mutant HLA-DR molecules which have lost the capacity to bind three bacterial toxins (*Staphylococcus aureus* enterotoxin A [SEA], *S. aureus* enterotoxin B [SEB], and toxic shock syndrome toxin 1 [TSST-1]). Cells expressing these mutated class II molecules efficiently presented two retroviral SAGs (*Mtv-9* and *Mtv-7*) to T cells while they were unable to present the bacterial SAGs. These results demonstrate that the binding sites for both types of SAGs can be dissociated.

Superantigens (SAGs) bind to MHC class II molecules and stimulate a large proportion of T cells in a Vβ-restricted manner (1–3). Bacterial SAGs are soluble proteins that bind in an unprocessed form to class II molecules (4). The toxins produced by *Staphylococcus aureus* (*S. aureus* enterotoxin A [SEA]; *S. aureus* enterotoxin B [SEB], and toxic shock syndrome toxin 1 [TSST-1]) have been the most studied and are responsible for food poisoning and toxic shock syndrome (5). Although these SAGs have affinities in the nanomolar range for class II molecules (5), mutation of a single residue on the α or β chain of HLA-DR is sufficient to completely abrogate the binding of TSST-1 or SEA (6–8). In contrast, retroviral SAGs (known as minor lymphocyte stimulating antigens or Mls) encoded by the open reading frame (ORF) in the 3′ LTR of mouse mammary tumor viruses (MMTV) (9–12) are type II transmembrane glycoproteins that likely encounter class II inside the cell (13, 14). Moreover, the primary sequences of bacterial and retroviral SAGs have no significant homology (1, 3, 5). Little is known about the interactions between retroviral SAGs and MHC class II molecules, except that a hierarchy exists in the ability of the different alleles and isotypes of human and murine class II molecules to present these SAGs (3, 5, 15). It is interesting to note that this hierarchy is not the same for the presentation of bacterial SAGs (2, 5). These differences between the two types of SAGs have prompted us to investigate whether they interact with similar sites on MHC class II molecules. Extensive site-directed mutagenesis studies have shown that only a few residues on MHC class II molecules are critical for the binding of bacterial SAGs (6–8). We show that these mutations on MHC class II molecules abrogate the binding of SEA, SEB, or TSST-1 but do not impair the presentation of two retroviral SAGs (*Mtv-7* and *Mtv-9*) to T cells. These results demonstrate that the binding sites for these two types of SAGs on MHC class II molecules can be dissociated.

Materials and Methods

Cell Lines. The murine T cell hybridoma Kmls 13.11 expresses a TCR Vβ6 chain that allows recognition of *Mtv*-7 SAG but not of SEA and TSST-1 (16). 3DT52.5.8 (Vβ1$^+$ and Vβ8.1$^+$) is a CD4$^-$ murine T cell hybridoma that recognizes SEA and SEB but not *Mtv*-7 SAG (17). KTS-104.3 is a murine T cell hybridoma expressing the TCR Vβ15 chain and is responsive to TSST-1. Vβ5#11

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is an *Mtv-9* SAG-reactive murine T cell hybridoma expressing Vβ5. DAP-3 is an MHC class II negative murine fibroblastic cell line (18). The hybridomas Kmls13.11 and KTS-104.3 were grown in DME supplemented with 10% FCS (Gibco Laboratories, Grand Island, NY), 4 mM dextrose (Sigma Chemical Co., St. Louis, MO), 2 mM L-glutamine, essential and nonessential amino acids, 1 mM sodium pyruvate (all from Gibco Laboratories), 10 μM β-mercaptoethanol (Sigma Chemical Co.), and 20 μg/ml gentamycin (Gibco Laboratories). 3DT52.5.8 and Vβ5#11 were grown in RPMI 1640 (Gibco Laboratories) supplemented with 10% FCS, 2 mM L-glutamine, 10 μM β-mercaptoethanol, and 20 μg/ml gentamycin. DAP-3 cells were cultured in DME supplemented with 5% FCS, 2 mM L-glutamine, and 20 μg/ml gentamycin.

Mutagenesis and Transfections. Site-directed mutagenesis of the DR1 α and β chain cDNAs was performed as previously described using the PCR overlap extension technique (6). Mutated DR1 β chain cDNAs were subcloned in RSV.3 vector and mutated DR α chain cDNA was subcloned in RSV.5neo. The following mutations were introduced simultaneously in the α chain: K39A, V42A, and E46R (DRα 39.42.46). The mutated cDNAs generated by PCR were entirely sequenced. Stable transfections of wild-type or mutated MHC class II cDNAs were carried out using the calcium phosphate coprecipitation technique (19). Briefly, DAP-3 cells were cotransfected with 10 μg of wild-type or mutated MHC class II α and β chain cDNAs in the presence of 2 μg of pSV2neo carrying the neomycin resistance gene. Stable transfectants were selected in 1 mg/ml of G418 (Gibco Laboratories). Homogeneous populations of DAP-3 cells expressing comparable levels of MHC class II molecules were obtained by aseptic cell sorting on a FACStar® Plus (Becton Dickinson Immunocytometry Systems, Mountain View, CA). MMTV sag genes cloned into the eukaryotic expression vector pHβ-Apr1-neo (20), were stably transfected as described above. Transfected cells were selected in 1 mg/ml of G-418 or in 10 μg/ml of puromycin (Sigma Chemical Co.). Clones were picked after 2 wk of selection and the remaining cells were kept as populations.

For transient transfections, the *Mtv-7 sag*, *Mtv-9 sag*, and B7 cDNAs were subcloned into the R3CPy-II eukaryotic expression vector containing the polyoma origin of replication (21). Transient transfections were performed using DEAE-dextran as described (22). Briefly, 2 × 10^6 DR1, DRα 39.42.46, and DR1 81A cells were plated in 10-cm petri dishes 24 h before incubation in DME containing 0.4 mg/ml DEAE-dextran (Pharmacia, Uppsala, Sweden), 10% Nuserum (Collaborative Research Incorporated, Waltham, MA) and 20 μg of DNA for 4 h at 37°C. Cells were then incubated in PBS-10% DMSO for 2 min and washed two times with DME without serum. Cells were kept in culture for 2 d, stained for class II expression and used in functional assays. Parallel transfections using the B7 cDNA confirmed the reproducibility of this assay.

Cytofluorometric Analysis of DAP-3 Cells Transfected with HLA-DR Molecules. Cells were stained with L-243 or 50D6, a mouse anti-human MHC class II antibody which recognizes all DR alleles except DR7 and DRw53, followed by GAM-FITC. As negative control, the different transfectants were stained with the secondary antibody alone. MHC class II expression was analyzed by flow cytometry using a FACScan® (Becton Dickinson Immunocytometry Systems).

Northern Blot Analysis. RNA was isolated using RNAzol (Cinna/Biotecx Laboratories, Friendswood, TX). 20 μg of RNA were separated on a 1.2% agarose-formaldehyde gel (23), transferred to a nylon membrane (Amersham Corporation, Oakville, Ontario, Canada), and hybridized at 42°C in 50% formamide with *Mtv-7 sag* and histone H3 cDNA (24) probes at the same time. The blot was washed at 65°C for 30 min in 2× SSC and exposed on a phosphorimager screen (Molecular Dynamics, Inc., Sunnyvale, CA) and then on Kodak XAR-5 film. Ratios of MMTV *sag* to histone H3 mRNAs were calculated using the Imagequant software (Molecular Dynamics, Inc.).

T Cell Stimulation. Stimulation of T cell hybridomas with SEA, SEB, and TSST-1 (Toxin Technology Inc., Sarasota, FL) was carried out as follows: 75 × 10^3 T cells/well were added to 2 × 10^4 DAP-3 fibroblasts expressing wild-type or mutated HLA-DR1 molecules with various concentrations of bacterial toxins (0–10 μg/ml). Presentation of MMTV SAGs by mutated MHC class II molecules was performed as follows: DAP-3 cells expressing MHC class II molecules were stably or transiently transfected with MMTV *sag* gene and cocultured in the presence of 6 × 10^4 MMTV SAG-reactive murine T cell hybridomas at different stimulator/effector ratios (1:1, 1:3, and 1:10). All the cocultures were done in a final volume of 200 μl in 96-well flat bottom plates for 18 h at 37°C, 5% CO_2.

IL-2 Production Measurements. IL-2 production was determined by the ability of the coculture supernatants to support the proliferation of the IL-2–dependent cell line CTLL.2 using the hexosaminidase colorimetric assay (25). A calibration curve using recombinant human IL-2 (Cetus Corp., Berkeley, CA) was performed in parallel.

Results and Discussion

With the knowledge that bacterial and retroviral SAGs have different primary structures, we tested the possibility that their binding sites on MHC class II molecules are separable. For this purpose, we generated DR1 mutants that do not bind the bacterial toxins SEA, SEB, and TSST-1. As illustrated in Fig. 1 *A*, the substitution of histidine 81 in the DRβ chain by an alanine (DAP DR1 81A) abolishes presentation of SEA to the 3DT52.5.8 T cell hybridoma confirming previously published results (6, 7). Control cells expressing either wild-type DR1 or DR1 mutated at positions 39, 42, and 46 in the α chain (DAP DRα 39.42.46) efficiently stimulated 3DT52.5.8 in the presence of increasing concentrations of SEA (Fig. 1 *A*). Cells expressing DRα 39.42.46 could not present SEB (Fig. 1 *B*) or TSST-1 (Fig. 1 *C*) to T cells, whereas the control DR1 and DR1 81A transfectants efficiently presented both toxins. This inability to present SEB and TSST-1 is due to a lack of binding of these toxins to MHC class II molecules (not shown). Lysine 39 on DRα had already been shown to be critical for binding and presentation of TSST-1 (8). These results indicate that TSST-1 and SEB binding sites are in close proximity (Thibodeau, J., N. Labrecque, and R.-P. Sékaly, manuscript in preparation).

Cells expressing class II molecules that fail to present SEA, SEB, and TSST-1 were transfected with *Mtv-7 sag* and analyzed for their ability to stimulate T cells. The Kmls13.11 hybridoma responded efficiently to DR1 mtv7 cells but not to control DR1 mtv9 or DR1 cells (Fig. 2 *A*). Cells expressing DRα 39.42.46, which can not present SEB and TSST-1, efficiently presented the *Mtv-7* SAG to this hybridoma even at the lowest dilution of APCs. Moreover, DR1 81A cells which fail to bind or present SEA (Fig. 1 *A*), stimulated Kmls 13.11 cells when transfected with the *Mtv-7 sag*. Similar results were obtained using a Vβ 8.1^+ and another Vβ 6^+ *Mtv-7* SAG-reactive T cell hybridoma (not shown). Fluctuations in

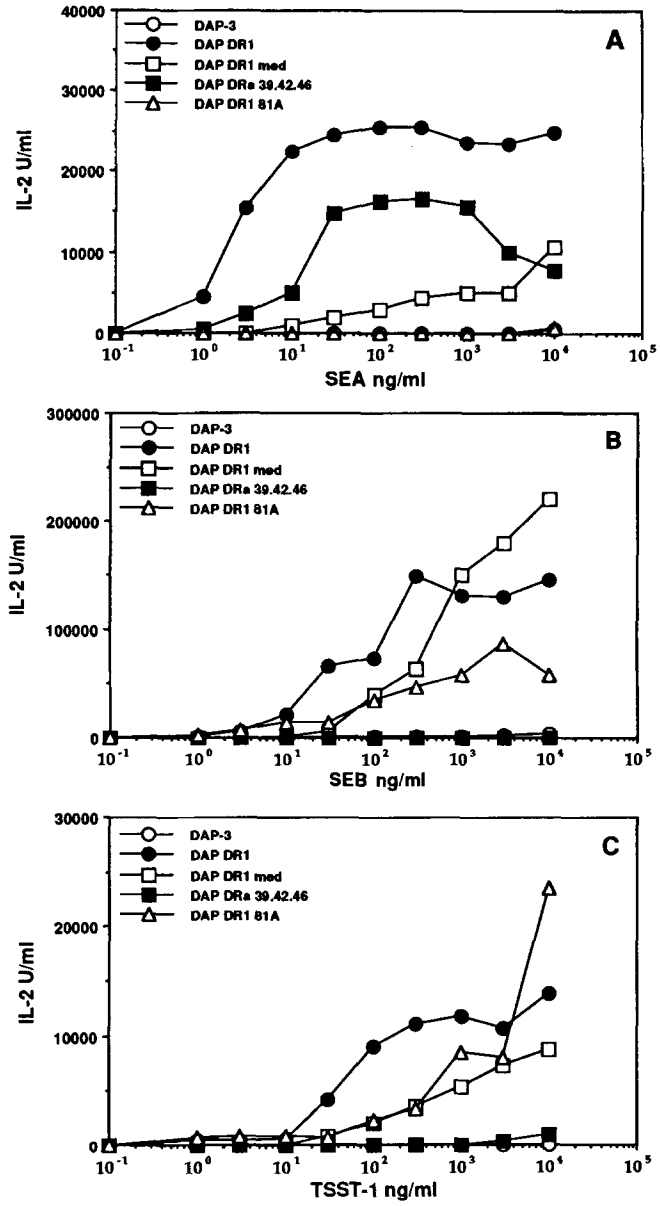

Figure 1. SEA, SEB, and TSST-1 presentation to T cells by HLA-DR mutants. (*A*) SEA presentation is abolished by mutation of residue β81 of HLA-DR1. Dose-response curve of SEA presented by DAP-3 cells expressing wild-type DR1 (DAP DR1 or DAP DR1 med which expresses lower levels of class II), DRα 39.42.46, and DR1 81A to the murine T cell hybridoma 3DT52.58. (*B*) Mutations at residues 39, 42, and 46 of HLA-DRα chain abrogate SEB presentation to T cells. Dose-response curve of SEB presented to 3DT52.5.8 by APCs expressing DR1 or DR1 med, DRα 39.42.46, and DR1 81A. (*C*) TSST-1 presentation is abolished by mutation of HLA DRα chain residues 39, 42, and 46. Dose-response curve of TSST-1 presented to the murine T cell hybridoma KTS-104.3 by untransfected DAP-3 cells, DAP DR1, DAP DR1 med, DAP DRα 39.42.46, and DAP DR1 81A. M.F.V. of class II expression using 50D6 mAb were DAP DR1, 125; DR1 med, 7; DAP DRα 39.42.46, 74; and DAP DR1 81A, 73.

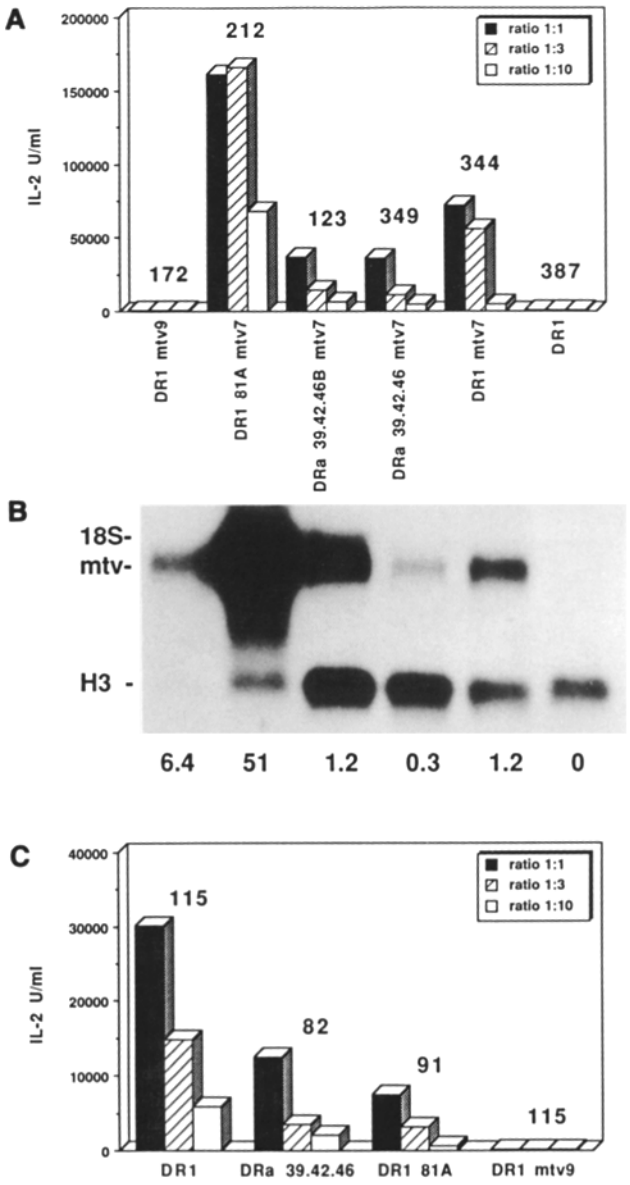

Figure 2. Presentation of *Mtv-7* SAG by mutant MHC class II molecules. (*A*) DAP-3 cells expressing wild-type or mutated class II molecules were stably transfected with *Mtv-7 sag* and used as APCs at different stimulator/effector ratios (1:1, 1:3, 1:10) for the stimulation of Kmls13.11 T cell hybridoma. Populations of cells were used except for DRα 39.42.46B and DR1 81A which have been cloned from the *Mtv-7* transfected population. Untransfected DR1 cells or transfected with *Mtv-9 sag* were used as negative controls. M.F.V. of class II expression as determined using 50D6 mAb is indicated on top of each histogram. (*B*) Northern blot showing the levels of *Mtv-7 sag* and histone H3 mRNA in the different transfectants. The ratio of *Mtv-7 sag* to histone H3 mRNA is indicated below each lane. (*C*) Stimulation of Kmls13.11 using DAP DR1 mutants transiently transfected with *Mtv-7 sag*. DR1 cells transfected with *Mtv-9 sag* were used as negative control in the stimulation assays. Parallel transfections using the B7 cDNA showed that 40–50% of the cells expressed B7 with a mean fluorescence value (M.F.V.) of 300. M.F.V. of class II expression on the different transfectants was determined using the 50D6 mAb and is indicated on top of each histogram.

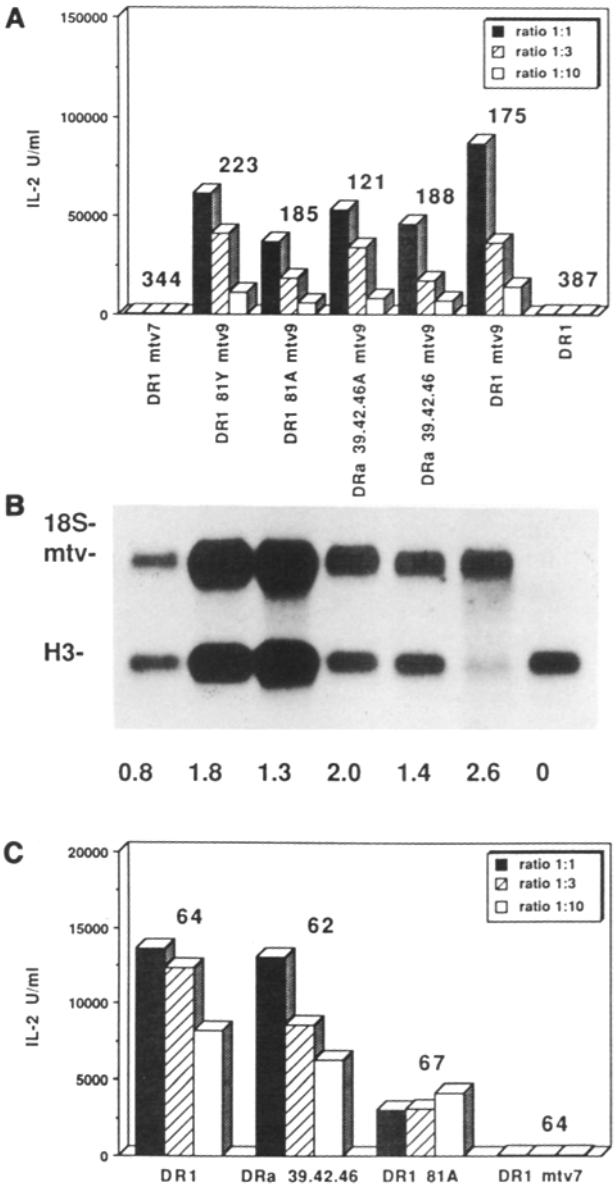

Figure 3. Presentation of *Mtv-9* SAG by mutant MHC class II molecules. (*A*) DAP-3 cells expressing DR1, DRα 39.42.46, DR1 81A, or DR1 81Y were stably transfected with *Mtv-9 sag* DNA and used as APCs to stimulate Vβ5#11 T cell hybridoma (Vβ5$^+$). Populations were used except for DRα 39.42.46A which has been cloned from the puromycin resistant population. DAP cells expressing DR1 alone or DR1 plus *Mtv-7* SAG were used as negative controls. M.F.V. of class II expression on the different transfectants as determined using 50D6 mAb is indicated on top of each histogram. (*B*) The levels of *Mtv-9 sag* and histone H3 mRNA in the different transfectants is shown. Northern blot analysis was carried out using the homologous *Mtv-7 sag* probe. The ratio of *Mtv-9* mRNA as compared with histone H3 mRNA is indicated for each transfectant. (*C*) Stimulation of Vβ5#11 T cell hybridoma using DAP DR1, DAP DRα39.42.46, and DAP DR1 81A transfected transiently with *Mtv-9 sag*. Control transfections using B7 cDNA showed that 20–30% of the cells expressed B7 with a M.F.V. of 200–400 (data not shown). M.F.V. of class II expression are indicated on top of each bar and was determined using 50D6 mAb.

levels of T cell stimulation were correlated to levels of *Mtv-7 sag* transcripts, as determined by Northern blot analysis (Fig. 2 *B*) and to class II levels, as monitored by flow cytometry (Fig. 2 *A*).

To minimize clonal variations, a transient transfection assay was used. Control transfections using the B7 cDNA reproducibly gave 40–50% of positive cells (not shown). DR1 cells transiently transfected with the *Mtv-7 sag* stimulated the Kmls 13.11 hybridoma (Fig. 2 *C*) whereas no response was observed with control DR1 cells transiently transfected with *Mtv-9 sag*. Cells expressing mutant class II molecules that did not present SEA (DR1 81A) or SEB and TSST-1 (DRα 39.42.46) stimulated Kmls13.11 after transient transfection with the *Mtv-7 sag* at all ratios of cells used (Fig. 2 *C*). These results show that mutation of the residues critical for the binding of bacterial toxins have no effect on the presentation of the *Mtv-7* SAG.

These observations were extended to another retroviral SAG (*Mtv-9*). Fig. 3 *A* shows that comparable levels of IL-2 production were obtained when the T cell hybridoma Vβ5#11 was cultured with cells expressing *Mtv-9* SAG and wild-type (DR1) or mutant (DR1 81A, DR1 81Y, and DRα 39.42.46) class II molecules (Fig. 3 *B*). These results were confirmed using the transient transfection assay (Fig. 3 *C*). Cells expressing wild-type or mutated DR1 were capable of presenting *Mtv-9* SAG, whereas control DR1 cells transfected with *Mtv-7 sag* failed to stimulate this hybridoma.

These results demonstrate that the residues that are critical for the binding of bacterial SAGs are not essential for the presentation of two different retroviral SAGs, *Mtv-7* and *Mtv-9*. On the basis of the mutations we have performed on MHC class II molecules, it is clear that the binding sites of both types of SAGs can be dissociated. This is not surprising since bacterial SAGs like SEA and SEB, which share strong homologies in their amino acid sequences (1, 5) and have a proposed similar three-dimensional structure (26), bind to different sites on MHC class II molecules (Fig. 1) (6–8, 27). Moreover, the hierarchy observed in the presentation of bacterial and retroviral SAGs by allelic and isotypic forms of class II molecules is different. Bacterial SAGs are efficiently presented by I-A, I-E, DR, and DQ, whereas I-E and DR are the most potent class II molecules for presentation of retroviral SAGs (3, 5, 15). We have recently shown that several DR alleles that can efficiently present SEA, SEB, or TSST-1 (DR2a, DR2b, DRw53) fail to present *Mtv-7 sag* to T cells, further confirming that these two types of SAGs interact differently with MHC class II molecules (15, 28). Torres et al. have recently reported that bacterial SAGs and the peptide 76-119 derived from *Mtv-1* SAG (ORF peptide) can compete for binding to class II, indicating that both types of SAGs share a common binding region (29). The peptide used in these competition experiments contains three putative glycosylation sites that are conserved in MMTV SAGs (13, 14). The use of such peptide might not reflect the natural interactions between MHC class II and retroviral SAGs. It is likely however that many regions are involved in the binding of retroviral SAGs to MHC class II molecules. Single point mutations may

not be sufficient to abrogate the interaction between these two molecules.

Results presented in this paper and others (6–8) have clearly demonstrated that SAGs (retroviral and bacterial) can interact with class II in many distinct ways and still stimulate T cells in a Vβ restricted fashion. Selective pressures must act on pathogens to code for SAGs that bind to different regions on class II in order to avoid competition with other endogenous or exogenous molecules.

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