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Tumor cells expressing a retroviral envelope escape immune rejection *in vivo*

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ABSTRACT **A model system for the *in vivo* control of tumor cell proliferation by the immune system has been used to assay for the possible immunosuppressive activity of retroviral proteins. Expression vectors for the entire or the transmembrane subunit of the Moloney murine leukemia virus envelope protein were constructed, as well as control vectors for irrelevant transmembrane proteins—or no protein. They were introduced either into MCA205 murine tumor cells, which do not proliferate upon s.c. injection into an allogeneic host, or into CL8.1 murine tumor cells, which overexpress class I antigens and are rejected in a syngeneic host. In both cases, expression of the complete envelope protein or of the transmembrane subunit resulted in tumor growth *in vivo*, with no effect of control vectors. Tumor cell growth results from inhibition of the host immune response, as the envelope-dependent effect was no more observed for MCA205 cells in syngeneic mice or for CL8.1 cells in x-irradiated mice. This inhibition is local because it is not observed at the level of control tumor cells injected contralaterally. These results suggest a noncanonical function of retroviral envelopes in the "penetrance" of viral infections, as well as a possible involvement of the envelope proteins of endogenous retroviruses in tumoral processes.**

Various strategies are used by viruses to invade their host. The mouse mammary tumor virus encodes a superantigen, which activates the natural target cells of the virus, thus favoring viral propagation (reviewed in refs. 1 and 2). Other viruses, such as the Epstein–Barr human herpes virus 4, encode proteins which have immunosuppressive properties *in vivo* (3, 4). It has been suggested that retroviruses also might encode immunosuppressive proteins. Indeed inactivated virions, transmembrane (TM) envelope proteins, and synthetic peptides from envelope domains conserved among retroviruses display immunosuppressive effects in *in vitro* assays (reviewed in refs. 5–7). These include inhibition of interleukin 2 (IL-2)-dependent lymphocyte proliferation (8), of alloantigen-stimulated proliferation of murine and human lymphocytes (8), of cytolytic activity of human natural killer cells (9), and of monocyte-mediated tumor cell killing (10), as well as modulation of cytokine synthesis (11). Immunosuppressive effects of retroviral proteins also have been reported under *in vivo* conditions. These were manifested by the retardation of delayed-type hypersensitivity, in mice injected with sheep erythrocytes in the footpad (12), by the inhibition of macrophage accumulation at the site of inflammation, triggered upon phytohemagglutinin injection in the mouse i.p. cavity (13), and by the abrogation of immunity to feline oncornavirus in vaccination trials (14). All of these results suggested an involvement of retroviral proteins on host immune functions [but see ref. 15 and *Discussion*]. In the present investigation, we show that the TM envelope protein of a "model" retrovirus, the Moloney murine leukemia virus (MoMLV), actually acts *in vivo* as an immunosuppressive agent involved in tumor cell proliferation, since its expression results in the proliferation of tumor cells engrafted into immunocompetent mice, that would otherwise be rejected because of the presence of tumor antigens or of allogeneic determinants on these cells. Such effects could be involved in the "penetrance" of retroviral infections as well as on the onset and/or progression of tumoral processes involving induction of endogenous retroviruses.

MATERIALS AND METHODS

Mice and Cell Lines. C57BL/6 and BALB/c mice, 8- to 12-wk-old, were obtained from Janvier (Laval, France). The Bosc23-packaging cell line (16) was from American Type Culture Collection, and the tumor cell lines MCA205 (17) and CL8.1 (18) were gifts from L. Zitvogel (Institut Gustave Roussy, Villejuif, France) and E. Gorelik (University of Pittsburgh, Pittsburgh, PA), respectively. Culture conditions were as indicated in the corresponding references.

Constructions. The full-length MoMLV envelope (env) expression vector (pDFG-env) was constructed on introduction of a *Pml*I- and Klenow-treated *Nhe*I envelope fragment from pMov3 (19) into the pDFG MoMLV-derived retroviral expression vector (3) opened at the homologous *Pml*I site and the Klenow-treated *Eco*RI site in pBR322, and then on introduction of the *Eco*RI–*Eco*RI fragment from pDFG into the reconstituted *Eco*RI site of this intermediate construct. Functionality of the envelope gene in this construct was verified by a rescue assay by using cells containing an env-defective marked recombinant MoMLV provirus (20). pDFG-TM was constructed by using the p15EP plasmid (21) that has an in-phase deletion within the envelope surface protein but still contains the envelope signal peptide and proteolytic cleavage site allowing normal export of the p15E protein to the cell surface [see ref. 21 and Fig. 1*C*]. This truncated envelope was PCR amplified with a primer at the envelope 5′-end and an *Eco*RI-containing primer at the envelope 3′-end; the PCR fragment was *Eco*RI-restricted and introduced into pDFG opened at the envelope ATG-containing *Nco*I site (Klenow-treated) and the *Eco*RI site in pBR322. The pDFG-TM vector was finally obtained upon introduction of the *Eco*RI–*Eco*RI pDFG fragment, as above. pDFG-mREpo was constructed upon introduction of a *Cla*I–*Kpn*I Klenow-treated fragment from pBTKSmREpo (22) into pDFG opened as above with *Pml*I and *Eco*RI, and then upon introduction of the *Eco*RI–*Eco*RI pDFG fragment. pDFG-mCD2 was constructed by a three-fragment ligation of a *Nco*I–*Pvu*II mCD2-containing fragment [from pMFG-mCD2;

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Abbreviations: TM, transmembrane; IL, interleukin; MoMLV, Moloney murine leukemia virus; env, envelope; SU, surface.
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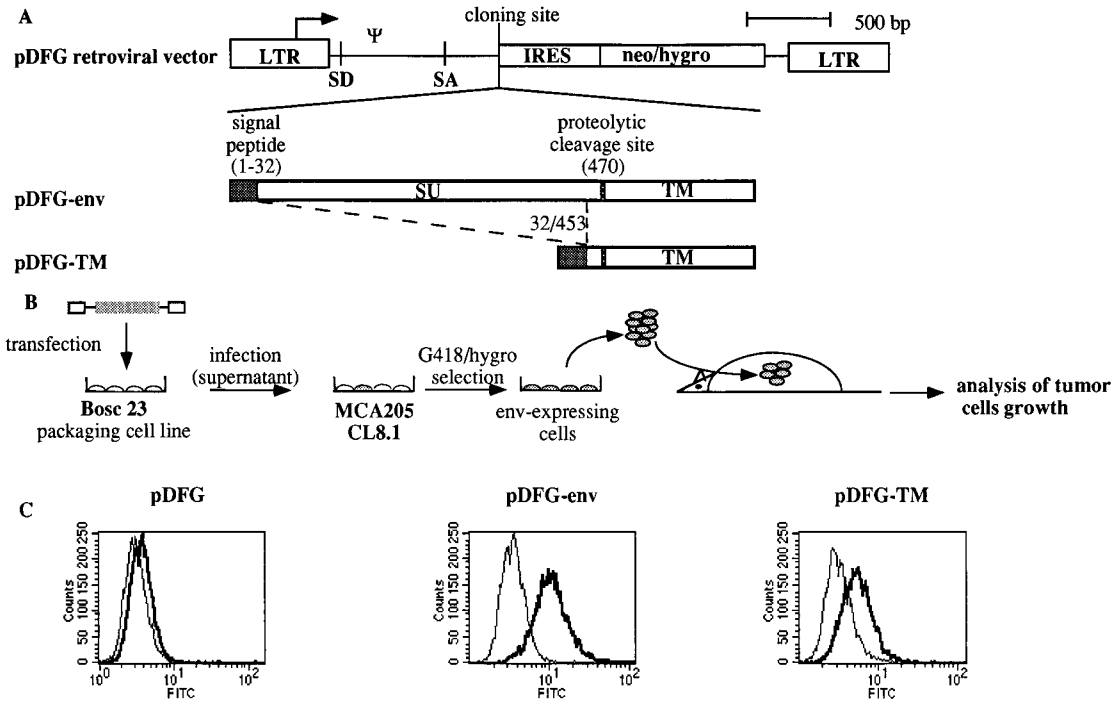

FIG. 1. Envelope-expressing vectors and rationale of the assay. (*A*) Structure of the expression vector and envelope constructs. The structure of the pDFG expression vector is indicated with the LTR promoter, the MoMLV envelope-associated splice sites for expression of the env genes, the Ψ sequence allowing packaging of the retroviral transcript, and an internal ribosome entry site (IRES), allowing translation of either the neomycin or hygromycin gene from the retroviral transcript; the env gene is schematized with the amino acid positions of the envelope signal peptide and of the proteolytic cleavage site between the surface (SU) and the TM envelope subdomains; structure of the envelope construct for expression of the TM protein (pDFG-TM) also is schematized, with amino acid positions for the SU deletion. (*B*) Rationale of the assay. Bosc23-packaging cell lines transfected with the envelope expression vectors are used for recombinant retrovirus production; supernatants, collected 2 days after transfection, are used for infection of the tumor cell lines MCA205 and CL8.1, which are then submitted to selection in either G418- or hygromycin-containing medium; envelope-expressing cells are then introduced s.c., and tumor cell growth is measured. (*C*) Envelope protein expression by the transduced tumor cells. Flow cytometry analysis of cells stained with an anti-MoMLV TM polyclonal antibody revealed with a fluorescein isothiocyanate-labeled goat anti rabbit antibody. Profiles for cells (MCA205) transduced with pDFG, pDFG-env, and pDFG-TM, stained with the fluorescein isothiocyanate-labeled second antibody alone (light lines), or together with the anti-MoMLV TM antibody (heavy lines). Similar profiles were observed for CL8.1 transduced cells.

(23)], with *Eco*RI Klenow-treated to *Bam*HI and *Bam*HI to *Nco*I fragments from pDFG. Introduction of the hygromycin gene in the pDFG vectors was achieved upon removal of the *Bst*XI–*Bam*HI neo-containing fragment and replacement with an hygromycin-containing fragment generated by PCR from pCEP4 (Invitrogen), by using primers containing *Bst*XI and *Bam*HI sites. pDFG-vIL10 was a gift from M. T. Lotze (University of Pittsburgh, Pittsburgh, PA).

Establishment of Envelope-Expressing Tumor Cells and *in Vivo* Assay. Retroviral expression vectors were first introduced into the Bosc23-packaging cell line by transient transfection (8 μg of DNA for 2×10^6 cells; calcium phosphate transfection method) and supernatants were recovered 2 days later as described (16). They were used for infection of the MCA205 and CL8.1 tumor cells (1 ml of supernatant for 5×10^5 cells, with 8 μg/ml polybrene). Cells were maintained in selective medium (600 μg/ml G-418 or 200 units/ml hygromycin) for 3 weeks. For *in vivo* assays, tumor cells were washed three times with PBS, scrapped without trypsination, and inoculated s.c. in the shaved area of the right flank (in some experiments also in the left flank to assay for contralateral effects). Tumor establishment was determined by palpation and tumor area (mm^2) was determined by measuring perpendicular tumor diameters.

Controls for Expression of Envelope Proteins. Analysis of envelope expression was performed by flow cytometry using a polyclonal (24) or the 4F5 monoclonal antibody (25) directed against the TM subunit of the MoMLV envelope protein and a FACScan flow cytometer (Becton Dickinson). For Western blot analyses, we had to use cell membrane preparations because envelope expression could not be detected in total cell extracts (even from the control Bosc23-packaging cell line) and an anti-gp70 antibody because available antibodies against the TM protein did not provide clear-cut signals in our assay of env-transduced cells (including for the Bosc23 cells). The antibody used was a goat antiserum raised against the Rausher leukemia virus gp70 (Quality Biotech, Camden, NJ) revealed by an anti-goat horseradish peroxidase-conjugated antibody (Sigma) and an enhanced chemiluminescence kit (Amersham). Cell membrane preparations were processed as described (26). In brief, dilacerated tumors and cells in culture were suspended in 2 ml of ice-cold hypotonic lysis solution (10 mM Tris, pH 7.4/2 mM MgCl2/1 mM CaCl2) containing 1 mM phenylmethylsulfonyl fluoride. After centrifugation at 2,000 rpm (4°C), the microsome-containing supernatant was kept and the pellet relysed in the same conditions. Both supernatants were combined and ultracentrifuged at 100,000 × *g* for 30 min at 4°C in a Beckman (Palo Alto, CA) precooled 70.1 Ti rotor (38,000 rpm). After slow deceleration, supernatant was discarded and excess fluid wiped out from tubes. Pellets were then resuspended in 10 mM Tris, pH 7.4, (100 μl) resulting in suspension of membrane fragments that were further solubilized in 0.1% SDS and analyzed by Western blot (30 μg of membrane proteins per lane) using standard procedures. A lysat of purified Rauscher murine leukemia virus particles (gift from G. Cianciolo, Duke University Medical Center, Durham, NC) was used as a control for envelope expression.

RESULTS

Rationale of the Assay and Constructs. To assay for the immunosuppressive effect of retroviral envelopes and its con-

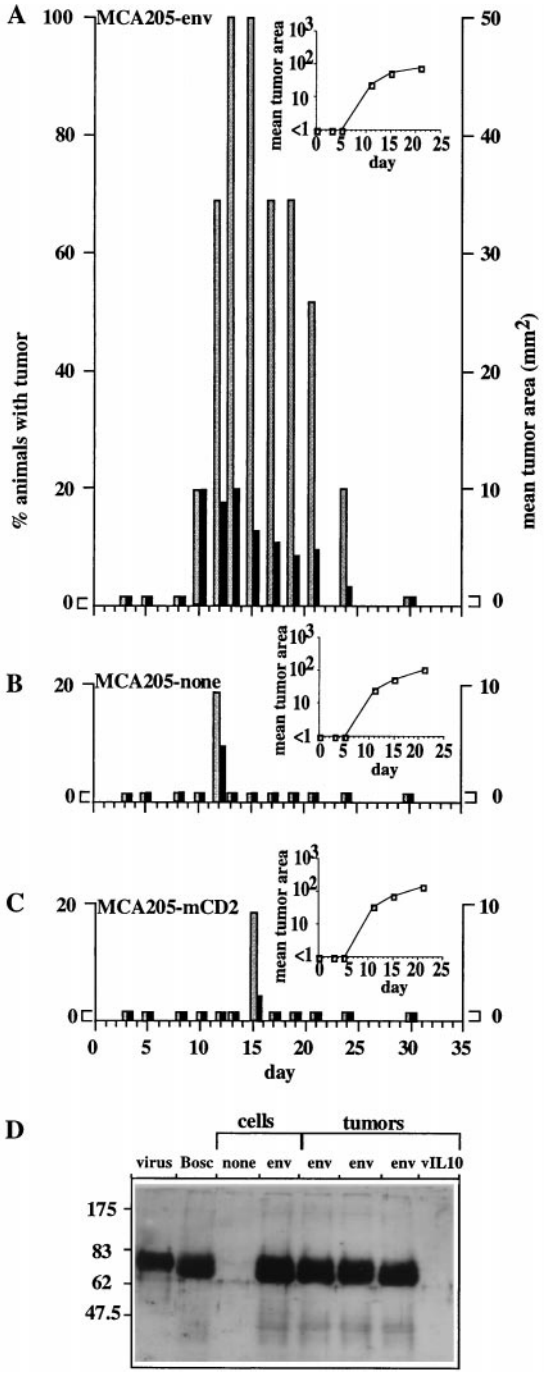

FIG. 2. Induction of tumor cell growth by retroviral envelope expression. (*A–C*): tumor cells (MCA205) transduced with the pDFG vector encoding the MoMLV envelope (*A*), no ORF (*B*), or mCD2 (*C*) were engrafted into allogeneic BALB/c mice (2×10^6 cells/mice; 6 mice/group), and tumor progression was assayed twice or thrice weekly. Shown are the percentages of animals with tumor (grey bars); mean tumor areas are indicated when >1 mm^2 (black bars). (*Insets*) Control growth of the transduced MCA205 cells engrafted under syngeneic conditions within C57BL/6 mice; mean tumor areas are indicated (6 mice/group, all animals developed tumors). (*D*) Western blot analysis of envelope protein expression in the transduced cells and tumors. Membrane preparations of control Bosc23-packaging cells (lane 2), MCA205-none (lane 3), and MCA205-env (lane 4) cells, and of three groups of MCA205-env tumors in BALB/c mice (5 mice/group, lanes 5–7), together with a control tumor (tumors generated by using MCA205 cells transduced with a pDFG vector for the Epstein–Barr vIL10 immunosuppressive protein, lane 8) were analyzed by SDS/PAGE (30 μg of membrane proteins per lane) and Western blotting with an anti-gp70 polyclonal antibody. Purified viral particles (RLV) were deposited in the first lane as a control.

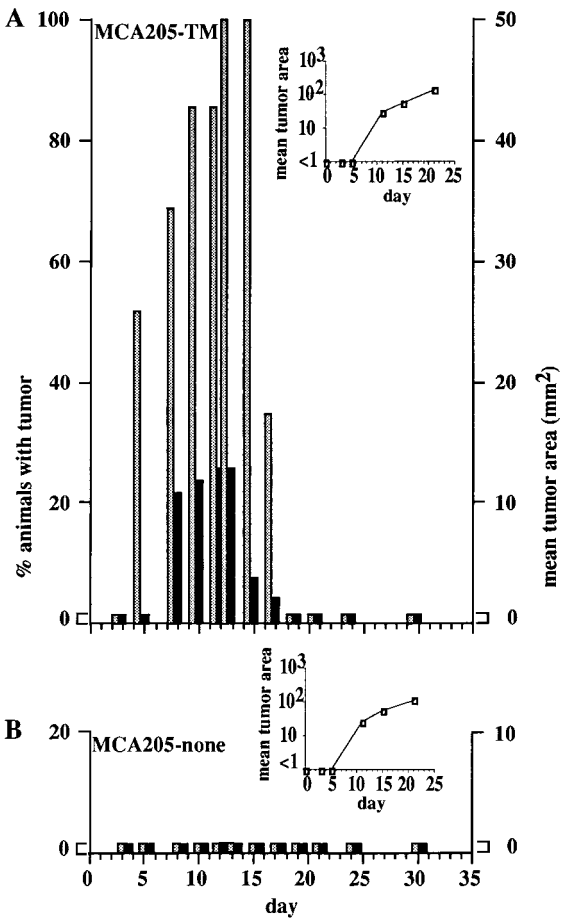

FIG. 3. Induction of tumor cell growth only requires the TM envelope protein. Same conditions as in Fig. 2, for MCA205 cells transduced with the pDFG vector encoding the MoMLV TM (*A*) and no ORF (*B*).

sequence on tumor growth, we used an *in vivo* model derived from that developed by Lotze *et al.* (3) for the Epstein–Barr virus vIL10 protein. This model uses tumor cell lines that are normally rejected by the immune system upon engrafting into immunocompetent mice, but which can grow into tumors following expression by the cells of immunosuppressive molecules. We constructed retroviral expression vectors that contained the entire (pDFG-env) or part (pDFG-TM) of the MoMLV envelope protein gene under control of the retroviral LTR and the neomycin or hygromycin resistance gene inserted 3′ to a ribosomal entry site (see Fig. 1*A*). For the TM construct, sequences for the signal peptide and the proteolytic cleavage site of the envelope polypeptide were maintained to allow processing of the recombinant envelope protein and export to the cell surface (ref. 21 and see below). These expression vectors were then used to generate recombinant retroviruses, upon transient transfection of Bosc23-packaging cell lines (see scheme in Fig. 1*B*). Recombinant retroviruses were used to infect murine tumor cells (MCA205 and CL8.1 cells) thus providing, after selection in either G418 or hygromycin medium, populations of envelope-expressing cells. An immunofluorescence assay by using an anti-TM polyclonal (ref. 24; Fig. 1*C*) or monoclonal antibody (ref. 25 and data not shown) demonstrated that pDFG-env and pDFG-TM both induced expression of the envelope domains at the cell surface, although to a smaller extent for the TM construct, and occurrence of a single peak indicated that all cells—as expected from the selection—were positive within the population. The ability of envelope expression to induce tumor cell proliferation in mice was then assessed by monitoring the onset

of tumors among a series of engrafted mice and the kinetics of their growth.

Induction of Tumor Cell Growth by the Envelope Protein in an Allogeneic Host. In a first series of experiments, we used methylcholanthrene-induced murine fibrosarcoma cells (MCA205; H-2^b haplotype). These tumor cells, when injected into an allogeneic host (BALB/c, H-2^d haplotype), lead to tumors (of reduced size), which can only be detected in a small fraction of the engrafted animals and are further very rapidly rejected by the host immune system (Fig. 2*B*). Under the same conditions, MCA205 cells expressing the envelope protein were able to form easily detectable tumors that persisted for at least 2 wk in a large fraction of the engrafted animals (Fig. 2*A*). This enhancement of tumor cell growth was not observed with any of the control expression vectors: either with "empty" vectors (Fig. 2*B*) or with vectors for other transmembrane proteins unrelated to retroviral envelopes, including the murine CD2 protein (Fig. 2*C*) or the murine receptor for erythropoietin (data not shown). As shown in the Western blot analysis in Fig. 2*D*, the envelope protein can be detected not only in the transduced MCA205-env cells, as expected from the analysis in Fig. 1*C*, but also in the resulting tumors, with a closely related intensity, whereas no signal could be detected in the controls (e.g., MCA205-none cells, and vIL10-induced tumors, see Fig. 2*D* and legend). This indicates that the env-tumors actually result from the proliferation of the engrafted MCA205-env cells and, in turn, that envelope expression is maintained in this *in vivo* process. Fig. 2 also shows that the envelope-mediated enhancement of tumor cell growth is not due to differences in proliferative rates between the envelope-transduced cells vs. control-transduced cells, as similar proliferation rates were measured in all cases when the cells were engrafted, under identical conditions, into syngeneic hosts (C57BL/6, H-2^b haplotype) (Fig. 2 *Insets*). Interestingly, enhancement of tumor cell growth also was observed with the pDFG-TM construct (Fig. 3*A*), a result consistent with previous *in vitro* data locating an immunosuppressive domain within the envelope TM protein. This expression vector therefore was used in all following experiments.

Induction of Tumor Growth by the Envelope Protein in a Syngeneic Host. A physiologically more relevant situation was studied in a second series of experiments, using CL8.1 tumor cells (H-2^b haplotype) that do not proliferate under syngeneic conditions due to an overexpression of MHC class I molecules (14). These cells were rejected when engrafted into an immunocompetent syngeneic host (C57BL/6, H-2^b haplotype; Fig. 4*B*), although they grew into large tumors in all cases when the mice were rendered immunodeficient by prior x-ray irradiation (Fig. 4*D*). By using TM-transduced CL8.1 cells, we showed that expression of the envelope TM resulted in tumor growth in the nonirradiated mice (Fig. 4*A*): a large fraction of the immunocompetent mice (>80%) developed tumors, which grew continuously, leading to animal death. Tumor growth is not due to differences in proliferative rates between the envelope-transduced and control cells, as similar profiles, both for the percentage of animals with tumors and for tumor size, were observed, under identical conditions, in irradiated hosts (Fig. 4 *C* and *D*). No significant differences were either observed for TM-expressing cells between immunodeficient (x-irradiated) and immunocompetent (nonirradiated) mice, thus suggesting that the TM has a very strong immunosuppressive effect. To examine whether TM expression induces a systemic immunosuppression of the animals, we finally made experiments under conditions in which tumor cells not expressing any envelope construct were engrafted contralaterally to those expressing the envelope TM domain. As illustrated in Fig. 5, tumor growth was observed for the envelope-expressing cells, as expected, whereas rejection was systematically observed for the cells injected contralaterally. Thus TM-mediated immunosuppression is locally restricted.

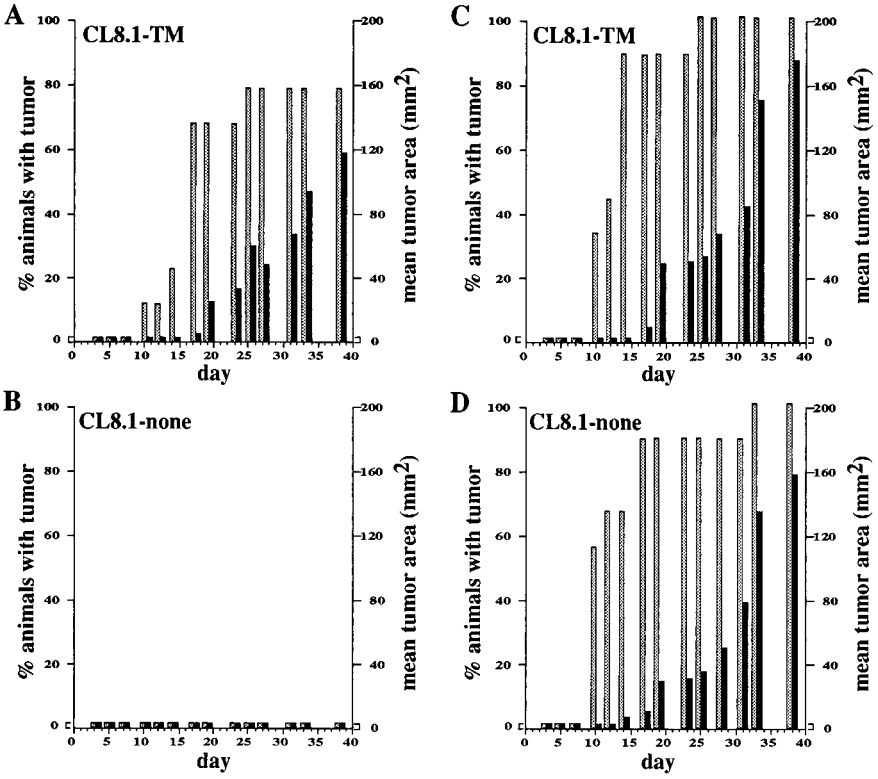

FIG. 4. Induction of tumor cell growth by the retroviral TM protein in a syngeneic host. Tumor cells (CL8.1) transduced with the pDFG vector encoding the TM retroviral envelope protein (*A* and *C*) or no ORF (*B* and *D*) were engrafted into immunocompetent (*A* and *B*) or x-ray irradiated (500 rads; *C* and *D*) syngeneic mice (5×10^4 cells; 6 mice/group). The percentages of animals with tumors and the mean tumor sizes are indicated (grey and black bars, respectively).

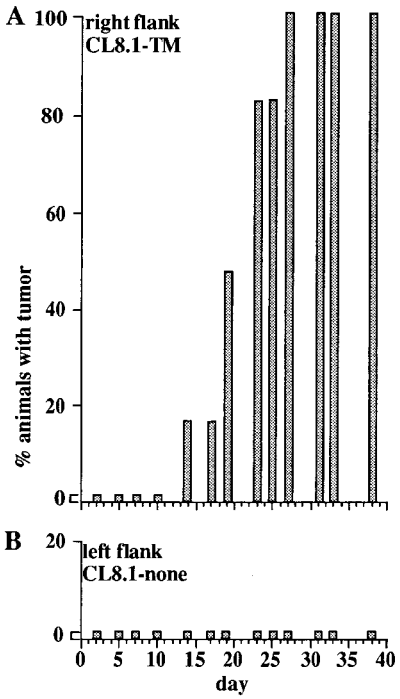

FIG. 5. TM expression does not affect rejection of nontransduced tumor cells at a distant site. Mice (C57BL/6; 6 mice/group) were engrafted with TM-expressing CL8.1 cells (5×10^4 cells) injected s.c. in the right flank and with control CL8.1 cells injected contralaterally (5×10^4 cells).

DISCUSSION

We have shown that the MoMLV envelope has immunosuppressive properties that enables tumor cells expressing this protein to escape rejection by the host immune system. This immunosuppressive effect is local as tumor cells engrafted contralaterally to envelope-expressing cells are not rejected. Yet, it is a strong effect as it can suppress the immune response not only to syngeneic determinants, but also to allogeneic determinants which usually trigger the most stringent immune responses. In the latter case, immunosuppression only delays rejection as in all cases tumor cells are finally rejected, whereas under the more physiological conditions of syngeneic engrafting, immunosuppression finally leads to the animal death through tumor overgrowth. Our data show that the domain(s) within the MoMLV envelope responsible for immunosuppression resides within the envelope TM moiety, a location compatible with previous data disclosing that a peptide corresponding to a conserved domain within retroviral TM proteins, namely the 17-mer CKS-17 peptide, mediates—by still unknown molecular intermediates and mechanisms—immunosuppressive effects *in vitro* (reviewed in refs. 5 and 7). Preliminary experiments involving deletion of this domain within the TM protein resulted in the loss of immunosuppression, but we were unable to demonstrate envelope expression under these conditions (for the effect of mutations on the expression of retroviral TMs, see refs. 27–29), and more refined experimental procedures will be required to unambiguously identify within the TM the "active domain" for the *in vivo* tumor-inducing effects. Our data also show that the entire envelope protein (SU + TM) has immunosuppressive effects similar to those of the TM alone. This result is noteworthy because in a previous attempt to identify possible immunosuppressive effects of retroviral envelopes, Schmidt and Snyderman (15) using transformed-3T3 cells that expressed the entire envelope protein, failed to demonstrate any immunosuppressive effects of the transduced cells, which still did not grow into tumors when engrafted into mice (15). They argued that one possible interpretation for this failure could be the presence of the highly immunogenic SU moiety that would counteract the immunosuppressive effect of the TM. Clearly, this cannot be the correct interpretation and more complex explanations for this failure have to be considered. Among those already proposed by the authors, one might suggest that the cells used in the assay, i.e., 3T3 cells transformed *in vitro* with activated oncogenes, are too far from naturally occurring tumor cells to be prone to immunosuppression, a property that actually might be shared by other transformed cell lines.

There are two possible consequences of the presently demonstrated immunomodulatory effects of the envelope TM domain. One concerns the pathological development of retroviral infections, including those by human retroviruses, because it is conceivable that immunosuppression of the host could be a general "strategy" of invading viruses and possibly be involved in their "penetrance." In this respect, it is worth mentioning that most oncoviruses exert immunosuppressive influences during *in vivo* infections (reviewed in refs. 7 and 30). In the cat, the feline leukemia virus induces leukemia but also a severe immunosuppression leading to death, without leukemia, in >30% of the infected animals (reviewed in ref. 31). Immunosuppression of the host could therefore be an important factor not only in the rate of the pathological development of viral infections, but also in the nature of the pathologies themselves. A second important consequence of the immunosuppressive effect of retroviruses concerns endogenous retroviruses. These elements are numerous (>1% of the mammalian genomes), and several families of such elements have been identified (referred to as HERV in the human genome, reviewed in refs. 32–34). Some of them contain env-like genes with a putative immunosuppressive domain (reviewed in refs. 7 and 35). For instance the HERV-H family, which comprises ≈100 copies of env-containing elements, shows almost perfect identity with the MoMLV sequence within the envelope-subdomain corresponding to the immunosuppressive peptide identified by using *in vitro* assays. The present assay will allow a characterization of the immunosuppressive effects of these endogenous elements under *in vivo* conditions and, consequently, of the incidence of their expression in the development of tumoral processes.

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