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Dissecting the Immune Response to Moloney Murine Sarcoma/Leukemia Virus-Induced Tumors by Means of a DNA Vaccination Approach

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The intramuscular inoculation of Moloney murine sarcoma/leukemia (M-MSV/M-MuLV) retroviral complex gives rise to sarcomas that undergo spontaneous regression due to the induction of a strong immune reaction mediated primarily by cytotoxic T lymphocytes (CTL). We used a DNA-based vaccination approach to dissect the CTL response against the Gag and Env proteins of M-MSV/M-MuLV in C57BL/6 (B6) mice and to evaluate whether plasmid DNA-immunized mice would be protected against a subsequent challenge with syngeneic tumor cells expressing the viral antigens. Intramuscular DNA vaccination induced CTL against both Gag and Env proteins. A detailed analysis of epitopes recognized by CTL generated in mice inoculated with the whole virus and with the Gag-expressing plasmid confirmed the presence of an immunodominant peptide in the leader sequence of Gag protein ($Gag_{85–93}$, CCLCLTVFL) that is identical to that described in B6 mice immunized with Friend MuLV-induced leukemia cells. Moreover, CTL generated by immunization with the Env-encoding plasmid recognized a subdominant Env peptide ($Env_{189–196}$, SSWDFITV), originally described in the B6.CH-2^{bm13} mutant strain. B6 mice immunized with the Gag-expressing plasmid were fully protected against a lethal tumor challenge with M-MuLV-transformed MBL-2 leukemia cells, while vaccination with the Env-expressing plasmid resulted in rejection of the tumor in 44% of the mice and in increased survival of an additional 17% of the animals. Taken together, these results indicate the existence of a hierarchy in the capacity of different structural viral proteins to induce a protective immune response against retrovirus-induced tumors.

Lymphoma and leukemia cell lines originally induced by the antigenically related Friend, Moloney, and Rauscher (FMR) murine leukemia viruses (MuLV) have been widely used to study the role of the T-cell-mediated immune response in the eradication of advanced disseminated malignancies (9, 20, 28). The importance of cellular immunity in controlling tumor growth has also been studied in physiologic models represented by the natural oncogenic process that follows FMR virus infection (11, 12, 21).

In particular, Moloney murine sarcoma virus (M-MSV) is a replication-defective, acutely transforming retrovirus whose replication defect can be overcome through the helper activity of chronic transforming M-MuLV, which encodes the viral envelope components that are necessary for cell infection. Intramuscular injection of the M-MSV/M-MuLV complex gives rise to sarcomas that develop at the inoculation site after a short latency period and regress spontaneously following the induction of a strong immune reaction, which is mediated primarily by cytotoxic T lymphocytes (CTL) (13–15).

As recently reviewed by Hasenkrug and Chesebro (21), studies aimed at identifying antigenic epitopes recognized by virus-specific effector cells have focused mainly on products of the *gag* and *env* structural genes (16, 22, 32).

In analyses of mice vaccinated with recombinant vaccinia virus expressing F-MuLV *env* and *gag* genes, $CD4^+$ cells were shown to be activated by immunization with an Env-expressing construct while CTL were activated by immunization with a Gag-expressing construct (20, 23). Their recognition by different T-cell subsets suggested that the *gag* and *env* gene products might undergo different antigen processing and presentation pathways (20). However, recent reports demonstrated that both *env*- and *gag*-encoded proteins of FMR-MuLV contained immunogenic peptides that could associate with both class I and class II major histocompatibility complex (MHC) molecules and thus were able to activate both CTL and $CD4^+$ cells (21).

To investigate the capacity of different M-MSV/M-MuLV proteins to induce CTL generation and confer protection against challenge with leukemia cells bearing the relevant antigens, we took advantage of DNA-based immunization, a recently developed procedure based on the intramuscular or intradermal transfer of a plasmid DNA expression vector coding for a specific antigen (8, 34). This vaccination approach affords long-lasting induction of a cellular as well as a humoral immune response against both infectious agents and tumor-associated antigens (18). We recently demonstrated that mice immunized with a plasmid expressing the tumor-specific antigen P1A generated a strong CTL response and were protected against a subsequent challenge with tumor cells expressing the relevant antigen (30).

In the present study, we evaluated the CTL response induced in B6 mice following DNA vaccination with plasmids encoding the M-MuLV *gag* and *env* genes in comparison with that induced following injection of the M-MSV/M-MuLV retroviral complex and carried out a detailed analysis of epitopes recognized by virus-specific CTL. We also tested whether the immunization achieved was capable of protecting against challenge with a tumor cell line expressing the viral antigens as

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tumor-associated antigens. The results showed that immunization with Gag-expressing plasmid mimicked the immune response to virus infection, in terms of both CTL generation and protection against tumor growth; although DNA vaccination with the Env-expressing vector was effective in generating CTL, it did not confer complete protection against tumor challenge.

MATERIALS AND METHODS

Mice. Female C57BL/6 (B6) mice, 5 to 6 weeks old, were purchased from Charles River Laboratories (Calco, Como, Italy). Procedures involving animals and their care conformed with institutional guidelines that comply with national and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, Dec. 12, 1987; *NIH Guide for the Care and Use of Laboratory Animals*, NIH Publication 85-23, 1985; *UKCCR Guidelines for the Welfare of Animals in Experimental Neoplasia* [35]). Mice used for the in vivo tumor growth experiments were examined daily, and in compliance with our normal practice, premoribund animals were sacrificed by being given an ethyl ether overdose.

Tumor cell lines. MBL-2 is a leukemia cell line (*H-2*^{b}) derived from an M-MuLV-infected B6 mouse; EL-4$^-$ (*H-2*^{b}) is a chemically induced thymoma, and EL4$^+$ is a variant infected by and expressing antigens of FMR-type viruses. 293D^b and 293K^b are derivatives of the transformed human embryonic kidney cell line 293, stably transfected with the murine MHC class I restriction elements *H-2D*^{b} and *H-2K*^{b}, respectively (kindly provided by D. Perry-Lalley, Surgery Branch, National Cancer Institute, Bethesda, Md.) (3). All tumor cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL, Paisley, United Kingdom) supplemented with 2 mM L-glutamine, 10 mM HEPES, 20 μM 2-mercaptoethanol, 150 U of streptomycin per ml, 200 U of penicillin per ml, and 5 or 10% heat-inactivated fetal bovine serum (FBS) (GIBCO BRL). 293D^b and 293K^b cells were cultured in the presence of 0.4 mg of G418 (GIBCO) per ml.

Plasmids. The *gag* gene of M-MuLV was derived from pMov-9, a biologically active molecular clone of M-MuLV in pBR322 (19), as an *Eag*I-*Asp*718 I^ restriction fragment and cloned into intermediate plasmid vectors. The insert was then transferred to the eukaryotic expression vector pcDNA3 (Invitrogen BV, Leek, The Netherlands) as an *Eco*RI-*Xba*I fragment, resulting in pcDNA3-*gag*, in which the *gag* coding sequence spans nucleotides 346 to 2554 of the viral genome. Plasmid pCMV-eco*env*-bpA (a gift of N. Somia, The Salk Institute for Biological Studies, La Jolla, Calif.) is based on Bluescript KS+ (Stratagene, La Jolla, Calif.) and contains the human cytomegalovirus early promoter, the ecotropic *env* gene of M-MuLV, and the polyadenylation signal/site of the bovine growth hormone gene. Mock plasmid pCMV-ecoΔ was derived from pCMV-eco*env*-bpA by digestion with *Xba*I to remove the *env* gene. Restriction enzymes were purchased from New England Biolabs (Hitchin, United Kingdom) and Boehringer (Mannheim, Germany). Plasmid DNA was purified with Qiagen columns (Qiagen GmbH, Hilden, Germany).

DNA immunization and virus inoculation protocols. Mice were anesthesized by ethyl ether inhalation and injected intramuscularly (i.m.) three times at 20-day intervals with 100 μg of plasmid in 100 μl of saline solution (50 μl was injected into each tibialis anterior muscle). Mice that underwent complete tumor regression following i.m. injection of 100 μl of cell extract containing defective M-MSV copelleted with its natural helper M-MuLV served as positive controls for CTL production. The M-MSV/M-MuLV cell extract was prepared from primary sarcomas induced by serial passages in 1-week-old BALB/c mice, which had an in vitro M-MSV titer of 3×10^5 PFU/ml on 3T3/FL cells.

MLTC and MLPC. At 20 days after the last inoculation with plasmid DNA, spleens were removed and 2.5×10^7 splenocytes were restimulated in vitro in a mixed leukocyte tumor culture (MLTC) with 10^6 syngeneic irradiated (60 Gy) MBL-2 cells or in a mixed leukocyte peptide culture (MLPC) with peptides corresponding to amino acids 85 to 93 of gPr80^{gag} (CCLCLTVFL [10]) or amino acids 189 to 196 of gp70^{env} (SSWDFITV [32]). Peptides were synthesized and purified by Tecnogen (Piana di Monte Verna, Caserta, Italy), dissolved to 1 mM in dimethyl sulfoxide (stock solution), and then diluted in tissue culture medium to a final concentration of 1 μM. The cultures were set up in 15 ml of DMEM–10% FBS, maintained in 25-cm^2 tissue culture flasks (Falcon, Becton Dickinson, Lincoln Park, N.J.) for 5 days at 37°C under 5% CO_2, and then tested for their lytic activity in a ^{51}Cr release assay.

Analysis of CTLp frequency in limiting-dilution assays and generation of specific CTL clones. Thirty replicate microcultures containing various numbers of responder spleen cells were stimulated with 3×10^4 irradiated (60 Gy) MBL-2 cells and 3×10^5 irradiated (30 Gy) syngeneic spleen cells in 200 μl of DMEM–10% FBS supplemented with 40 U of recombinant interleukin-2 (Boehringer) per ml. On day 7, the cultures were divided into three aliquots and tested for cytotoxic activity in ^{51}Cr release assays (see below) with MBL-2, EL-4$^-$, and $Gag_{85–93}$-pulsed EL-4$^-$ cells as targets. Positive cultures were defined as those in which the experimental release values exceeded the spontaneous release values by 3 standard deviations (SD). Minimal estimates of CTL precursor (CTLp) frequencies were calculated from the zero-order term of the Poisson distribution with linear regression analysis by the least-squares method (15). Microcultures of spleen cells from mice in which an M-MSV/M-MuLV-induced sarcoma regressed, as well as from pcDNA3-*gag*-injected animals and pCMV-eco*env*-bpA-injected animals, were also subjected to limiting-dilution cloning for the generation of specific CD8$^+$ CTL clones. These clones were restimulated once a week with irradiated MBL-2 cells and syngeneic spleen cells under the conditions indicated above and assayed for peptide specificity in ^{51}Cr release assays.

^{51}Cr release assay. Cytolytic activity was tested by using a short-term incubation assay. Briefly, 2×10^3 ^{51}Cr-labeled target cells were incubated with effector cells at various effector-to-target-cell ratios in 96-well microplates. After 4 or 6 h of incubation at 37°C, supernatants were harvested and radioactivity was counted in a microplate scintillation counter (Top-Count; Packard Instrument Co., Meriden, Conn.). For peptide pulsing, 10^6 ^{51}Cr-labeled 293D^b, 293K^b or EL-4$^-$ cells per ml were incubated for 30 min at 37°C with $Gag_{85–93}$ or $Env_{189–196}$ peptide at a final concentration of 0.5 and 5 μM, respectively, and then washed three times before use.

Tumor protection assay. At 5 weeks after the last DNA immunization, B6 mice were challenged subcutaneously (s.c.) with 2×10^5 MBL-2 cells and then monitored for a total of 120 days after tumor inoculation. Mice injected with the mock plasmid and nonimmunized animals served as negative controls. Statistical analysis of differences among experimental groups was carried out by the Mantel-Haenszel test.

RESULTS

The $Gag_{85–93}$ peptide is the immunodominant epitope recognized by CTL from M-MSV/M-MuLV-induced tumor regressor mice. It was recently reported that most CTL from B6 mice immunized with FBL-3 tumor cells, a murine leukemia cell line originally induced by F-MuLV, recognize a single antigenic peptide (CCLCLTVFL), which is localized within the leader sequence and has been mapped to amino acids 85 to 93 of gPr80^{gag}, in the transmembrane domain of the protein (10). This antigenic epitope is restricted by the *H-2D*^{b} molecule and is shared by FMR-induced leukemias (5, 10). To evaluate whether this peptide represents the major epitope recognized by CTL of B6 mice inoculated with M-MSV/M-MuLV, we obtained splenocytes from mice in which an M-MSV/M-MuLV-induced sarcoma had regressed after retroviral complex inoculation (referred to hereafter as tumor regressor mice) and performed limiting-dilution analysis to compare the frequency of CTLp specific for M-MuLV antigen(s) and for the relevant nonapeptide. As shown in a representative experiment (Fig. 1), we detected a high frequency of CTLp against FMR-MuLV-negative EL-4$^-$ cells pulsed with $Gag_{85–93}$ peptide (1 in 2,100), which was comparable to the frequency of CTLp (1 in 1,700) against MBL-2 tumor cells, a leukemia cell line derived from an M-MuLV-infected mouse. Moreover, the results of cold-target competition assays carried out on MLTC bulk cultures from tumor regressor mice showed that the addition of increasing numbers of unlabeled MBL-2 or EL-4$^-$ cells pulsed with $Gag_{85–93}$ peptide reduced the lysis of ^{51}Cr-labeled MBL-2 target cells by a similar extent (data not shown). Taken together, these data strongly suggest that the $Gag_{85–93}$ peptide represented the immunodominant epitope for CTL generated during the course of the immune response to M-MSV/M-MuLV virus infection, although we cannot rule out the possibility that restimulation with MBL-2 leukemia cells does not reveal some epitopes unique to the defective MSV component.

CTL generation in B6 mice immunized with plasmid DNA expressing M-MuLV Gag. The results described above prompted us to test whether the injection of plasmid DNA coding for M-MuLV Gag protein would generate a strong CTL response against the immunodominant *gag*-encoded antigen. To this end, mice were inoculated i.m. three times with 100 μg of pcDNA3-*gag* plasmid at 20-day intervals; animals injected with the same vector lacking the insert (pcDNA3) served as negative controls, while tumor regressor mice were used as positive controls. At 20 days after the last plasmid DNA inoculation, splenocytes were stimulated in MLTC with irradiated

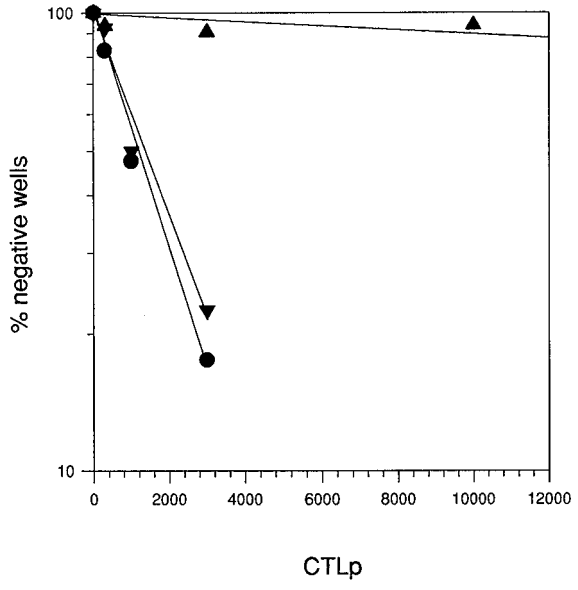

FIG. 1. Frequency of CTLp against the Gag_{85-93} CCLCLTVFL peptide in tumor regressor B6 mice. Minimal frequencies of CTLp specific for MBL-2 cells (●), Gag_{85-93} peptide-pulsed EL-4$^-$ cells (▼), and FMR-MuLV-negative EL-4$^-$ cells (▲) were estimated by linear-regression analysis. Limiting numbers of responder spleen cells from virus-induced tumor regressor B6 mice were cultivated as reported in Materials and Methods and divided into three portions for testing in ^{51}Cr release assays.

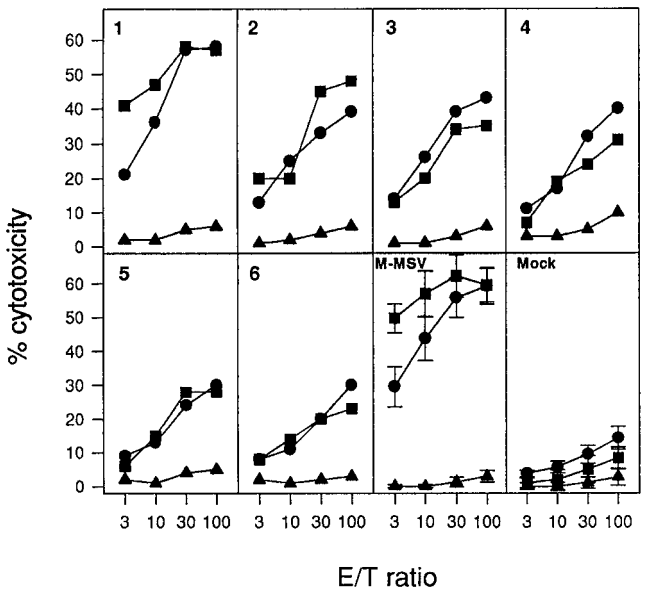

FIG. 2. CTL generation in B6 mice immunized with pcDNA3-*gag*. Plots 1 to 6 show the cytotoxicity of MLTC from splenocytes of six pcDNA3-*gag*-injected mice after restimulation in vitro with MBL-2 tumor cells. Lytic activity was evaluated with ^{51}Cr-labeled FMR-MuLV-positive MBL-2 cells (●), EL-4$^+$ cells (■), and FMR-MuLV-negative EL-4$^-$ cells (▲) as targets. The cytotoxicities of MLTC generated from four tumor regressor mice (plot M-MSV) and from six pcDNA3-injected mice (plot Mock) are shown as mean and SD in the bottom right-hand plots. The lytic activity of CTL from immunized mice was considered positive when it exceeded the mean lysis values of mock-injected animals + 3SD. E/T, effector/target.

syngeneic MBL-2 leukemia cells, and lytic activity was evaluated 5 days later in a short-term ^{51}Cr release assay. As shown in Fig. 2, all pcDNA3-*gag*-injected mice generated CTL that efficiently lysed MBL-2 target cells, as well as EL-4$^+$ cells, a syngeneic leukemia subline expressing FMR-MuLV-induced antigens. The observation that the parental cell line EL-4$^-$, which lacks the relevant viral antigens, was resistant to lysis indicated that the cytotoxic activity was virus specific. As expected, this cytolysis was MHC restricted, since allogeneic (*H-2^d*) LSTRA target cells expressing M-MuLV antigens were not killed (data not shown).

Next, we analyzed whether vaccination with pcDNA3-*gag* would result in the generation of CTL specific for the Gag_{85-93} antigenic peptide. Thus, spleen cells from another group of B6 mice immunized with pcDNA3-*gag* as described above were stimulated in MLPC by adding 1 μM of Gag_{85-93} peptide to the cultures. As shown in Fig. 3, a very strong cytotoxic response was generated in all instances; it is noteworthy that stimulation with Gag_{85-93} peptide generated CTL that not only killed EL-4$^-$ cells pulsed with Gag_{85-93} but also efficiently lysed EL-4$^+$ target cells expressing the FMR-MuLV antigens. This result suggests that DNA immunization might mimic the natural immune response to viral infection and hence could provide a useful procedure to define the role of the different epitope(s) within the M-MSV/M-MuLV sequence that elicits an efficient CTL response to the virus.

CTL generation in B6 mice immunized with plasmid DNA expressing M-MuLV Env. It was recently reported that M-MSV/M-MuLV infection in the B6-derived mutant B6.CH-2^{bm13} strain of mice, whose class I D^b antigen-presenting groove is partly shaped by a class I K^b-encoded sequence, induces a strong CTL response against an immunodominant K^b-restricted peptide that spans amino acids 189 to 196 of the M-MuLV Env protein (i.e., SSWDFITV). However, although $Env_{189-196}$-specific CTL could be induced in normal B6 mice by peptide immunization, the frequency of CTLp recognizing the K^b-restricted SSWDFITV peptide in B6 tumor regressor mice is very low (32).

To assess whether immunization with plasmid DNA expressing Env would also result in the induction of a strong CTL response, B6 mice were injected three times with pCMV-eco*env*-bpA as reported above and sacrificed 20 days after the last plasmid DNA injection, and spleen cells were stimulated in MLTC with MBL-2 cells. Mice receiving repeated injections with the pCMV-ecoΔ mock plasmid were used as negative controls, while tumor regressor mice served as positive controls. As shown in a representative experiment (Fig. 4), spleen cells from five of seven pCMV-eco*env*-bpA-injected mice showed high lytic activity against EL-4$^+$ and MBL-2 target cells, while in two mice the cytotoxicity was less than 3 SD above the mean value obtained in MLTC of mock-treated control mice. These results indicate that a DNA-based immunization approach is capable of expanding a CTL population specific for an M-MSV/M-MuLV epitope that is poorly recognized in the course of the virus infection.

Analysis of MHC restriction of Gag- and Env-specific CTL. To finely dissect the CTL response in tumor regressor and plasmid-inoculated mice and to assess the restriction specificity of the antigenic epitopes recognized by Gag- and Env-specific CTL, the human embryonic cell lines 293D^b and 293K^b were pulsed with Gag_{85-93} and $Env_{189-196}$ peptides, respectively, and used as targets of CTL generated from spleens of tumor regressor mice or mice injected with the Gag- or Env-expressing plasmids. CTL were generated in MLTC with MBL-2 cells or in MLPC following addition of Gag_{85-93} (CCLCLTVFL) or $Env_{189-196}$ (SSWDFITV) antigenic peptides. As reported in Fig. 5, MLTC from tumor regressor mice efficiently lysed both

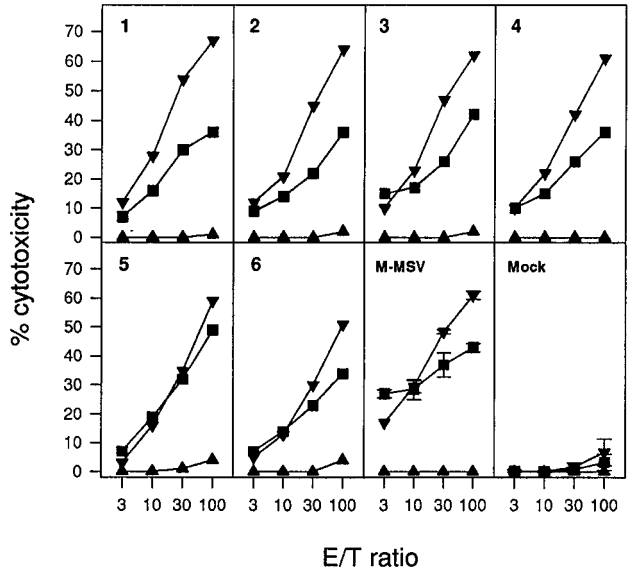

FIG. 3. CTL activity of splenocytes from pcDNA3-*gag*-injected mice following in vitro restimulation with the Gag_{85-93} CCLCLTVFL peptide. Plots 1 to 6 show the lytic activity of MLPC from splenocytes of six pcDNA3-*gag*-injected mice against ^{51}Cr-labelled EL-4$^+$ cells (■), EL-4$^-$ cells (▲), and Gag_{85-93} peptide-pulsed EL-4$^-$ cells (▼). The cytotoxicities of MLPC generated from four tumor regressor mice (plot M-MSV) and from six pcDNA3-injected mice (plot Mock) are shown as mean and SD in the bottom right-hand plots. E/T, effector/target.

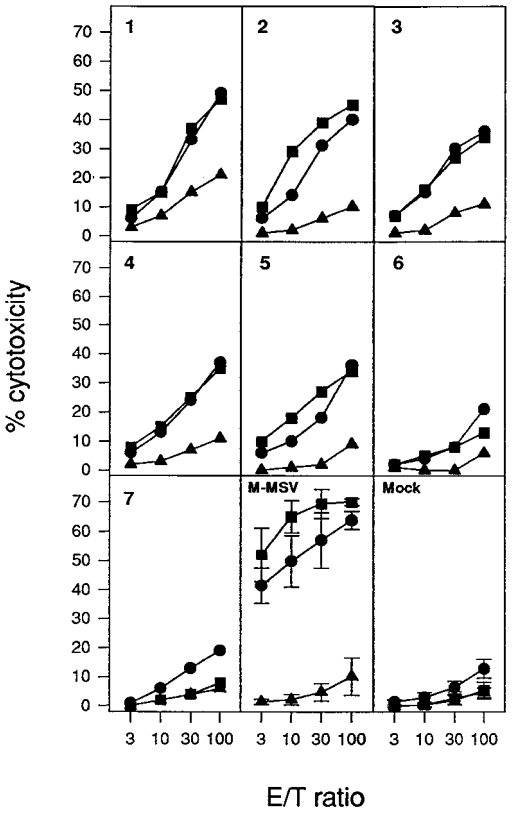

FIG. 4. CTL generation in B6 mice immunized with pCMV-eco*env*-bpA. Plots 1 to 7 report the lytic activity of MLTC from splenocytes of seven pCMV-eco*env*-bpA-injected mice, after restimulation in vitro with MBL-2 tumor cells, against ^{51}Cr-labeled FMR-MuLV-positive MBL-2 cells (●), EL-4$^+$ cells (■), and FMR-MuLV-negative EL-4$^-$ cells (▲). The cytotoxicities of similar MLTC generated from five tumor regressor mice (plot M-MSV) and from eight pCMV-ecoΔ-injected mice (plot Mock) are depicted as mean and SD in the bottom right-hand plots. E/T, effector/target.

MBL-2 and Gag_{85-93}-pulsed 293D^b cells and displayed weak cytotoxic activity against 293K^b cells loaded with $Env_{189-196}$. However, $Env_{189-196}$-loaded 293K^b target cells were killed to a much greater extent by MLTC from pCMV-eco*env*-bpA-injected mice; this cytotoxic activity was highly specific, since lysis of MBL-2 cells but not against 293D^b cells pulsed with Gag_{85-93} peptide was also detected. As expected, the cytotoxic activity of MLTC from pcDNA3-*gag*-injected mice was directed only against the Gag_{85-93} peptide and was D^b restricted. MLTC from animals injected with either of the two mock plasmids did not show lytic activity against any target (data not shown).

These data were further confirmed by analysis of lytic activities of MLPC prepared with the Gag_{85-93} and $Env_{189-196}$ synthetic peptides as stimulators. MLPC of Gag_{85-93}-stimulated splenocytes from both tumor regressor and pcDNA3-*gag*-injected mice exhibited very high cytotoxic activity against MBL-2 and Gag_{85-93}-loaded 293D^b cells but no cytolytic activity against $Env_{189-196}$-pulsed 293K^b cells. Splenocytes from mice inoculated with pCMV-eco*env*-bpA showed no lytic activity after in vitro stimulation with Gag_{85-93} peptide.

On the other hand, splenocytes stimulated with $Env_{189-196}$ peptide generated CTL specific for the Env-derived epitope in all of the pCMV-eco*env*-bpA-inoculated mice, while only few tumor regressor mice showed substantial cytotoxic activity against $Env_{189-196}$-pulsed 293K^b cells. Splenocytes derived from animals injected with pcDNA3-*gag* and restimulated in MLPC with the $Env_{189-196}$ peptide did not exhibit any cytotoxicity against the panel of target cells.

Analysis of CTL clones derived from tumor regressor and plasmid-injected mice. We next derived a large panel of CTL clones from splenocytes of tumor regressor B6 mice by limiting-dilution cloning with MBL-2 tumor cells as stimulators and tested them for their specificity against syngeneic virus-transformed cells and FMR-MuLV-negative Gag_{85-93} peptide-pulsed tumor cells. Of 97 CTL clones analyzed, 89 (92%) were indeed specific for the Gag-derived epitope associated with D^b MHC class I, as illustrated by the results for the representative clone 76GM shown in Fig. 6, while 8 clones killed MBL-2 cells but not Gag_{85-93} peptide-pulsed cells. These eight clones were found to recognize the $Env_{189-196}$ peptide in the K^b context (e.g., clone 13/1 [Fig. 6]). These observations indicate that the few CTL emerging from tumor regressor mice that were not specific for the immunodominant Gag epitope were directed against this subdominant Env peptide. Moreover, a set of CTL clones obtained from mice immunized with pcDNA3-*gag* exhibited the same specificity as the majority of CTL clones from tumor regressor mice and killed MBL-2 cells, EL-4$^+$ cells, and EL-4$^-$ cells pulsed with Gag_{85-93} peptide but did not kill EL-4$^-$ cells (data not shown).

Figure 6 also shows the cytotoxicity of two clones (123 and 167) that were representative of more than 30 clones derived from mice injected with pCMV-eco*env*-bpA. These clones efficiently lysed MBL-2 cells and showed no cytotoxic activity against 293D^b or 293K^b cells. Clones 123 and 167 also killed $Env_{189-196}$-pulsed 293K^b cells but were inactive against Gag_{85-93}-loaded 293D^b cells, thus showing the same specificity as clone 13/1.

Protection against challenge with an M-MuLV-induced MBL-2 leukemia cell line in mice immunized with pcDNA3-*gag* or pCMV-eco*env*-bpA. Our previous finding that CTL play an

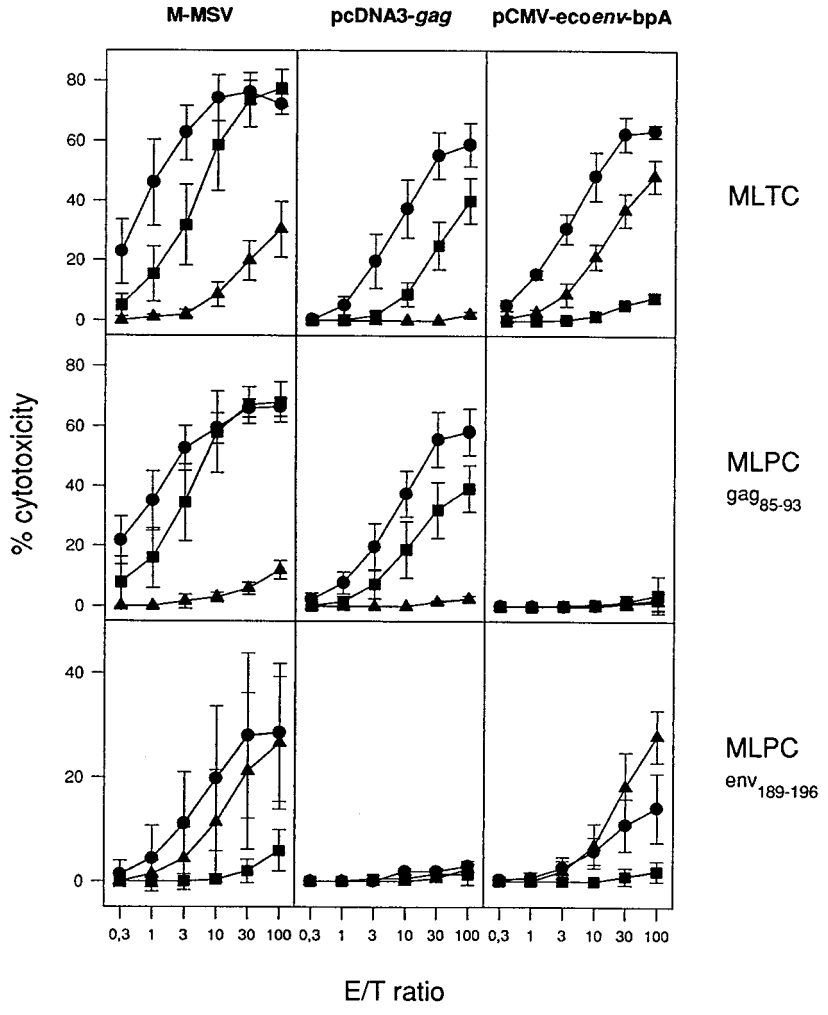

FIG. 5. Peptide specificity and MHC restriction of CTL induced by immunization of B6 mice with M-MSV/M-MuLV, pcDNA3-*gag*, or pCMV-eco*env*-bpA. Splenocytes from five tumor regressor mice and five mice vaccinated with either pcDNA3-*gag* or pCMV-eco*env*-bpA were restimulated in vitro with MBL-2 tumor cells (MLTC) or with Gag_{85-93} or $Env_{189-196}$ peptide (MPLC). The lytic activity of the cultures, reported as the mean and SD, was assayed against FMR-MuLV-positive MBL-2 cells (●) Gag_{85-93} peptide-pulsed $293D^b$ cells (■), and $Env_{189-196}$ peptide-pulsed $293K^b$ cells (▲). No lysis was observed against $293D^b$ and $293K^b$ cells without peptide, $293D^b$ cells plus $Env_{189-196}$ peptide, or $293K^b$ cells plus Gag_{85-93} peptide. E/T, effector/target.

essential role in the immune response against M-MSV/M-MuLV-induced tumors led us to evaluate whether CTL generation in mice immunized with plasmid DNA coding for individual structural viral proteins would confer resistance to a subsequent challenge with a lethal dose of tumor cells expressing virus antigens. Therefore, B6 mice vaccinated with pcDNA3-*gag* were challenged with 2×10^5 MBL-2 leukemia cells 5 weeks after the last immunization; nonimmunized mice and mice injected three times with pcDNA3 vector were used as negative controls. As shown in a representative experiment reported in Fig. 7, all the mice vaccinated with pcDNA3-*gag* were protected ($P < 0.001$) while all the nonimmunized and mock-inoculated mice rapidly developed tumors and had to be sacrificed, with mock-injected mice showing a slightly increased survival time compared to nonimmunized mice ($P = 0.02$). Interestingly, in one experiment, two pcDNA3-*gag*-immunized mice that had rejected the primary tumor graft developed a secondary tumor that appeared 6 weeks after tumor regression at the site of the original inoculum. However, these tumor cells were not lysed by Gag-specific CTL obtained in MLTC or by CTL clones that specifically recognized the Gag peptide; moreover, cytofluorimetric analysis revealed that these tumor cells were not stained by an anti-gp70 monoclonal antibody (MAb) but expressed K^b and D^b molecules at levels comparable to those of the parental MBL-2 cell line (data not shown).

Figure 8 shows the cumulative results obtained with 18 B6 mice that were vaccinated with pCMV-eco*env*-bpA and whose survival was compared with that of 31 nonimmunized mice and 18 pCMV-ecoΔ-injected mice. The pCMV-eco*env*-bpA-immunized mice showed increased survival compared to the nonimmunized and mock-injected animals ($P < 0.001$); no difference in survival was observed between the two control groups ($P = 0.139$). Specifically, vaccination with the Env-encoding vector fully protected 8 of 18 mice (44%) against challenge with MBL-2 leukemia cells and prolonged the survival time of 3 other vaccinated animals (17%).

DISCUSSION

In the present study, we demonstrate that DNA immunization with an M-MSV/M-MuLV Gag-expressing plasmid that

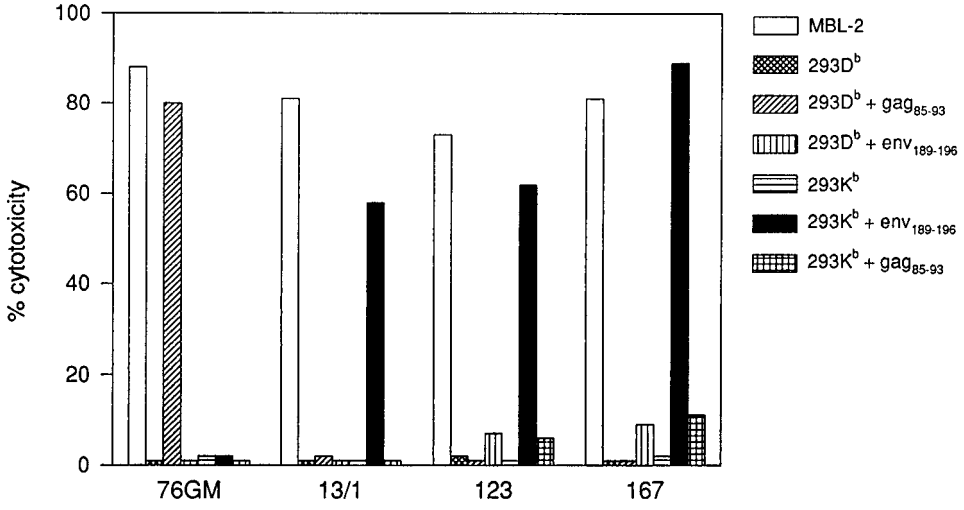

FIG. 6. Cytotoxic activity of CD8$^+$ CTL clones derived from tumor regressor mice and from mice immunized with pCMV-eco*env*-bpA. The specificity of lysis of two representative CTL clones derived from tumor regressor mice (76GM and 13/1) and two CTL clones derived from pCMV-eco*env*-bpA-injected mice (123 and 167) was evaluated against the indicated target cells at an effector-to-target-cell ratio of 10.

included the naturally occurring immunodominant epitope conferred complete protection against subsequent challenge with tumor cells bearing the relevant virus antigens; substantial therapeutic efficacy was also achieved by immunizing mice with a plasmid containing the *env* gene, which expresses a subdominant epitope that is weakly immunogenic during virus infection.

Our data extend the results of studies by Chen et al. (10) conducted with B6 mice that demonstrated that the majority of CTL produced in response to immunization with an F-MuLV-induced leukemia cell line were specific for a Gag epitope ($Gag_{85–93}$; CCLCLTVFL). Our findings indicate that $Gag_{85–93}$ also represents the immunodominant epitope during the course of the natural immune response to M-MSV/M-MuLV infection in mice having the *H-2*^{b} haplotype. Analyses of tumor regressor mice showed that (i) the frequency of CTLp against MBL-2 leukemia cells almost completely paralleled that against EL4$^-$ leukemia cells pulsed with $Gag_{85–93}$ nonapeptide, (ii) the majority of the CTL clones from virus-injected mice were specifically directed against target cells loaded with this peptide, and (iii) the cytotoxic activity of bulk MLTC against MBL-2 target cells was inhibited in a dose-dependent manner in the presence of $Gag_{85–93}$-pulsed EL-4$^-$ cells used as cold targets. On the contrary, in the F-MuLV model, mice infected with the retrovirus generated a CTL response directed predominantly against a determinant(s) in the F-MuLV envelope protein (21).

Moreover, we observed that immunization with the Gag-expressing plasmid brought about a striking induction of CTL that were specific for the immunodominant peptide recognized by CTL from tumor regressor mice and, more importantly, resulted in full protection against tumor challenge. The therapeutic effects we obtained are particularly relevant in light of

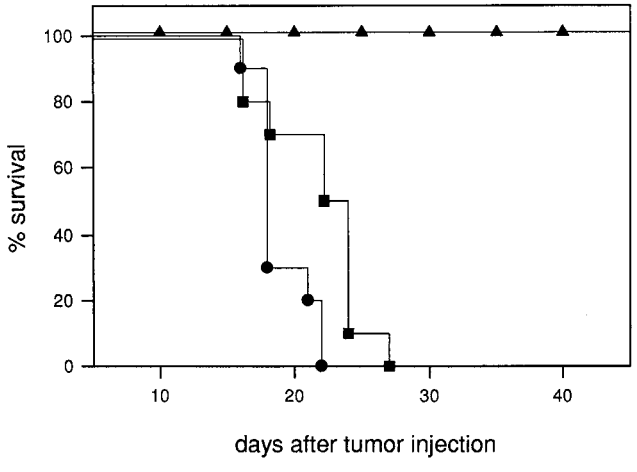

FIG. 7. Protection of B6 mice vaccinated with pcDNA3-*gag* from challenge with MBL-2 tumor cells. Shown is a representative survival experiment in which two groups of 10 B6 mice were injected three times with either 100 μg of pcDNA3-*gag* (▲) or mock plasmid pcDNA3 (■) and then challenged with 2×10^5 MBL-2 leukemia cells s.c. 5 weeks after the last plasmid immunization. Ten nonimmunized mice (●) served as controls.

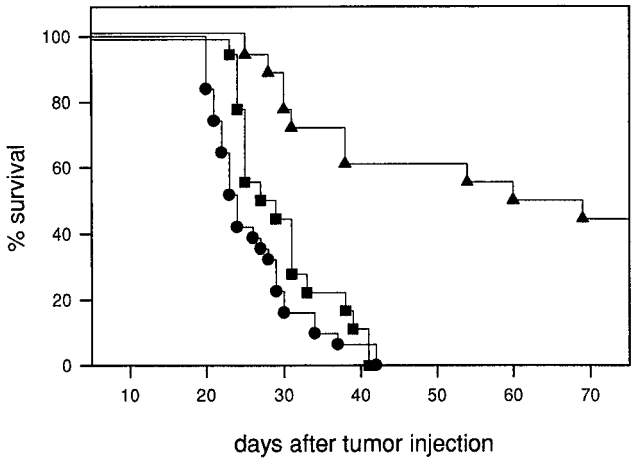

FIG. 8. Survival of pCMV-eco*env*-bpA-immunized B6 mice after challenge with MBL-2 tumor cells. B6 mice inoculated three times with 100 μg of pCMV-eco*env*-bpA ($n = 18$) (▲) or with mock plasmid pCMV-ecoΔ ($n = 18$) (■) were challenged with 2×10^5 MBL-2 leukemia cells s.c. 5 weeks after the last immunization. Nonimmunized mice ($n = 31$) (●) were used as controls.

a recent report showing that immunization of B6 mice with the $Gag_{85–93}$ peptide alone protected only 20 to 40% of animals against tumor development and that the association of this CTL epitope with a virus-specific Env-derived T-helper epitope was needed to achieve nearly complete protection (27).

Two hypotheses may explain the higher protective efficacy of DNA vaccination than of vaccination with a synthetic Gag peptide. It was recently reported that CTL induction requires the participation of dendritic cells that are activated and "licensed" by $CD4^+$ T cells (1, 29, 31). The fact that plasmid DNA is endowed with immunostimulatory properties capable of inducing IL-12 production (24) and dendritic-cell maturation (33) leads to the suggestion that it is able per se to provide dendritic cells with the necessary signal to properly present the *gag*-derived epitope to CTL.

However, the observation by Ossendorp et al. (27) that administration of the immunodominant Gag peptide in combination with a non-virus-specific T-helper epitope failed to confer protection argues against the hypothesis that bystander help favors the induction of CTL against M-MSV/M-MuLV tumors.

Alternatively, the *gag* sequence itself might contain a T-helper epitope(s). In this regard, it is noteworthy that a protective epitope capable of activating $CD4^+$ T-helper cells was identified by Miyazawa et al. (26) following immunization with recombinant vaccinia virus expressing the F-MuLV *gag* gene. This epitope, whose precise sequence has not yet been identified, is located in the N-terminal region of the Gag precursor and presents strong homology to the M-MuLV sequence. Given that DNA vaccination has also been reported to induce the sensitization of $CD4^+$ Th1 cells (7), it is quite likely that immunization with the Gag-expressing plasmid activates specific $CD4^+$ cells which are required for optimal CTL induction and protection against tumor challenge. In fact, we previously observed that in vivo inactivation of $CD4^+$ cell function by treatment with anti-CD4 MAb leads to progressive M-MSV/M-MuLV-induced tumor growth due to the lack of tumor-specific CTL generation (2); accordingly, recent experiments have shown that anti-CD4 MAb treatment during pcDNA3-*gag* immunization counteracts protection (data not shown).

Previous studies of the M-MuLV system identified a peptide spanning amino acids 189 to 196 of the Env precursor that displays enhanced recognition in B6.C-H-2^{bm13} mice but behaves as an immunorecessive epitope in B6 mice (32). In agreement with these findings, we observed weak cytotoxic activity against $293K^b$ cells loaded with this octapeptide in MLTC obtained from tumor regressor mice (Fig. 5). Moreover, only a few CTL clones from tumor regressor mice were able to recognize target cells pulsed with $Env_{189–196}$ peptide. In contrast, most of the mice that were vaccinated with the Env-expressing plasmid generated CTL that not only recognized $Env_{189–196}$-pulsed target cells but also efficiently lysed MBL-2 leukemia cells.

Notwithstanding, protection against MBL-2 tumor cell challenge was achieved in less than 50% of the mice vaccinated with pCMV-eco*env*-bpA, thus indicating the existence of a hierarchy in the capacity of different antigenic epitopes to induce a therapeutic immune response against viruses and possibly tumors. The observed limited protection afforded by the Env-encoding plasmid is in line with the results of a study by Ossendorp et al., which showed that the $Env_{189–196}$ peptide was less effective than the $Gag_{85–93}$ peptide in inducing protection in experiments involving these peptides combined with the virus-specific helper epitope (27).

The present findings lead to hypotheses regarding the development of an effective DNA-based vaccine against retroviruses or tumors. The possibility of DNA immunization to induce a potentially protective immune response against recessive epitopes might be very advantageous when the immunodominant epitope is lost or the presenting class I molecule is down-regulated. Furthermore, DNA vaccination allows the expression of selected subunits of the infectious agent, thus bypassing the potential risks associated with the use of attenuated viruses, such as reversion to virulence and recombination with endogenous retroviral sequences resulting in the emergence of new infectious viruses.

DNA-based immunization also appears to be more straightforward than epitope-focused immunization procedures (17), such as those involving synthetic peptides, given that DNA vaccination does not require prior identification of the antigenic epitopes and allows the MHC haplotype of the recipient organism to determine which protein will present the immunodominant peptides to effector cells of the immune system.

The encouraging results achieved in DNA-immunized chimpanzees challenged with high human immunodeficiency virus doses (4), as well as very recent reports showing the induction of humoral and cell-mediated immune responses in humans immunized with plasmids encoding human immunodeficiency virus proteins (6, 25), provide tangible evidence that DNA immunization represents a viable approach for vaccination of humans against retroviruses.

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