Paracrine Transfer of Mouse Mammary Tumor Virus Superantigen

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Summary

Transfer of vSAG 7, the endogenous superantigen encoded in the Mtv7 locus, from MHC class II+ to MHC class II− cells has been suggested to occur both in vivo and in vitro. This transfer usually leads to the activation and deletion of T cells expressing responsive Vβs. However, there is no direct molecular evidence for such a transfer. We have developed an in vitro system which confirms this property of vSAGs. vSAG 7 was transfected into a class II− murine fibroblastic line. Coculture of these cells with class II+ cells and murine T cell hybridomas expressing the specific Vβs led to high levels of IL-2 production which was specifically inhibited by vSAG 7- and MHC class II− specific mAbs. Moreover, injection of vSAG 7+ class II− cells in mice led to expansion of Vβ6+ CD4+ cells. We show that this transfer activity is paracrine but does not require cell-to-cell contact. Indeed, vSAG 7 was transferred across semi-permeable membranes. Transfer can occur both from class II+ and class II− cells, indicating that MHC class II does not sequester vSAG 7. Finally, competition experiments using bacterial toxins with well defined binding sites showed that the transferred vSAG 7 fragment binds to the α1 domain of HLA-DR.

Superantigens (SAGs) are a family of proteins which can induce the stimulation, followed in vivo by the deletion of a subset of T cells sharing particular TCR Vβ segments. These SAGs can be either integrated in the host genome (endogenous superantigens) or can be produced by an increasing array of pathogens (exogenous superantigens). These include microorganisms such as bacteria (staphylococci [1], streptococci [2, 3]), or viruses (MMTVs [4, 5], rabies virus [6]). The fact that they are conserved in such a wide array of pathogens indicates that they might play a pivotal role in the pathogenic process. Indeed, in the case of mouse mammary tumor virus (MMTV) encoded superantigens, it has been clearly demonstrated that viral infection is directly associated with the expansion of cells carrying the Vβ elements responding to the SAGs (7–9). Moreover, the presence of Vβ6+ cells which are responsive to the rabies virus SAG is required to induce paralysis of rabies-infected mice (6, 10).

Endogenous superantigens are encoded in the mammalian genome by retroviruses (MMTVs) that have integrated in the germline host DNA. Most of these integrated proviruses have lost their ability to form virions, but continue to express viral proteins. More than 30 sites of integration (Mv) for distinct MMTVs on different chromosomes are known, with most of the common murine strains possessing two to eight of these loci [11, 12]. The 3′ LTR of Mtv contains an open reading frame encoding the viral superantigen (vSAG) [13–15], which has been characterized as a type II transmembrane protein (16) with five glycosylation sites and three furin-like cleavage sites [17].

The primary sequence of different vSAGs is highly conserved except in the COOH terminus region (18, 19), which imparts Vβ specificity (20, 21). When vSAG 7, the 3′ LTR product of Mtv 7 is presented at the cell surface by MHC class II molecules, T cells expressing TCR Vβ 6, 7, 8.1, or 9 chains are triggered to proliferate in vitro or are deleted in vivo (4, 5, 22, 23).

Different studies have demonstrated that vSAG presentation is not strictly restricted to particular alleles of class II. However, some murine alleles of MHC class II do present vSAGs better than others, and the hierarchy was established as I-E > I-A > I-Aq (24–27). As demonstrated by Labrecque et al. (28), human DR alleles can also present vSAGs to human PBMCs. Moreover, human class II alleles and isotypes show a hierarchy in vSAG presentation (29).

Several reports have suggested that vSAGs can be inter-
cellularly transferred from class II− cells that express vSAGs to class II+ cells (30-33): first, it was shown that the response of a T cell clone to minor lymphocyte stimulatory (Mls) required the presence of B cells and of splenic adherent cells (SAC) (30), suggesting that SAC cells present an Mls fragment transferred from B cells. However, these experiments failed to demonstrate whether spleen cells provide a costimulatory signal or if they stimulate the T cell clone after the transfer of vSAG7 from B cells. It was then shown that F1→parent parent chimeras between mtv7+/I-E− mice from the H-2b haplotype (which do not delete Vβ6 cells) and mtv7+/I-Ed mice leads to the elimination of Vβ6+ cells (31). Lastly, injection of mtv7− mice with CD8+ cells from mtv7+ mice leads to the activation and expansion of the mtv7 responsive Vβ6+ cells; it is noteworthy that the magnitude of the activation of Vβ6+ cells was comparable after the injection of CD8+ cells which are class II− and B cells which express high levels of class II, suggesting the transfer of vSAG from CD8+ cells to class II+ cells (32, 33).

These in vivo studies have provided strong arguments for the transfer of vSAGs from cells which do not express class II (or which express class II isotypes that fail to present vSAG7) to class II+ cells proficient in vSAG presentation. However, it has never been possible to exclude that undetected expression or passive acquisition of MHC class II by T cells rather than vSAG7 transfer is responsible for the observed Vβ-specific expansions. In this report we have developed an in vitro system to confirm at the molecular level mechanisms leading to the intercellular transfer of vSAGs.

Materials and Methods

Mice. 5-wk-old CBA/CaJ female mice were purchased from Jackson Laboratories (Bar Harbor, ME).

Culture Media. PBMCs were cultured in RPMI 1640 supplemented with 5% FCS, 10 mM Hepes, 10 mM 2-mercaptoethanol, 20 μg/ml gentamycin (all Gibco BRL, Gaithersburg, MD). Fibroblasts were cultured in DMEM supplemented with 10% calf serum, 10−2 M Hepes (all Gibco BRL), and 20 μg/ml gentamycin.

Cell hybridomas were grown in DMEM supplemented with 5% FCS, 10 mM Hepes, 10 μM β-mercaptoethanol, 20 μg/ml gentamycin, 4 mM Dextrose (Sigma Chemical Co., St. Louis, MO), essential and nonessential amino acids (Gibco BRL), 1 mM Sodium pyruvate (Gibco BRL), and 10 mM Sodium Bicarbonate (Sigma).

Cell Lines. We used DAP DR1 cells (DAP cells transfected with MHC class II DR 1α and β chains) (34), DAP vSAG7 cells (DAP cells transfected with vSAG7 gene) and 3B2, called DAP DR1 vSAG7 cells thereafter (DAP cells transfected both with DR 1 and vSAG7 genes [28]). A panel of T cell hybridomas, Kmls 13.11, Kmls 12.6 (kindly provided by Drs. J. Kapppler and P. M arrack, National Jewish Hospital and Howard Hughes Institute, Denver, CO), R G17 (kindly provided by Dr. B. H uber, Tufts University, Boston, MA), and KR 3 (kindly provided by Dr. O. Kanagawa, Washington University, St. Louis, MO) were used in these experiments. Kmls 13.11, Kmls 12.6, and R G17 express Vβ6. KR 3 expresses Vβ8.1.

Monoclonal Antibodies. The mAb specific for the murine Vβ3 (K25) was purified and biotinylated using standard procedures (35). The mAb directed against Vβ6 (R R 4-7) was used as a supernatant. The CD4 specific mAb (GK1-5) was purified and FITC-conjugated using standard procedures (35). FITC-mAb directed against human Vβ12 and Vβ13 were purchased from Immunotech (Marseille, France). OT145, a mAb directed against Vβ6, is a kind gift from Dr. D. Posnett (The New York Hospital, Cornell Medical Center, New York, NY). PE-conjugated anti-CD4 (leu-3a) mAb was purchased from Becton Dickinson (Mountain View, CA).

FITC-conjugated goat anti-mouse Ig antibodies and biotinylated goat anti-rat Ig antibodies were, respectively, purchased from Caltag (San Fransisco, CA) and Vector Laboratories (Burlingame, CA).

X D5.117, an IgG1 mAb specific for the human class II molecule was used as a supernatant. HU T78, a mAb specific for human Vβ23, was purified in the laboratory and used as an isotypic control for the mAb X D5.117. Purified mAb directed against vSAG7 (6E1) was kindly provided by Dr. H. Acha-O’Rea (University of Lausanne, Switzerland) (36).

Transfections. DAP cells were transfected using the Calcium Phosphate precipitation technique (37) with the vSAG7 gene cloned in the expression vector pH β-AP1-neo (38). G418 resistant clones were grown and screened for the expression of vSAG7 by Northern blot analysis.

PCR Analysis. RNA was isolated using RNAzol B (Cinna Biotech Laboratories, Friendswood, TX). 20 μg of RNA were fractionated on 1.2% formaldehyde-agarose gels (39), transferred onto nylon membranes (Amersham Corporation, Oakville, Ontario, Canada) and hybridized at 42°C in 50% formamide with vSAG7 (EcoR I-BglII fragment of 0.9 kb) (30) or actin (PstI fragment of 1.1 kb) (40) probes labeled using a random priming kit (Pharmacia, Uppsala, Sweden). Blots were washed at 65°C in 5× SSC, 1× SSC and finally in 0.1× SSC solutions, and exposed on Kodak XAR-5 film for 24 h.

Functional Assays. 60 × 104 DAP DR 1 cells were cocultured with 60 × 104 T cell hybridomas and various concentrations of DAP vSAG7 for 24 h in 250 μl of DMEM supplemented with 10% calf serum. Supernatants were harvested, and IL-2 production was measured using the CTLL hexoaminidase assay (41).

Transfer experiments were carried out using transwells (Nunc, Naperville, IL) with 0.2-μm pores. 60 × 104 DAP DR 1 and 60 × 104 hybridoma cells were added in the lower compartment, while various amounts of DAP vSAG7 cells were added in the upper compartment.

PBMC Stimulation. Blood was obtained from different healthy donors, and PBMCs were purified using ficoll-hypaque gradients (Pharmacia) as previously described (28). One million PBMCs in 1 ml of RPMI 10% FCS were incubated for 7 d in 24-well plates (Falcon, Becton Dickinson, Plymouth, UK). In the presence of either DAP vSAG7 cells treated with mitomycin C (100 μg/ml, 1 h at 37°C) or for 4 d in the presence of the bacterial superantigen Staphylococcal enterotoxin B (SEB) (Toxin technologies, Sarasota, FL), viral or bacterial superantigens were either put in the same chamber as PBMCs, or were separated from PBMCs by a transwell (Costar, Cambridge, MA) with 0.4-μm pores. Cells were then harvested and tested by flow cytometry for Vβ expression.

Cytotoxicity. For human TcR Vβ repertoire studies, 2-5 × 105 cells were stained with anti-Vβ antibodies at previously defined optimal titers for 20 min at 4°C in the dark and washed in PBS containing 2% FCS. When necessary cells were then incubated with FITC-conjugated goat anti-mouse mAb, and 10%
normal mouse serum (Jackson Laboratories) in PBS. After washing, cells were finally incubated with PE- or PerCP-conjugated anti-CD4 for 20 min in the dark. For murine TcR Vß repertoire studies, cells were first incubated with biotinylated anti-Vß mAbs. In a second step, they were incubated with FITC-conjugated anti-CD4, and finally, with PE-conjugated streptavidin. For RR4-7, two additional steps (incubation with biotinylated goat anti-rat and then with normal rat serum 10% in PBS) were made before adding FITC-conjugated anti-CD4. Acquisition and analysis of cells were carried out using a FACScan® and the Lysis II software (Becton Dickinson, Mountain View, CA). For each analysis a minimum of 1 × 10⁴ live cells gated by forward and side scatter were analyzed.

Results

vSAG7 Is Transferred In Vitro and Stimulates T Cells.
The vSAG7 gene was transfected into the MHC class II-DAP3 murine fibroblastic line. Seven clones of G418-resistant cells were analyzed by Northern Blot. Equal loading of all wells was confirmed by comparable levels of actin mRNA in all clones tested (Fig. 1 a). When compared to other clones, clones 17 and 18 showed significantly higher levels of vSAG7 mRNA (Fig. 1 b). Clone 2 also expressed vSAG7 mRNA, although at lower levels. To verify the capacity of MHC class II- vSAG7+ cells to transfer vSAGs, we developed a transfer assay in which equal numbers (6 × 10⁴) of DAP DR1 cells and the Vβ6– vSAG7-responsive Kmls 13.11 hybridomas were cocultured for 16 to 20 h together with various amounts of the DAP vSAG7 cells. T cell stimulation was assessed by measuring IL-2 present in the supernatants. The three vSAG7+ clones (clones 2, 17, and 18) were able to induce a dose-dependent T cell activation in several independent experiments. A representative experiment is shown in Fig. 1 c where increases in the production of IL-2 ranged from 10- to 20-fold as compared to cocultures of DAP vSAG7 cells and untransfected DAP cells. The latter indicated the absolute prerequisite for MHC class II molecules in order to obtain vSAG7 presentation.
Levels of IL-2 production were directly correlated with the number of cells expressing vSAG7 or with the levels of vSAG7 expressed by the class II+ fibroblasts. Indeed, little or no stimulation was observed with less than $2 \times 10^5$ DAP vSAG7 cells, whereas maximal response was obtained with $5 \times 10^5$ DAP vSAG7 cells per well.

Controls were performed to eliminate the possibility that this activity could be caused by the fusion of DAP DR1 with DAP vSAG7 cells. A flow cytometric assay was developed in which two different fibroblastic lines expressing distinct surface markers were cocultured for the duration of the above described functional assay (16 to 20 h). Results indicate that the percentage of fused cells was below 1% and fusion could thus not account for the superantigenic activity (data not shown). Our experiments thus indicate that a fragment of the vSAG7 protein carrying superantigenic activity is transferred intercellularly. Additional experiments were performed to compare the superantigenic activity in the transfer assay and in the direct presentation assay. Results illustrated in Fig. 1 (d and e) show that DAP vSAG7 and DAP DR1 vSAG7 express comparable levels of vSAG7 mRNA. The levels of stimulation induced by vSAG7 in transfer assays or in direct presentation assays are similar (Fig. 1, d–f), indicating that transfer is an efficient process.

A similar strategy was used to monitor the transfer capacity of the exogenous vSAG GR. Results indicate that transfer of vSAG GR can occur from DAP DR1 vSAG GR to CH12 cells and lead to the stimulation of KOX15, a Vb15-expressing hybridoma (data not shown).

Transfer Is Inhibited by Anti-vSAG7 and Anti-Class II MAbs. To further demonstrate the requirement for vSAG7 in order to obtain T cell stimulation we used the vSAG7-specific mAb 6E1 to inhibit stimulation in the transfer as-
lates Vb superantigenic activity is thus transferred from class II molecules expressed at the surface of DAP DR1 cells.

Transfer of vSAG7 Stimulates T Cells Bearing Distinct TCRs and Occurs between Cell Lines of Different Origins. To further demonstrate that the transfer activity bears physiological relevance, experiments were set up to verify if transfer of vSAG7 from DAP cells is also able to stimulate PBMCs in a Vb-restricted manner. DAP vSAG7 (2x10^6) were cocultured with PBMCs (2.5x10^6) for 10 days. PBMCs were then harvested and titrated experiments involving coculture of PBMCs with decreasing numbers of DAP vSAG7, efficient stimulation of T cell hybridomas (Korman, A., personal communication). Titration experiments involving coculture of PBMCs with decreasing numbers of DAP vSAG7 (2x10^6) were repeated four times and led to similar results.

Figure 4. DAP vSAG7 induces Vb-specific expansion in vivo. 3x10^6 DAP vSAG7 cells in 30μl of PBS were injected in the hind footpad. 5 days later, lymph nodes were taken and CD4^+ lymphocytes were analyzed by flow cytometry for Vb3 and Vb6 expression. Live cells were gated by forward and side scatter. Percentages of Vb3- and Vb6-expressing cells among CD4^+ cells were respectively 11, 25, and 23%, respectively, after DAP DR1, DAP vSAG7, and DAP DR1 vSAG7 injection. This experiment was repeated four times and led to similar results.

The percentage of Vb6.7 cells, which are not responsive to vSAG7, remained unchanged.

Characterization of the Intercellular Transfer of vSAG7. Whereas the above results undoubtedly showed that transfer of vSAGs is possible, they did not address the requirement for cellular interactions in this process. We thus performed transfer assays in which DAP vSAG7 cells were separated from DAP DR1 cells and Kmls 13.11 hybridomas by a semi-permeable membrane. No stimulation could be observed under these conditions (Fig. 6a) even with concentrations of DAP vSAG7 cells as high as 2x10^6 per ml. Conversely, SEB, even at low concentrations (1.5 ng/ml), was able to cross this membrane and stimulate the Vb6 hybridoma. These results raised the hypothesis that transfer is mediated by an insoluble or unstable fragment. However, we were not able to exclude the possibility that a small proportion of vSAG7 molecules are able to cross the membrane.

For this purpose, we tried to identify a more sensitive readout and compared stimulation of human PBMCs to stimulation of T cell hybridomas (Korman, A., personal communication). Titration experiments involving coculture of PBMCs with decreasing numbers of DAP vSAG7 cells indicated that stimulation of PBMCs required as few
as 250 DAP vSAG7 cells (Fig. 6 b), while the stimulation of T cell hybridomas was repeatedly shown to require at least 2 \times 10^5 DAP vSAG7 cells (Fig. 1 c). The use of human PBM C s thus provides a readout which is 10-fold more sensitive than that obtained using T cell hybridomas.

In vitro assays were then established in which DAP vSAG7 cells were separated from PBMCs by a 0.4-μm semi-permeable membrane. A threefold enrichment in CD4^{+} blasts expressing Vβ12 was observed by flow cytometry (Fig. 6 b). However, this enrichment was only detected when high amounts (>3 × 10^4) of DAP vSAG7 cells were used. This result clearly indicates that the fragment of vSAG7 carrying the superantigenic activity is able to cross the semi-permeable membrane. Our results also show that at least 100-fold more DAP vSAG7 cells are required when cells are separated by a transwell as compared to direct co-culture of DAP vSAG7 cells and PBMCs in order to obtain the same levels of Vβ12 expansion. It is thus possible to estimate that only ~1% of the soluble vSAG7 molecules are able to efficiently cross membranes. In comparison, SEB was shown to freely cross the membrane, as the SEB-induced stimulation of PBMCs is not altered by this compartmentalization, even at the lowest concentrations of bacterial toxin (data not shown).

Similar experiments were performed using DAP DR1 vSAG7 cells in the upper compartment. Our results (Fig. 7) show that the transferred fragment of vSAG7 crosses the membrane and stimulates efficiently Vβ12^{+} PBMCs. These results were obtained using two different DAP DR1 vSAG7 clones, 3B2 and 3A5. The results obtained using DAP vSAG7 or DAP DR1 vSAG7 (Figs. 6 b and 7) were very similar: in both cases a small and comparable proportion of vSAG molecules were able to cross the membrane and stimulate PBMCs. These results indicate that the MHC class II molecules expressed by DAP DR1 cells are not interfering with the transfer of vSAG7 molecules.

vSAG7 interacts with the HLA-DR α1 chain. Bacterial toxins bind to well-identified sites on MHC class II. To provide a molecular characterization of the class II site which is involved in the interaction with vSAG7, we have performed inhibition experiments of both vSAG7 in direct and transfer presentation assays using bacterial toxins. We
show in Fig. 8 that the DAP DR1 vSAG7-induced stimulation of Kmls 13.11 is not inhibited by addition of SEA, even at the highest concentration tested (50 \( \mu \text{g/ml} \)). On the other hand, intercellular transfer of vSAG7 is significantly affected by the simultaneous incubation of cells with high concentrations of SEA (Fig. 8). This inhibition of vSAG7-dependent T cell stimulation was directly correlated with the amount of SEA. Addition of increasing concentrations of SEA led to up to 95% inhibition of T cell stimulation. These experiments allowed us to suggest that the molecular interactions of the whole vSAG7 or of the transferred fragment with MHC class II are different.

SEA binds to the \( \alpha \) and \( \beta \) chains of MHC class II molecules (41a). We were thus interested to determine if the binding sites for the bacterial toxin were also involved in the binding of vSAG7. To address this question, we used a mutant of SEA, SEA F47, which has lost its ability to bind MHC class II through the \( \alpha \) chain but is still able to bind with high affinity to the \( \beta \) chain of MHC class II (41a, 42). We show here (Fig. 9) that SEA F47 partially blocks the activity of vSAG7 in the transfer assay, as compared to the 20-fold inhibition induced by SEA wt. Indeed, the addition of high concentrations of SEA F47 decreases T cell stimulation by less than a twofold (~40% of inhibition). We concluded from these studies that vSAG7 interacts mainly with MHC class II through its \( \alpha \) chain. This result was further confirmed by the fact that high concentrations of TSST-1, a toxin known to bind exclusively to the \( \alpha \) chain of MHC class II (43, 44), also exerted a partial (~60%) but reproducible inhibition of vSAG7 presentation in the transfer system (Fig. 9).

Discussion

In this report we provide in vitro and in vivo evidence indicating that a soluble form of vSAG7 can activate primary T cells of human or murine origin. This transfer is absolutely dependent on the presence of class II\(^+\) cells, and is specific for the vSAG, as shown by inhibition experiments. Our demonstration that this transfer can occur across a semi-permeable membrane clearly rules out the possibility that fusion between class II\(^+\) and class II\(^-\) cells is responsible for this effect.

Moreover, the use of the semi-permeable membrane provided conclusive evidence that cell-to-cell contact is not required for intercellular transfer of vSAG7. In these conditions, however, transfer is not very efficient and involves only a small proportion (~1%) of vSAG7 molecules. Indeed, titration curves of vSAG7 in the presence or in the absence of a semi-permeable membrane shows that comparable V\( \beta \) specific expansion requires 100-fold more vSAG7 cells in the presence of a semi-permeable membrane. The fact that vSAG7 molecules can only cross membranes with low efficiency suggests that vSAG7 is weakly soluble, and that most of the transferable molecules remain uncovalently bound at the cellular membrane to its NH\(_2\)-terminal part.

Figure 7. vSAG7 can be transferred despite the presence of MHC class II molecules on donor cells. Various concentrations of DAP DR1 vSAG7 cells clone 3B2 or 3A5 were disposed in the upper compartment. The experiment was then performed as described in Fig. 6.

Figure 8. vSAG7 transfer is inhibited by SEA. 6 \( \times \) 10\(^4\) DAP DR1 cells, 6 \( \times \) 10\(^4\) Kmls 13.11 cells and 5 \( \times \) 10\(^4\) DAP vSAG7 cells were co-cultured overnight in presence of different concentrations of SEA. IL-2 released in supernatants was measured 24 h later.

Figure 9. vSAG7 binds to the DR \( \alpha \) chain. To standard conditions of transfer assay were added various concentrations of either SEA, SEA F47, or TSST-1. IL-2 was measured in supernatants as previously described.
Tese features leads us to conclude that vSAG7 transfer does not require strict cell-to-cell contact but rather cellular proximity. It can thus be considered as a paracrine phenomenon. Tese results also provide convincing evidences that vSAG7 molecules can reach the cell surface in the absence of MHC class II molecules and are biologically active.

Titration experiments involving DAP DR1 vSAG7+ cells in the presence or in the absence of a semi-permeable membrane indicate that DR molecules do not sequester vSAGs. Indeed, the Vβ skewing obtained using DAP DR1 vSAG7 and DAP vSAG7 shows that vSAG7 can be transferred efficiently from both cells. Tis suggests that vSAG molecules are not sequestered by MHC class II at the surface of DAP DR1 vSAG7 cells. Alternatively, vSAGs could be loosely associated to class II enabling the dissociation of the transferred fragment and subsequent binding to another class II+ cell. It is possible that the transferred fragment has never been associated with class II molecules. Tese observations raise the hypothesis that the soluble molecule involved in the transfer phenomenon is a fragment of vSAG7 obtained by cleavage of vSAG7 at one of the three furin-like cleavage sites. Tese observations raise the hypothesis that the soluble molecule involved in the transfer phenomenon is a fragment of vSAG7 obtained by cleavage of vSAG7 at one of the three furin-like cleavage sites. Tese features lead us to conclude that vSAG7 transfer does not require strict cell-to-cell contact but rather cellular proximity. It can thus be considered as a paracrine phenomenon. Tese results also provide convincing evidences that vSAG7 molecules can reach the cell surface in the absence of MHC class II molecules and are biologically active.

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