

Production and Characterization of an Mls-1-specific Monoclonal Antibody

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Summary

Superantigens (SAGs) represent a new class of antigens, characterized as T cell receptor (TCR) $V\beta$ -reactive elements. Bacterial toxins constitute the major group of exogenous SAGs, while the mouse mammary tumor virus (MMTV)-encoded Mls molecules represent the endogenous SAGs. Mls-1 is the prototype of the latter SAGs, because it elicits a very potent T cell stimulatory response in vitro in unprimed T cells expressing the TCR $V\beta$ 6 or 8.1 chains. In vivo, Mls-1 causes deletion of immature T cells bearing the $V\beta$ 6, 7, 8.1, or 9 chains. Although Mls-1 was functionally discovered >20 yr ago, it has not been possible to raise antibodies against this molecule. We have previously cloned and sequenced the *Mtv-7 sag* gene, which encodes Mls-1. Sequence comparisons with other MMTV *sag* genes suggested that the polymorphic 3' end encodes the TCR $V\beta$ specificity of these SAGs. We have, therefore, immunized hamsters with a 14-amino acid peptide from the deduced COOH-terminal sequence of the *Mtv-7 sag* gene. We describe here the production of a monoclonal antibody (mAb), 3B12, which is peptide specific and reacts with a recombinant baculovirus product of *Mtv-7 sag*. This mAb blocks Mls-1-specific T cell recognition and detects the Mls-1 protein on the surface of the B cell hybridoma LBB.A, but not on LBB.11, which is an *Mtv-7* loss variant of LBB.A. Transfection of the *Mtv-7 sag* gene into LBB.11 renders this cell functionally Mls-1⁺ as well as positive for 3B12 binding, confirming the specificity of this mAb. It is well documented that B cells and CD8⁺ T cells express T cell stimulatory Mls-1 determinants, and we show here that this functional profile correlates with the expression of MMTV-specific mRNA. However, primary lymphocytes derived from Mls-1⁺ mice do not stain with 3B12, even after in vitro activation with mitogens or phorbol ester.

A number of bacterial and viral proteins have been classified recently as superantigens (SAGs)¹ (1–6). The hallmark of a SAG is the ability to stimulate a significant proportion of naive T lymphocytes. While conventional antigens are recognized by the TCR antigen recognition site, SAGs interact primarily with particular $V\beta$ chains of the TCR (7). Furthermore, SAGs are not processed into peptides (8) and are believed to associate with MHC class II molecules outside the peptide binding groove (9).

A number of groups have shown that the retrovirus mouse mammary tumor virus (MMTV) encodes a SAG (4–6). MMTVs exist as both exogenous infectious viruses and as endogenous inherited elements in the germline. About 20 polymorphic endogenous MMTVs have been found in mice. The MMTV SAG is encoded within the U3 region of the viral 3' LTR (10–12), the open reading frame of which is capable of encoding a protein of ~300 amino acids. Our lab-

oratory has recently shown that the open reading frame of the endogenous virus *Mtv-7* encodes the Mls-1 protein and has named this gene *Mtv-7 sag* (12). Mls-1 is the prototype of the endogenous SAGs, as it elicits a strong in vitro proliferative response in CD4⁺ T cells bearing the TCR $V\beta$ 6 or 8.1 chains (13–16). Furthermore, it causes in vivo deletion of immature T cells bearing the appropriate TCR $V\beta$ chain.

Although the Mls molecules were functionally defined >20 yr ago (17), it has not been possible to raise antibodies against this strong T cell stimulatory determinant, despite efforts of many research groups. Thus, biochemical analysis of this molecule has been impaired. In this paper we describe the generation and characterization of a mAb, 3B12, that is specific for Mls-1.

Materials and Methods

Mice. DBA/2 (Mls-1⁺, H-2^d) mice were purchased from Taconic Farms (Germantown, NY). B6.C-H-2^{bm12} (bm12) mice were bred at the animal facilities at Tufts University School of Medicine. All mice were at least 6–8 wk of age. Armenian hamsters,

¹ Abbreviations used in this paper: MMTV, mouse mammary tumor virus; SAG, superantigen.

6 wk of age, were purchased from Cytogen Research and Development (West Roxbury, MA).

Reagents and Media. Complete medium was RPMI 1640 (Gibco Laboratories, Grand Island, NY), supplemented with 2 mM L-glutamine, 1 mM HEPES, 50 μ M 2-ME, 100 μ g/ml penicillin, 100 U/ml streptomycin, and 10% FCS (Hazelton Biologics, Lenexa, KS). All supplements were purchased from JRH Biosciences (Lenexa, KS). TNM-FH medium (Sigma Chemical Co., St. Louis, MO) plus 10% FCS was used for propagation of Sf9 insect cells. PMA and Con A were purchased from Sigma Chemical Co. Protein A was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden).

Cell Lines. The V β 6 T cell hybridoma 18bbm19 (Mls-1 reactive, I-A^{bm12} alloreactive) was provided by Dr. E. Palmer (Denver, CO). The V β 6 T cell hybridoma RG17 (Mls-1 reactive), the Mls-1-expressing B cell hybridoma LBB.A (18), and its Mls-1⁻ variant LBB.11 (4, 18) were gifts from Dr. A. Glasebrook (Indianapolis, IN). The V β 8.1⁺ T cell hybridoma, KJ.16, was provided by Dr. P. Marrack (Denver, CO). HT-2, Sf9, and P3X63-Ag8.653 cell lines were obtained from American Type Culture Collection (ATCC; Rockville, MD). Puro 14 is a cell line derived by transfection of LBB.11 with the *Mtv-7 sag* gene (12). This clone was chosen because it expresses high levels of Mls-1 in functional assays.

Antibodies. The following mAb-producing B cell hybridomas were obtained from ATCC: RL172 (rat IgM) specific for murine CD4, TIB 211 (rat IgM) specific for murine CD8, and 1452C11 (hamster IgG) specific for murine CD3. T7-Tag antiserum was purchased from Novagen Inc. (Madison, WI). The mAb 2F9 (hamster IgG), which showed no detectable binding to Mls-1⁺ cells, was used as a control Ab.

Peptides. Two peptides of 21 and 14 amino acids (peptide 21 and peptide 14) were synthesized according to the deduced amino acid sequence of the 3' polymorphic region of the *Mtv-7 sag* gene (Fig. 1) by solid-phase peptide synthesis. Peptide 21 (Ac-IAKILYNMKYTHGGRVGFDPF) was synthesized by Dr. J. Gariépy (Toronto, Canada). An additional cysteine was added to the NH₂ terminus of peptide 14 (CMKYTHGGRVGFDPF) for conjugation with carrier proteins and was synthesized at the Tufts Protein Synthesis facility. Peptide 14 was conjugated to KLH and OVA (Sigma Chemical Co.), using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Sigma Chemical Co.) by standard procedures (19).

Immunization. Armenian hamsters were immunized subcutaneously with 50 μ g of KLH-peptide 14, emulsified in Hunter's TiterMax (CytRx Corporation, Norcross, GA). After 2 wk, boosters were given intraperitoneally in IFA (Sigma Chemical Co.). 3 d before fusion, 20 μ g of KLH-peptide 14 was administered intravenously.

Production and Screening of mAb. Fusions were carried out by standard methods (20), using P3x63-Ag8.653 myeloma cells as a fusion partner. Hybrids secreting peptide-specific mAbs were detected using an ELISA. Briefly, 96-well microtiter plates (Corning Laboratory Sciences Company, Corning, NY) were coated with OVA-peptide 14 (100 ng/well) to capture peptide-specific mAbs. Alkaline phosphatase-conjugated anti-hamster IgG (Jackson ImmunoResearch, West Grove, PA) was added after washing. Enzyme activity was visualized by the addition of *p*-nitrophenylphosphate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). The plates were read at 405 nm, using a microplate reader (MR 700; Dynatech, Chantilly, VA).

Construction of Recombinant Transfer Vectors and Production of Recombinant Baculovirus. The recombinant transfer vector pVL1392-T7-TagSAG was constructed from the transfer vector pVL1392 (Invitrogen, San Diego, CA) and the plasmid pET-11aSAG (Fig.

2), which was produced by PCR cloning of the full-length *Mtv-7 sag* gene from a genomic clone (12) into the BamHI site of pET-11a (Novagen Inc., Madison, WI), using the following primers: 5' primer GGGGATCCATGCCGCGCCTGCAG, and 3' primer GGGCTAGCGGATCCGCAAGTAGACCTG. The correct orientation of the fragment was confirmed by restriction endonuclease digestion. The pET-11aSAG vector was digested with XbaI and EcoRV, and the 1.2-kb fragment containing the *Mtv-7 sag* gene was cloned into the XbaI/SmaI sites of the pVL1392 transfer vector.

The recombinant baculovirus AcMNPV-T7-TagSAG was produced by homologous recombination after cotransfection of the recombinant transfer plasmid pVL1392-T7-TagSAG with circular wild-type AcMNPV viral DNA (Invitrogen). Screening and enrichment for recombinant virus was carried out by three rounds of limiting dilution in 96-well tissue culture plates. Recombinant viruses were identified by dot-blot DNA hybridization of alkali-lysed cells (21), using a ³²P-labeled probe of *Mtv-7 sag*. The putative AcMNPV-T7-TagSAG viral isolates were used to infect Sf9 cells. Infected cell extracts were tested for expression of the T7-TagSAG protein by Western blot analysis, using a polyclonal antibody directed against the first 12 amino acids of the T7 bacteriophage gene 10 protein (Novagen). Final purification of the AcMNPV-T7-TagSAG virus was achieved by two rounds of plaque purification (21).

Western Blotting. Sf9 cells were infected at a multiplicity of infection of 1 and harvested after 60–96 h. Protein extracts were prepared by the solubilization of infected Sf9 cells in SDS-PAGE sample buffer with 2-ME and boiled for 5 min before electrophoresis on a 10% SDS-PAGE gel (22). The proteins were electrotransferred to a PVDF membrane (Millipore Corp., Bedford, MA) and probed with the 3B12 mAb or T7-Tag antiserum, as described previously (23). Blocking experiments with peptide 21 were performed by preincubating the 3B12 mAb with peptide 21 (100 μ g/ml) for 15 min at room temperature.

Purification of Splenic B Lymphocytes and LPS Stimulation. Single-cell suspensions of murine spleens were treated with Tris-buffered ammonium chloride to lyse RBC. The cells were incubated for two cycles with a cocktail containing RL172 (anti-CD4), TIB 211 (anti-CD8), and guinea pig complement (Pel-freeze Biologicals, Rogers, AR) for 15 min at 37°C to eliminate T cells. The B cell-enriched population was suspended at 2×10^6 cells/ml and stimulated with LPS (10 μ g/ml, *Escherichia coli* 055:B5; Sigma Chemical Co.) for 40–48 h.

Flow Cytometry. The Mls-1-specific, protein G-purified mAb 3B12 (Hamster IgG, 1 mg/ml) was biotinylated using the succinimide ester of biotin, as described by Goding (24). Cells (5×10^5) in PBS, pH 7.2, supplemented with 1% FCS and 0.1% NaN₃ (Sigma Chemical Co.), were incubated with biotinylated 3B12 mAb in the presence or absence of unconjugated 3B12 or peptide. The cells were washed and incubated with Streptavidin-conjugated PE (Southern Biotechnology Associates, Inc., Birmingham, AL). The cells were then analyzed by FACSscan® (Becton Dickinson & Co., Mountain View, CA).

Stimulation of T Cell Hybridomas. T cell hybridomas (2×10^4) were incubated with varying concentrations of B cell hybridomas (LBB.A, LBB.11) for 24 h at 37°C in an atmosphere of 5% CO₂ in the presence or absence of antibodies or peptides in a final volume of 200 μ l in 96-well microtiter plates. For allostimulation, splenic B cells (5×10^5) from bm12 mice were incubated with 18bbm19 (2×10^4) cells. For anti-CD3 assay, protein A (100 μ g/ml) was coated on microtiter plates to capture the CD3 mAb. RG17 cells (2×10^4) were added to these wells in the presence or absence of 3B12 or control mAbs. The IL-2 content of the resulting super-

Mtv 7 IYLGTM-NF WGIKIFDYTEE GAIKILYNN KYTHGGRVGE DPF*
 Mtv 1IH ...V.YNSR. E.KRHIEHI .ALP*
 Mtv 8VH..K. ...V.RQ.EHI SADTF.MSYN G*
 Mtv 9VH..K. ...V.RL.EHI SADTF.MSYN G*
 Mtv11VH..K. ...V.RQ.EHI SADTF.MSYN G*
 MMTV C3HH.H..K. .TV.GLIEHY SPKTY.MSY Y E*
 MMTV BR 6SSIH..K. RTV.ALIEHY SAKTY.MSY Y D*
 MMTV GRH.V.H..K.GLIEHY SAKTY.MSY Y D*

Figure 1. Comparison of the deduced amino acid sequences of the COOH terminus of various MMTV *sag*-encoded molecules.

natants from the above assays was determined in a bioassay using HT-2 cells. The proliferation of HT-2 cells after incubation with cell-free supernatants for 18–20 h was assessed by addition of [³H]TdR (ICN Biochemicals, Irvine, CA) during the last 6 h of culture, and the cells were harvested using a semiautomated cell harvester (PHD cell harvester; Cambridge Technology, Cambridge, MA). [³H]TdR incorporation was measured by liquid scintillation counting (LS 7000; Beckman Instruments, Inc., Irvine, CA). Values are expressed as means \pm SD of counts per minute of quadruplicate determinations.

RNA Preparation and Northern Blot Analysis. Total cellular RNA was extracted from lymphocytes using the guanidium isothiocyanate method (25). RNA (20 μ g) was run on formaldehyde gels, blotted onto nylon membrane, and hybridized as previously described (26) to a ³²P-labeled MMTV LTR probe (27). After exposure, the blot was stripped and rehybridized to a ³²P-labeled GAPDH probe to evaluate uniform mRNA loading.

Results

Production of mAbs. A comparison of the predicted amino acid sequences of various MMTV *sag* genes is shown in Fig. 1. *Mtv-7* SAG differs completely from other MMTV SAG sequences in the last 20 amino acids. This sequence was, therefore, used for synthesizing peptide 14 and for immunizing hamsters. Peptide specificity of the elicited serum antibodies was determined by an OVA-peptide 14 ELISA. Spleen cells

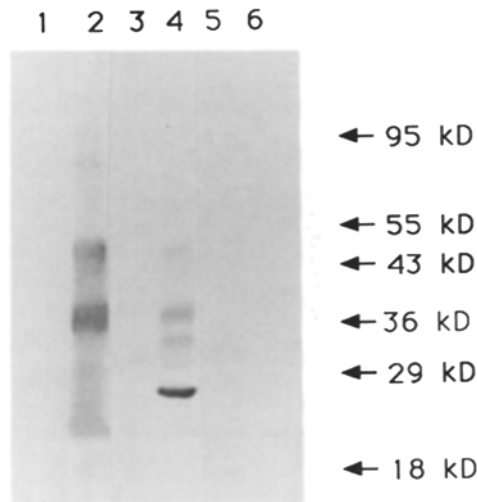


Figure 3. Western blot analysis of baculovirus-infected cell extracts. Lanes 1, 3, and 5 are wild-type baculovirus-infected Sf9 cell extracts. Lanes 2, 4, and 6 are AcMNPV-T7-TagSAG-infected Sf9 cell extracts. Lanes 1 and 2 show binding of the T7-Tag antiserum, lanes 3 and 4 show binding of the 3B12 mAb, and lanes 5 and 6 show the absence of binding of the 3B12 mAb after preincubation with peptide 21.

of hamsters with a positive serum titer were fused to myeloma cells, resulting in \sim 500 hybridomas, of which 15 hybridomas secreted peptide 14-specific mAbs. These mAbs were further tested in a Western assay for binding to recombinant *Mtv-7* SAG. Only one of the 15 peptide 14-specific mAbs, designated as 3B12, reacted with the SAG protein in this assay (see below) and was used for further studies.

Recognition of *Mtv-7* SAG by 3B12 mAb To produce large amounts of the Mls-1 protein, the *Mtv-7 sag* gene was expressed in Sf9 insect cells, using the recombinant baculovirus AcMNPV-T7-TagSAG. A fusion protein was generated with the first 12 amino acids of the T7 bacteriophage gene 10 pro-

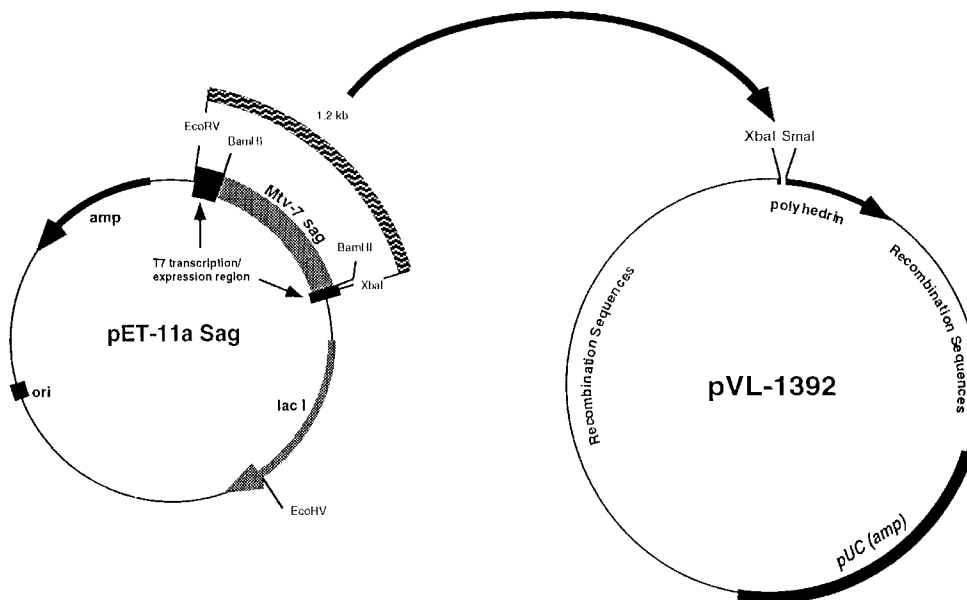


Figure 2. Construction of the recombinant transfer vector pVL-1392-T7-TagSAG. The PCR product of the full-length *Mtv-7 sag* gene was first cloned into the bacterial expression vector pET-11a. The resulting plasmid, pET-11aSAG, was used to derive the fragment T7-TagSAG, which was subcloned into the transfer vector pVL-1392.

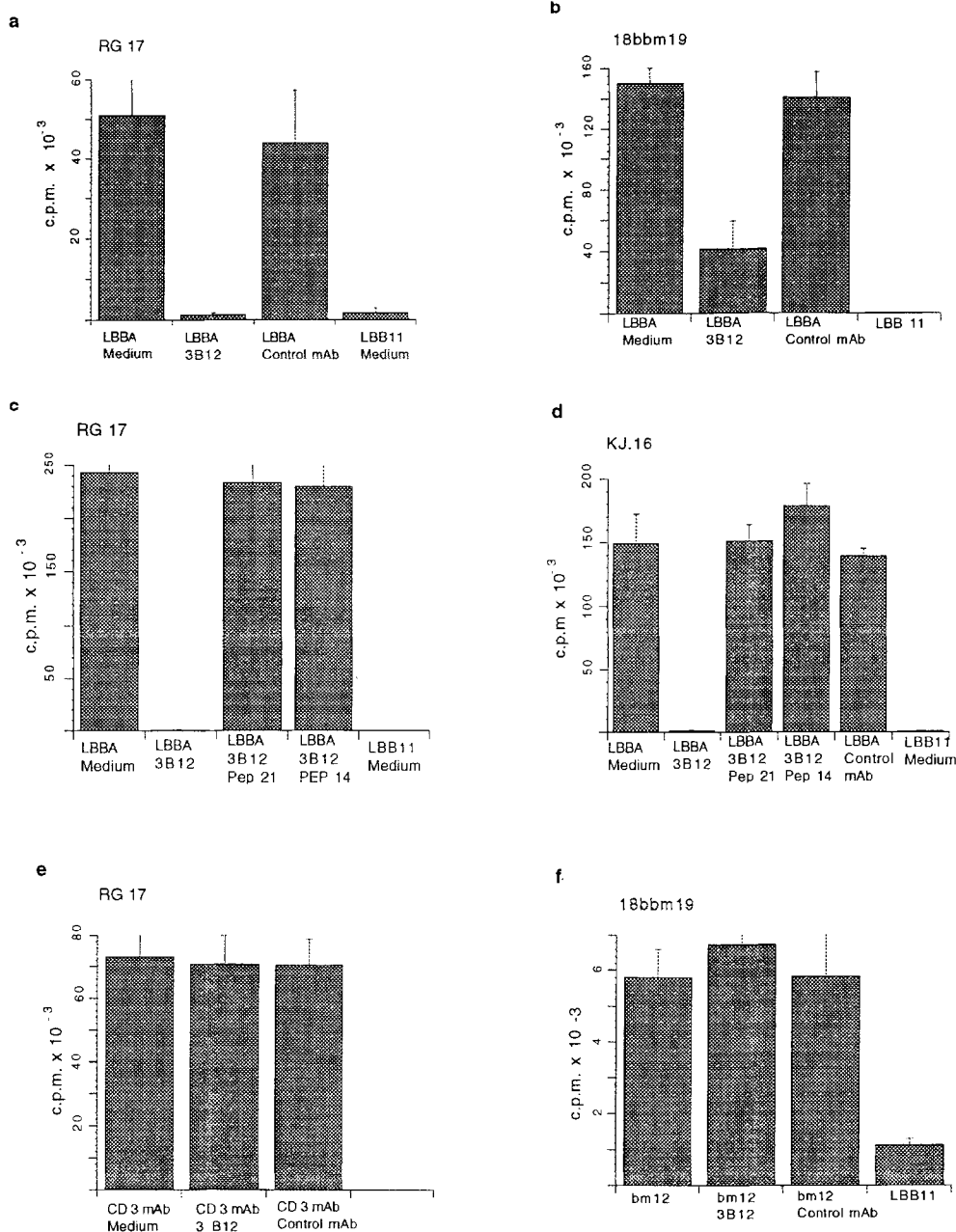


Figure 4. T cell proliferation assay. RG17, 18bbm19, or KJ.16 T cell hybridomas were cultured with the stimulator B cell hybridoma LBB.A. After 24 h, the supernatants were removed and tested in a bioassay on IL-2-dependent HT-2 indicator cells. (a and b) In the presence or absence of 3B12 mAb and control mAb; (c and d) 3B12 mAb in the presence or absence of peptide 21 or peptide 14; (e) anti-CD3 stimulation in the presence or absence of 3B12 or control mAb; (f) I-A^{bm12} allostimulation by bm12 B cells in the presence or absence of 3B12 or control mAb.

tein to serve as an epitope tag (Fig. 2). The predicted fusion product is a protein of 37 kD. Two prominent bands corresponding to 48 and 37 kD were observed in Western blot analysis of AcMNPV-T7·TagSAG-infected Sf9 cell extracts, using the T7·Tag antiserum (Fig. 3). Extracts from cells infected with wild-type virus showed no significant binding of this antiserum. The 3B12 mAb recognized the 48- and 37-kD products in AcMNPV-T7·TagSAG-infected Sf9 cells, although the ability of 3B12 to bind to the 48-kD protein was greatly reduced compared with the 37-kD form. No binding of 3B12 mAb to extracts of wild-type virus-infected cells was observed. Two other immunoreactive products were detected with the 3B12 mAb, namely a faint band of 33 kD

and a prominent band of 27 kD (Fig. 3). These bands may represent breakdown products of the T7·TagSAG fusion protein or indicate the use of alternate methionine initiation sites. The recognition of the T7·TagSAG protein by 3B12 mAb was completely blocked by the addition of peptide 21 (Fig. 3).

Inhibition of Mls-1-specific T Cell Activation. Two different T cell hybridomas bearing V β 6 TCRs (RG17 and 18bbm19) and a V β 8.1⁺ T cell hybridoma (KJ.16) were stimulated with the Mls-1-expressing B cell hybridoma LBB.A. The addition of 3B12 mAb to the cultures resulted in inhibition of IL-2 production in response to Mls-1 stimulation (Fig. 4). The inhibition was reversed by the addition of either peptide 14 or peptide 21. On the other hand, the 3B12 mAb

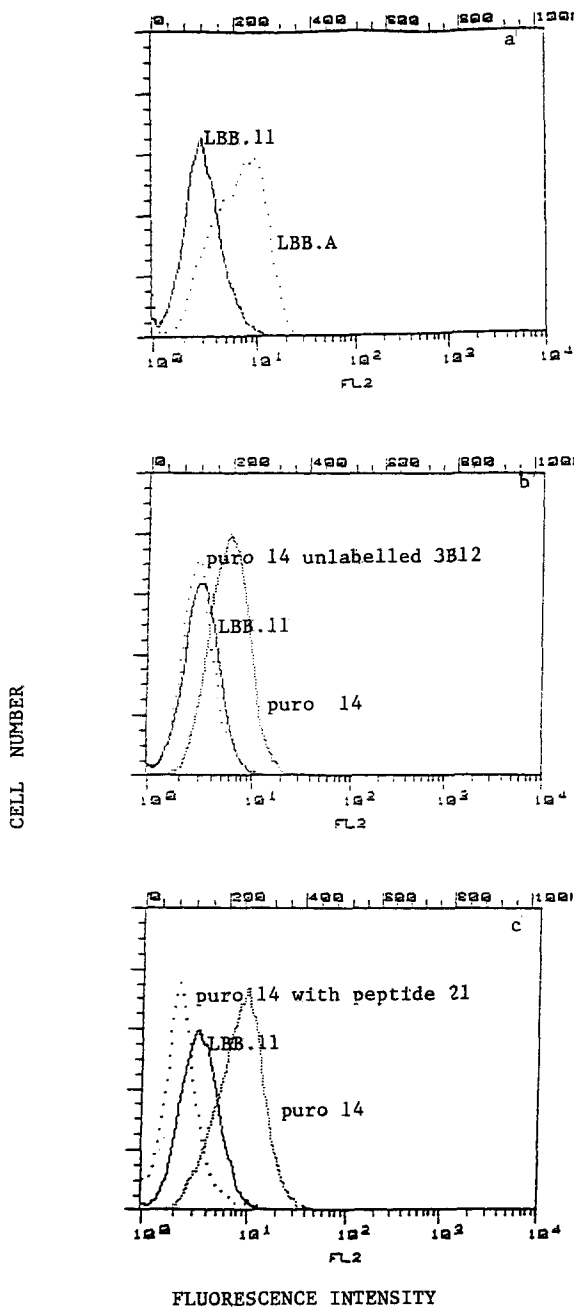


Figure 5. Surface expression of Mls-1 on LBB.A and puro 14. Indirect immunofluorescence was performed using biotinylated 3B12 and Streptavidin PE as second step. (a) LBB.A and LBB.11 cells; (b) puro 14 in the presence or absence of unlabeled 3B12; (c) puro 14 in the presence or absence of peptide 21.

did not block IL-2 production by the 18bbm19 T cell hybridoma in response to allostimulation with bm12 splenic B cells, nor did it block IL-2 production by T cell hybridomas in response to activation with anti-CD3 mAb (Fig. 4).

Surface Staining of Mls-1 by 3B12 mAb Biotinylated 3B12 mAb stained the Mls-1⁺ B cell hybridoma LBB.A, as determined by flow cytometry, whereas LBB.11, an Mls-1 loss variant of LBB.A, was not stained (Fig. 5 a). The *Mtv-7 sag*

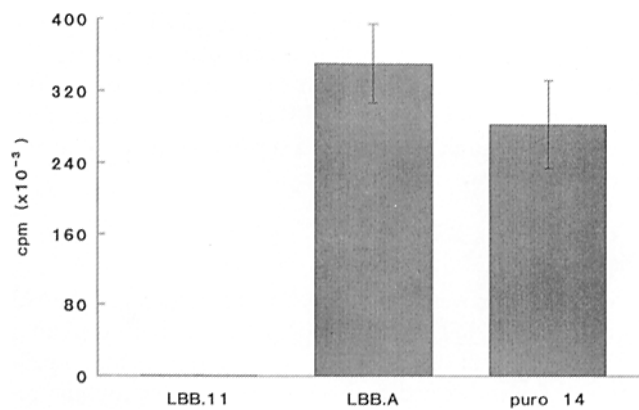


Figure 6. T cell proliferation assay. RG17 cells were cultured with LBB.A, LBB.11, or the *Mtv-7 sag*-transfected LBB.11 cell line (puro 14), and IL-2-containing supernatants were assayed in a HT-2 cell bioassay.

transfectant, puro 14, which activated Mls-1-reactive T cells equally well as LBB.A (Fig. 6), stained brightly with 3B12 mAb (Fig. 5 b). The staining of LBB.A and puro 14 cells was inhibited by the addition of unlabeled 3B12 mAb or peptide 21 (Fig. 5 c), confirming the specificity of the reaction.

Primary lymphocytes from four Mls-1⁺ mouse strains, CBA/J, DBA/2, SWR, and NZB, could not be stained with 3B12 mAb. Neither LPS activation of B cells, nor stimulation of T cells with Con A or PMA, resulted in a specific staining profile. Furthermore, resting as well as PMA or Con A-activated thymocytes were negative.

Northern Blot Analysis. Unstimulated B lymphocytes from DBA/2 mice showed the presence of LTR-specific mRNA, which was significantly increased upon LPS stimulation (Fig. 7). Furthermore, LTR-specific transcripts were detected only in PMA-stimulated CD8⁺ cells but not in CD4⁺ cells. Virtually no LTR-specific transcripts were observed in unstimulated CD4⁺ or CD8⁺ cells.

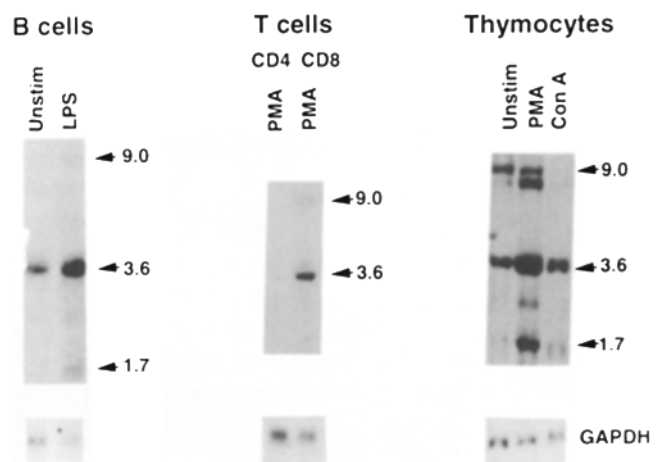


Figure 7. Northern hybridization analysis of MMTV expression. Total RNA from the indicated cell types, cultured in the presence or absence of stimulants for 16–18 h, was subjected to electrophoresis, transferred to nylon membrane, and probed with an MMTV-LTR probe (top), or the GAPDH probe (bottom).

Analysis of unseparated thymocytes revealed the presence of LTR-specific mRNA, even in unstimulated cells (Fig. 7). While Con A stimulation leads to a small increase in the amount of 3.6- and 1.7-kb MMTV transcripts in the thymocytes, PMA stimulation caused a substantial increase in the level of these two mRNA species.

Discussion

Since the discovery of the open reading frame in the U3 region of the MMTV LTR, virologists have been searching for the product of this gene. Racevskis (28) generated a peptide antiserum directed against a conserved hydrophilic region of the open reading frame gene product and was able to immunoprecipitate *in vitro* translation products of this gene. However, with the exception of phorbol ester-treated T cell thymomas, which have amplified MMTV, no protein expression was detected. Similar negative results were obtained by many other research groups. More recently, Brandt-Carlson and Butel (29) have raised antisera against a recombinant baculovirus product of the infectious MMTV C3H *sag* gene. While these investigators were able to visualize the recombinant protein from infected Sf9 insect cells in Western blots, no protein was detected in mammary tumor cells or lymphocytes. Numerous immunologists have attempted in vain to raise antisera to Mls-1 by immunization with Mls-1-expressing lymphocytes. These observations indicate that the MMTV SAG is expressed in minute quantities and, although efficiently recognized by T cells, is insufficient for eliciting an antibody response. Therefore, standard biochemical tests may not be sensitive enough to detect the low level of expressed SAG molecules.

We have previously shown that *Mtv-7 sag* encodes Mls-1 (12). The comparison of the predicted amino acid sequences of the various MMTV *sag* genes suggests that the TCR V β specificity is conferred by the COOH terminus (see Fig. 1), since the major polymorphism is restricted to this part of the molecule. It has recently been reported that the MMTV SAG is a type II transmembrane glycoprotein, because the COOH terminus is extracellular, as deduced from *in vitro* translation studies in the presence of microsomes (30–32). We have, therefore, synthesized peptides specific for the COOH-terminal region of the *Mtv-7 SAG*, to produce Mls-1-specific antibodies.

Among the 15 mAbs that reacted with the *Mtv-7 SAG*-specific peptide (peptide 14) in an ELISA, only one was able to detect the baculovirus-derived recombinant *Mtv-7 SAG* protein in Western blots. This mAb, called 3B12, reacted with 48- and 37-kD products (Fig. 3), which were also visualized with the T7-Tag antiserum. It is likely that the 48-kD product is a glycosylated form of the recombinant molecule, in agreement with previous reports (29), since the overall structure of the MMTV *sag* gene is very conserved. The 37-kD band, on the other hand, may represent the unglycosylated precursor molecule (29). Unlike the T7-Tag antiserum, the 3B12 mAb recognizes the 37-kD molecule more efficiently than the 48-kD protein. A possible explanation is that the Sf9 insect cell

glycosylation interferes with 3B12 binding. Two additional bands of 33 and 27 kD, respectively, recognized by 3B12, are either breakdown products of the T7-TagSAG fusion protein, or products that indicate the use of alternate methionine initiation sites, since they are not seen in cell extracts from wild-type-infected Sf9 cells. These bands are not detected with the T7-Tag antiserum, which is directed against an NH₂-terminal determinant. The addition of peptide 21 abrogated 3B12 binding, confirming the specificity of the 3B12 mAb for the T7-TagSAG protein.

To test whether this mAb could detect the native Mls-1 molecule expressed on murine B cells, we carried out blocking experiments, using TCR V β 6⁺ and V β 8.1⁺ T cell hybridomas as responders and Mls-1-expressing B cell hybridomas as stimulator cells. While this interaction was blocked by the addition of 3B12 mAb, no effect was seen on allorecognition or anti-CD3 stimulation of the same T cell hybridoma. Since the peptide sequence used for immunization is unique to the *Mtv-7*-derived SAG molecule, no blocking of any other SAG responses is expected. Furthermore, the specificity of the blocking reaction was again confirmed by the addition of peptide 14 or peptide 21, which eliminated the inhibition of IL-2 production.

The experiments outlined so far confirm the Mls-1 specificity of the 3B12 mAb. It was of interest, therefore, to carry out immunofluorescence experiments. The LBB.A B cell hybridoma, which stimulates Mls-1-reactive T cells efficiently, was clearly positive for surface staining with 3B12, compared with the *Mtv-7* loss variant of LBB.A (LBB.11), which was negative. To unequivocally prove that this staining reaction was specific for Mls-1, we tested *Mtv-7 sag* gene transfectants of LBB.11 and found that some of these had a bright staining profile.

It is well documented that B cells (33, 34) and CD8⁺ T cells (35), but not CD4⁺ T cells, are capable of stimulating an Mls-1-specific response. This stimulatory capacity is further enhanced after *in vitro* culture with various mitogens (28, 36). Our results confirm the above observations, since MMTV-specific steady-state mRNA was detected in unstimulated and LPS-stimulated B cells. Furthermore, only PMA-treated CD8⁺, but not CD4⁺, cells revealed the presence of MMTV-specific mRNA. When these lymphocyte subsets were tested for staining with the 3B12 mAb, they were all negative, even after *in vitro* stimulation with mitogens. Overall, we have repeatedly tested four *Mtv-7*-positive mouse strains of various H-2 types and could see no difference in the staining profile compared to lymphocytes from *Mtv-7*-negative strains. Furthermore, all thymocyte subsets tested were negative, although they have considerable MMTV-specific mRNA, as detected in Northern analyses. In agreement with this finding, we observed many *Mtv-7 sag* transfectants that showed good functional Mls-1 reactivity in terms of T cell stimulation, but were negative when stained with the 3B12 mAb. On the other hand, their Mls-1 stimulatory capacity could be blocked with this mAb (data not shown).

Overall, these experiments indicate that though Mls-1 expression is minimal, it is sufficient to cause T cell stimula-

tion, but not for detection with this mAb. This is a general feature of antigenic determinants recognized by T cells, as it has been shown that minute quantities of peptide are

sufficient for T cell stimulation (37, 38). The availability of *Mtv-7 sag* transfectants expressing high levels of Mls-1 protein will now facilitate biochemical analysis of this molecule.

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