Brief Definitive Report

Inhibition of Mouse Mammary Tumor Virus–induced T Cell Responses In Vivo by Antibodies to an Open Reading Frame Protein

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Summary

Minor lymphocyte stimulating (Mls) antigens specifically stimulate T cell responses that are restricted to particular T cell receptor (TCR) β chain variable domains. The Mls phenotype is genetically controlled by an open reading frame (orf) located in the 3' long terminal repeat of mouse mammary tumor virus (MMTV); however, the mechanism of action of the orf gene product is unknown. Whereas predicted orf amino acid sequences show strong overall homology, the 20–30 COOH-terminal residues are strikingly polymorphic. This polymorphic region correlates with TCR V_β specificity. We have generated monoclonal antibodies to a synthetic peptide encompassing the 19 COOH-terminal amino acid residues of *Mtv*-7 orf, which encodes the Mls-1^a determinant. We show here that these antibodies block Mls responses in vitro and can interfere specifically with thymic clonal deletion of Mls-1^a reactive $V_\beta6^+$ T cells in neonatal mice. Furthermore, the antibodies can inhibit $V_\beta6^+$ T cell responses in vivo to an infectious MMTV that shares orf sequence homology and TCR specificity with *Mtv*-7. These results confirm the predicted extracellular localization of the orf COOH terminus and imply that the orf proteins of both endogenous and exogenous MMTV interact directly with TCR V_β.

Expression of minor lymphocyte stimulating (Mls) antigens in mice leads to thymic clonal deletion of Mls-reactive T cells. In contrast to conventional antigen recognition where both the TCR α and β chains contribute, T cells interacting with a particular Mls antigen are characterized by the TCR V_β chain they express on the cell surface (1, 2). Thus, to assure self-tolerance in mice expressing Mls-1^a, the majority of T cells expressing TCR V_β6, 7, 8.1, and 9 are deleted from the mature $CD4^+8^-$ and $CD4^-8^+$ thymocytes as well as from peripheral T cells (1–4). Encounter of Mls antigens later in life can lead to an initial activation of the reactive T cells followed by induction of unresponsiveness and deletion (5–9).

Mls phenotypes are encoded by open reading frames (orf) in the 3' LTRs of endogenous mouse mammary tumor virus (MMTV) (10–14). Transfection studies showed that expression of *Mtv*-7 orf leads to stimulation of TCR $V_\beta6^+$ T cells (14).

Additionally, we have recently described infectious MMTV(SW) with properties of Mls-1^a such as activation and deletion of the same subsets of T cells and strong orf sequence homology (9).

Amino acid sequence comparisons of MMTV orf molecules suggest a correlation between the highly polymorphic COOH-terminal 20–30 amino acids and the TCR V_β specificity (10–12). In vitro translation studies suggested that orf molecules are type II membrane proteins (15–17). Thus, the COOH terminus would be accessible to the TCR. There is no experimental evidence, however, that MMTV orf molecules directly interact with the V_β element of the TCR.

Herein we describe the characterization of five mAbs specific for an *Mtv*-7 orf peptide encompassing the 19 COOH-terminal amino acids. These mAbs specifically block anti-Mls-1^a responses in vitro, rescue $V_\beta6^+$ T cells from clonal deletion in vivo, and strongly inhibit the $V_\beta6^+$ T cell response induced by MMTV(SW) infection in vitro. These results suggest that the viral superantigen directly interacts with the V_β chain of the TCR.

Materials and Methods

Production of Anti-Mtv-7 orf mAbs. For production of the mAbs described in this study, 8-wk-old BALB/c mice were immunized intraperitoneally with 50 μg of the COOH-terminal *Mtv*-7 orf peptide KILYNMKYTHGGRVGFDPF (9) coupled to albumin in CFA and boosted 4 wk later in IFA. The mouse having the strongest titer in serum, as judged by inhibition of an anti-Mls-1^a response, was reimmunized intravenously and fusions with the myeloma X63Ag8 were performed 3 d later. Five mAbs were selected that exhibited strong binding to the peptide used for immunization as

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determined in solid phase ELISA: 6B9.4 (IgG1), 2E5.6 (IgG1), 4D1.3 (IgG1), 6E1.8 (IgG2b), and 1G12.7 (IgM).

Inhibition of Mls Response In Vitro. Mls-1^a LBB cells (5–500 × 10^3, irradiated with 10^3 rad using a cobalt source) were cultured with 2 × 10^4 $V_\beta6^+$ T hybridoma (RG17) cells for 24–36 h (18). Supernatants were tested at different dilutions for IL-2 production using CTLL cells as indicator cells. Different doses of protein G–Sepharose (Pharmacia, Uppsala, Sweden) –purified antibodies and *Mtv*-7 orf peptide were added from the onset of culture. All five anti-*Mtv*-7 mAbs were similarly efficient in inhibiting an anti-Mls-1^a response in vitro (data not shown).

Expression of Mtv-7 orf in Baculovirus. *Mtv*-7 orf DNA was obtained by PCR amplification from DNA of BALB.D2 mice using the 5′ oligonucleotide GATCGTCGACATGCCGCGCCTGCAGCAGA and the 3′ oligonucleotide GTGTCGACCCAAACCAAGTCAGGAAACCACTTG (9). The start codon is underlined. The product was cut with the restriction enzyme SalI and ligated into the SalI-digested vector pGem3ZF$^+$ (Promega Biotec, Madison, WI). The insert was excised using the restriction enzymes BamHI and HindIII and ligated in the BglII-EcoRI-digested vector pCS1392 (obtained from F. Godeau). After 10-h ligation and blunting the ends with Klenow fragment the vector was recircularized by ligation and transfected together with wild-type baculovirus DNA into SF9 cells using standard methods. Colonies containing recombinant baculovirus were plaque purified twice. SF9 cells were infected with a multiplicity of infection of 5 and analyzed 36 h later.

FACS® Analysis. FACS® (Becton Dickinson & Co., Mountain View, CA) analysis of infected SF9 cells was performed using an amplification system consisting of 6 μg of biotinylated antibody 6E1.8 followed by PE-streptavidin (Caltag Laboratories Inc., Burlingame, CA), 1.25 μg/ml biotinylated antistreptavidin (Vector Laboratories Inc., Burlingame, CA), and PE-labeled streptavidin (19). Dead cells were excluded using forward scatter and side scatter analysis.

For three-color analysis, thymocytes were stained with FITC-conjugated anti-$V_\beta6$ (44-22-1; 20) or anti-$V_\beta8.2$ (F23.2; 21) antibodies, PE-conjugated anti-CD4 (GK1.5; Becton Dickinson & Co.), and biotinylated anti-CD8 followed by avidin-tandem (Southern Biotechnologies, Birmingham, AL). Two-color analysis of thymocytes (after CD8 depletion with antibody 3.168.1 and rabbit complement) (22) or lymph node cells was carried out with FITC-conjugated anti-V_β antibodies and PE-conjugated anti-CD4. Dead cells were gated out using forward scatter and side scatter analysis on a FACScan® flow cytometer.

Virus Isolation and Purification. Virus was purified from milk of BALB/c mice carrying MMTV(SW) as described (9). The virus was titered using a sandwich ELISA and independently by injection into the foot pads of BALB/c mice and measuring the lymph node $V_\beta6^+CD4^+$ T cell response. Limiting virus doses (~10^7 virus particles) giving an increase of $CD4^+$ $V_\beta6^+$ T cells from 10 to 25% were used in these experiments.

Results and Discussion

Based on the orf sequences of *Mtv*-7 (Mls-1^a) and the related infectious MMTV(SW) (9), mouse anti-orf mAbs specific for a peptide corresponding to the unique 19 COOH-terminal amino acid residues were produced. Five mAbs were selected based on their strong binding to the *Mtv*-7 orf peptide. As shown for the representative antibody 6E1.8 in Fig. 1 *a*, all five mAbs inhibited the in vitro anti-Mls-1^a response of the $V_\beta6^+$ T cell hybrid RG-17. This inhibition was specific since addition of the *Mtv*-7 orf peptide restored a normal

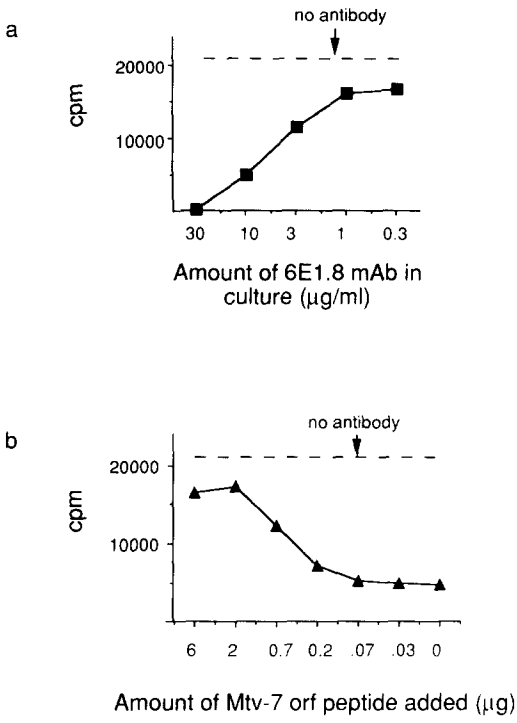

Figure 1. Inhibition of Mls-1^a response by anti-*Mtv*-7 orf mAbs. (*a*) The Mls-1^a-reactive T cell hybrid (RG17) was cultured with Mls-1^a-expressing LBB cells (18) in the absence or presence of different doses of mouse anti-orf mAb 6E1.8. After 36 h of coincubation, IL-2 production was assessed in the supernatants by measuring proliferation of IL-2-dependent CTLL cells. (*b*) Same as *a*, except using 10 μg/ml of mAb 6E1.8 and graded doses of *Mtv*-7 orf peptide.

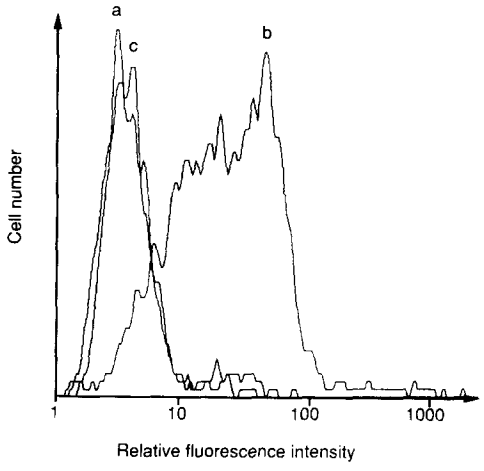

Figure 2. Flow microfluorometric analysis of baculovirus-infected SF9 cells expressing *Mtv*-7 orf. SF9 cells infected with either wild-type baculovirus or recombinant baculovirus were stained with biotinylated anti-*Mtv*-7 orf mAb 6E1.8. The signal was amplified with PE-streptavidin, biotinylated anti-streptavidin, and PE-streptavidin as described in Materials and Methods. (*a*) Wild-type baculovirus-infected SF9 cells, (*b*) *Mtv*-7 orf-expressing SF9 cells, and (*c*) same as *b*, but in the presence of 10 μg of the peptide used for immunization.

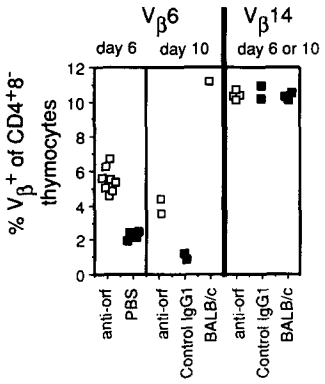

Figure 3. Inhibition of thymic clonal deletion by anti-orf mAbs. $V_\beta6$ and $V_\beta8.2$ expression was measured in $CD4^+CD8^-$ thymocytes 6 or 10 d after birth in anti-orf–treated or control mAb–treated BALB.D2 (Mls-1^a) mice or in untreated BALB/c (Mls-1^b) mice. All the IgG anti-orf mAbs gave comparable results and are pooled in this figure. As control IgG1, antibody B8-24-3 (anti-H-2 K^b) (25) was used.

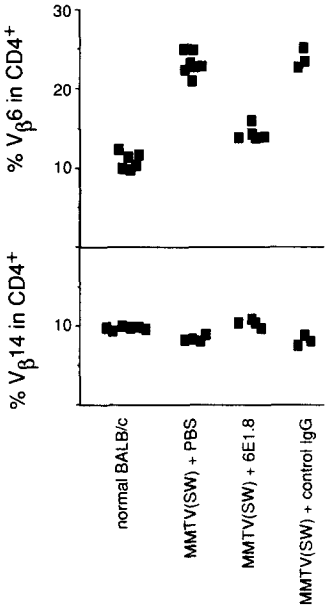

Figure 4. Inhibition of anti-MMTV(SW) T cell response by anti-orf mAbs. Adult (5–8 wk old) BALB/c mice were injected with 10^7 purified MMTV(SW) virus particles as described (9) into the left hind footpad. After 12 h and every 12 h thereafter, the mice were injected in the same footpad with 50 μg of either isotype-matched control antibody (SJL2-4) (26) or anti-orf mAb 6E1.8 (IgG2b). After 4 d the draining lymph nodes were removed and single cell suspensions were analyzed for $V_\beta6^+CD4^+$ or $V_\beta14^+$ $CD4^+$ T cells (27).

response (Fig. 1 *b*). Furthermore, since RG-17 cells express a second TCR V_β chain ($V_\beta1$, provided by the BW fusion partner), the response could be restored in the presence of staphylococcal enterotoxin A (SEA), which crosslinks $V_\beta1$ with MHC class II molecules (data not shown).

Flow microfluorometric analysis of SF9 cells overexpressing the *Mtv*-7 orf molecule 36 h after infection with recombinant baculovirus showed strong and specific staining with all the anti-orf mAbs. This staining could be completely blocked by addition of the *Mtv*-7 orf peptide (Fig. 2). The orf molecule in SF9 insect cells might therefore be expressed at the cell surface confirming in vitro translation studies (15–17). Similar analysis of Mls-1^a LPS induced B cell blasts showed very weak to undetectable staining (data not shown). Immunoprecipitation of *Mtv*-7 orf molecules from ^{35}S-methionine-labeled infected SF9 cells gave a very similar band pattern after SDS-PAGE to that observed by Brandt-Carlson and Butel (23). SF9 cells infected with recombinant baculovirus expressing the control orf molecule of MMTV (GR) did not yield any detectable bands (data not shown), consistent with the fact that MMTV(GR) orf has a completely different COOH-terminal sequence.

In vivo, most Mls-1^a mouse strains intrathymically delete Mls-1^a-reactive mature $V_\beta6^+$ T cells within the first 10 d after birth (24). This deletion occurs at the transition from $CD4^+8^+$ to either $CD4^+8^-$ or $CD4^-8^+$ mature thymocytes (1). To see whether anti-orf mAbs are able to rescue $V_\beta6^+$ T cells from deletion, we injected neonatal Mls-1^a mice with the different anti-*Mtv*-7 orf peptide–specific mAbs. As shown in Fig. 3, all four IgG antibodies tested were able to rescue a considerable proportion (40–60%) of $V_\beta6^+$ mature thymocytes from clonal deletion after either two intraperitoneal injections of 70–320 μg of antibody on days 0 and 4 or after nine daily injections with 100 μg starting within the first 24 h after birth. The percentage of T cells expressing TCR molecules not interacting with Mls-1^a ($V_\beta8.2$, $V_\beta14$) did not change, and isotype-matched control antibody did not interfere with the clonal deletion patterns (Fig. 3).

We recently described the characterization of infectious MMTV(SW) that has the same TCR specificity as Mls-1^a (*Mtv*-7 orf) and displays a very similar COOH-terminal orf amino acid sequence (9). Thus, we attempted using the anti-orf mAbs to interfere with a proliferative $V_\beta6^+$ $CD4^+$ response induced after MMTV(SW) infection in vivo. Preliminary experiments determined the minimal virus dose ($\sim10^7$ particles) required for a significant and specific (2.5-fold) increase in $V_\beta6^+CD4^+$ T cells in the draining lymph nodes. We injected 50 μg of anti-orf mAb 6E1.8 (IgG2b) 12 h after virus challenge into the hind footpad and repeated the antibody injections every 12 h until day 4, when the lymph node cells were isolated. As can be seen in Fig. 4, a strong and specific inhibition of the local increase of $V_\beta6^+CD4^+$ T cells is observed in anti-orf–treated but not control IgG2b-treated mice. This effect likely represents blocking of orf presentation to $V_\beta6^+$ T cells by MMTV(SW)-infected B cells, since the antibody should not interfere with MMTV infection per se.

In conclusion, the results described here indicate that MMTV orf molecules are expressed on the cell surface and that the orf COOH terminus is accessible to antibodies, thus confirming predictions made from sequence comparisons and in vitro translation studies. Furthermore, the in vivo blocking data indicate that MMTV-induced T cell stimulation and deletion both depend upon direct interaction of TCR V_β with the orf protein.

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