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Abstract: Immune surveillance against tumors usually depends on T cell recognition of tumor antigens presented by major histocompatibility complex (MHC) molecules, whereas MHC class I- tumors may be controlled by natural killer (NK) cells. Perforin-dependent cytotoxicity is a major effector function of CD8+ MHC class I-restricted T cells and of NK cells. Here, we used perforin-deficient C57BL/6 (PKO) mice to study involvement of perforin and Fas ligand in tumor surveillance in vivo. We induced tumors in PKO and normal C57BL/6 mice by (a) injection of different syngeneic tumor cell lines of different tissue origin in naive and primed mice; (b) administration of the chemical carcinogens methylcholanthrene (MCA) or 12-O-tetradecanoylphorbol-13-acetate (TPA) plus 7,12-dimethylbenzanthracene (DMBA), or (c) by injection of acutely oncogenic Moloney sarcoma virus. The first set of models analyzes the defense against a tumor load given at once, whereas the last two sets give information on immune defense against tumors at the very moment of their generation. Most of the tumor cell lines tested were eliminated 10-100-fold better by C57BL/6 mice in an unprimed situation; after priming, the differences were more pronounced. Lymphoma cells transfected with Fas were controlled 10-fold better by PKO and C57BL/6 mice when compared to untransfected control cells, indicating some role for FasL in tumor control. MCA-induced tumors arose more rapidly and with a higher incidence in PKO mice compared to C57BL/6 or CD8-deficient mice. DMBA+TPA-induced skin papillomas arose with similar high incidence and comparable kinetics in both mouse strains. C57BL/6 and PKO mice have a similar incidence of Moloney murine sarcoma and leukemia virus-induced sarcomas, but tumors are larger and regression is retarded in PKO mice. Thus, perforin-dependent cytotoxicity is not only a crucial mechanism of both cytotoxic T lymphocyte- and NK-dependent resistance to injected tumor cell lines, but also operates during viral and chemical carcinogenesis in vivo. Experiments addressing the role of Fas-dependent cytotoxicity by studying resistance to tumor cell lines that were stably transfected with Fas neither provided evidence for a major role of Fas nor excluded a minor contribution of Fas in tumor surveillance.

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Decreased Tumor Surveillance in Perforin-deficient Mice

By Maries F. van den Broek,* David Kägi, Ferry Ossendorpf, René Toes, Spiros Vamvakas, Werner K. Lutz, Cornelis J.M. Melief, Rolf M. Zinkernagel,* and Hans Hengartner*

From the *Institute of Experimental Immunology, University of Zürich, CH-8091 Zurich, Switzerland; †Department of Toxicology, University of Würzburg, D-97078 Würzburg, Germany; and ‡Department of Immunohematology and Bloodbank, University Hospital Leiden, NL 2333 AA Leiden, The Netherlands

Summary

Immune surveillance against tumors usually depends on T cell recognition of tumor antigens presented by major histocompatibility complex (MHC) molecules, whereas MHC class I tumors may be controlled by natural killer (NK) cells. Perforin-dependent cytotoxicity is a major effector function of CD8+ MHC class I-restricted T cells and of NK cells. Here, we used perforin-deficient C57BL/6 (PKO) mice to study involvement of perforin and Fas ligand in tumor surveillance in vivo.

We induced tumors in PKO and normal C57BL/6 mice by (a) injection of different syngeneic tumor cell lines of different tissue origin in naïve and primed mice; (b) administration of the chemical carcinogens methylcholanthrene (MCA) or 12-O-tetradecanoylphorbol-13-acetate (TPA) plus 7,12-dimethylbenzanthracene (DMBA), or (c) by injection of acutely oncogenic Moloney sarcoma virus. The first set of models analyzes the defense against a tumor load given at once, whereas the last two sets give information on immune defense against tumors at the very moment of their generation. Most of the tumor cell lines tested were eliminated 10-100-fold better by C57BL/6 mice in an unprimed situation; after priming, the differences were more pronounced. Lymphoma cells transfected with Fas were controlled 10-fold better by PKO and C57BL/6 mice when compared to untransfected control cells, indicating some role for FasL in tumor control. MCA-induced tumors arose more rapidly and with a higher incidence in PKO mice compared to C57BL/6 or CDS-deficient mice. DMBA+TPA-induced skin papillomas arose with similar high incidence and comparable kinetics in both mouse strains. C57BL/6 and PKO mice have a similar incidence of Moloney murine sarcoma and leukemia virus-induced sarcomas, but tumors are larger and regression is retarded in PKO mice.

Thus, perforin-dependent cytotoxicity is not only a crucial mechanism of both cytotoxic T lymphocyte- and NK-dependent resistance to injected tumor cell lines, but also operates during viral and chemical carcinogenesis in vivo. Experiments addressing the role of Fas-dependent cytotoxicity by studying resistance to tumor cell lines that were stably transfected with Fas neither provided evidence for a major role of Fas nor excluded a minor contribution of Fas in tumor surveillance.

Tumor cells arise in individuals as a result of exposure to mutagenic chemicals, radiation, viruses, or spontaneous mutations (1). Whether or not an individual succumbs to the tumor depends on the balance between growth kinetics and metastatic capacity of the tumor versus the ability of the host to control the tumor. For this, the host has two major mechanisms at its disposal: innate and acquired immunity. Innate immunity (e.g., NK cells) is not antigen specific and is able to act quickly and without priming. Acquired immunity depends on priming, is therefore relatively slow but highly specific, and has a memory. A tumor can prime acquired immunity if it presents tumor-specific antigens in the context of MHC class I or class II molecules to CD8+ or CD4+ T cells, respectively (1–3). Furthermore, expression of costimulatory molecules (e.g., B7, intracellular adhesion molecule-1, and LFA-3) has been shown to dramatically increase the efficiency of priming in certain instances (4–7). Under selective pressure, however, tumor escape variants that lack MHC class I or II molecules, costimulatory molecules, or tumor-specific antigens (8–11)
may arise. Some escape variants that had lost MHC class I expression are efficiently controlled in vivo by NK cells (12, 13) in a strictly perforin-dependent way (13).

Perforin-deficient (PKO) mice have been generated on a C57BL/6 genetic background (H-2b) by homologous recombination and are healthy under specific pathogen-free conditions (14). PKO mice completely lack perforin-dependent cytotoxicity, whereas the Fas (CD95)/FasLigand (FasL)-dependent cytotoxic pathway is unimpaired (14–22). Therefore, PKO mice allow the assessment of the relative contribution of each of the two major cell-mediated cytotoxic pathways to a variety of immune responses in vivo and in vitro (14, 15, 17–22). We have shown previously that the defense against infection with the noncytopathic lymphocytic choriomeningitis virus (LCMV) or, to a lesser extent, with the intracellular bacterium *Listeria monocytogenes* depends on perforin-dependent cytotoxicity (14, 17, 23).

Control of syngeneic MC57G fibrosarcoma cells was 10-fold better in C57BL/6 mice compared to PKO mice (14). Also, the development of insulin-dependent diabetes mellitus in a transgenic model (24) is perforin dependent. On the other hand, PKO mice were normal with respect to control of infection with cytopathic viruses like vaccinia virus (VV) or Semliki forest virus (25). Finally, a role for perforin-dependent cytotoxicity was demonstrated in rejection of MHC class I-disparate allografts such as heart (26) and allogeneic tumor cell lines (27), as well as in mixed lymphocyte reactions in vitro (14, 15), and in GVHD (28).

Here, we evaluate the role of perforin- and Fas-dependent cytotoxicity in the control of a variety of established, transplantable syngeneic MHC class I+ tumor cells in vivo and assess the susceptibility of PKO mice to primary individually arising tumors induced by chemical carcinogens and by an oncogenic virus.

**Materials and Methods**

**Mice**

Inbred C57BL/6 mice were obtained from the Institute für Zuchthygiene (University of Zürich, Switzerland), PKO (14), CD8-deficient mice (CD8-/-; 29), and RAG-2-deficient mice (RAG2-/-; 30) have been described before and were bred under specific pathogen-free conditions in our own facilities. PKO mice had a C57BL/6 (H-2b) background. Mice at least 7 wk old were used in all experiments that were performed according to cantonal and federal laws on animal protection, which requires use of minimal numbers of experimental animals.

**Cell Lines, Antibodies, and Viruses**

RMA is a mutagenized RBL-5 (Rauscher virus–induced T lymphoma [12]) cell line. EL-4 is a 9,10-dimethyl-1,2-benzanthracene-induced T lymphoma (American Type Culture Collection [ATCC], Rockville, MD). B16 is a spontaneous melanoma, MC57G is a methylcholanthrene-induced fibrosarcoma (31), and MBL-2 is Moloney virus–induced lymphoma. N1 are EL-4 cells stably transfected with the nucleoprotein (NP) of vesicular stomatitis virus (VSV), and V1 is a mock-transfected control (N1 and V1 were originally obtained from L. Lefranc, Division of Rheumatology, Connecticut Health Center, Farmington, CT; 32).

SR23B adenovirus type 5 early region 1 (Ad5E1) + Ejas transformed tumor cell line was generated from mouse embryonic cells as described previously (33) and contains an Ad5E1b-encoding CTL epitope. All cell lines were of C57BL/6 origin and were maintained in IMDM supplemented with 5% FCS and antibiotics.

Monoclonal rat anti-mouse CD4 (YTS191.1) and monoclonal rat anti-mouse CD8 (YTS169.4) were originally obtained from Dr. H. Waldmann (Dunn School of Pathology, Oxford, UK; 34). Treatment involved injection of 2 mg i.p. of the appropriate antibody. Efficiency of depletion was checked by FACS® analysis (Becton Dickinson & Co.). Purified monoclonal hamster antimouse TNF-α TN3-19.12 has been described (35) and was kindly provided by Dr. R. Schreiber (Washington School of Medicine, St. Louis, MO). TN3-19.12 was used in vivo at 0.25 mg/wk (two injections maximal) to neutralize TNF-α.

VV Indiana (Mudd-Summer isolate) was originally obtained from Dr. D. Kolakofsky (University of Geneva, Geneva, Switzerland) and were grown on BHK21 (baby hamster kidney, ATCC CRL 8544) cells infected with low multiplicity of infection (moi) and plated on Vero cells. VV expressing the NP of VSV (VV-VSV-NP) was kindly given by Dr. B. Moss (National Institutes of Health, Bethesda, MD; 36). VV expressing FasL has been described (37). All VV stocks were made on BSC40 cells, LCMV, isolate WE (LCMV-WE), was initially obtained from Dr. F. Lehmann-Grube (Heinrich-Pette Institut für Experimentelle Virologie und Immunologie, Universität Hamburg, Germany; 38) and was propagated in L929 fibroblast cells. Moloney murine sarcoma and leukemia virus (MoMSV) complex was prepared and titered as described previously (39).

**Transfection of Cell Lines with Fas**

MBL-2 cells were stably transfected with a construct carrying the mouse Fas gene and the neomycin resistance gene (kindly provided by Dr. P. Golstein, Centre d’Immunologie INSERM-CNRS, Marseille, France; 22) by electroporation and were subsequently selected with 0.9 mg/ml genetin (G418; Gibco BRL, Basel, Switzerland). G418-resistant cells were checked for Fas expression by FACS® analysis: 1 million cells were incubated with 10% normal hamster serum (15 min at 4°C) followed by biotin-labeled hamster anti-mouse Fas (Jo-2; PharMingen, San Diego, CA). Cells were stained with streptavidin–FITC or streptavidin–PE. Fas+ cell lines were subcloned by limiting dilution. MBL-2 and MBL-2.Fas cells were insensitive to TNF-mediated cytotoxicity in vitro (data not shown).

**Fas-dependent Cytotoxicity Assay**

To test whether the transfected Fas was functional, Fas-transfected (Fas+) and control (Fas−) cell lines were labeled with Na251CrO4 and were used as targets for Fas± effectors. Fas+ effectors were generated by infecting MC57G fibroblasts with VV-FasL (37) for 2 h with a multiplicity of infection (moi) of 2. Effectors and targets (104 per well) were incubated for 5 h at 37°C in a standard 51Cr release assay. In addition, PKO and control
Table 1.  C57BL/6-derived Tumor Cell Lines

| Tumor           | Class I* | Class II* | Fas* | Fas-mediated lysis |
|-----------------|----------|-----------|------|--------------------|
| MC57G           | MCA fibrosarcoma | 330 | 3 | 8 | 0 |
| MBL-2           | Moloney B lymphoma | 1,100 | 3 | 20 | 0 |
| MBL-2.Fas       | Moloney B lymphoma, Fas transfected | 1,200 | 3 | 250 | 54 |
| EL-4            | DMBA T lymphoma | 1,500 | 3 | 28 | 4 |
| RMA             | Rauscher T lymphoma | 1,300 | 3 | 33 | 6 |
| B16             | Melanoma | 40 | 3 | 10 | 0 |
| SR.23B          | Adeno-transfected embryonic cells | 75 | 3 | 10 | 0 |

*Cells were stained with biotin-labeled anti-MHC class I, anti-class II or anti-Fas mAb, followed by streptavidin-PE. Surface expression of all cell lines was measured by FACS® (CellQuest software) in one experiment and given in arbitrary units with the fluorescence of second stage antibody alone set at 3.

1 Percentage specific killing by VVFasL-infected MC57G effector cells at an E/T ratio of 10 in a 5-h 51Cr release assay.

C57BL/6 mice were infected intravenously with 200 PFU LCMV-WE. 8 d later, their spleens were tested in a CTL assay for LCMV-specific cytotoxicity on 51Cr-labeled, LCMV peptide (GP33, immunodominant CTL epitope in H-2d mice)-loaded targets (40). Percentage of specific 51Cr release = [(experimental release - spontaneous release)/(maximal release - spontaneous release)] × 100%.

Tumor Induction In Vivo by Injection of Tumor Cell Lines

Normal Untreated Mice. PKO and C57BL/6 mice were injected intraperitoneally with different amounts of viable tumor cells (Table 1). Mice were monitored daily for tumor growth by determining their weight; they were killed if the weight increased to more than 15% compared to noninjected controls. In case of SR.23B cells, subcutaneous tumor volume was determined with a caliper measuring two perpendicular diameters.

Effect of Priming with Viral Antigens. PKO and C57BL/6 mice were primed intravenously with 2 × 10^6 PFU VSV, with 2 × 10^6 PFU VV-VSV-NP, or were left unprimed. After 14 d, mice were injected intraperitoneally with different numbers of N1 (EL-4 thymoma expressing VSV-NP) or V1 (control EL-4), and tumor growth was monitored.

Effect of Priming with Tumor Antigens. PKO and C57BL/6 mice were primed intraperitoneally with 5 × 10^6 irradiated (100 Gy) MBL-2 cells in 0.2 ml PBS on days 0 and 7. On day 14, mice were injected intraperitoneally with different numbers of viable MBL-2 or MBL-2.Fas cells and were monitored for tumor growth. Alternatively, mice were primed subcutaneously with 10^6 irradiated (25 Gy) SR.23B cells in 0.1 ml PBS on days 0 and 14 and challenged subcutaneously on day 28 with the same number of SR.23B cells.

Male mice were used for all experiments to avoid possible tumor rejection caused by T cells recognizing the male antigen H-Y.

Tumor Induction by Tumorigenic Agents

Carcinogenic Chemicals. Groups of 10 C57BL/6, PKO, and CD8^-/- mice were injected subcutaneously in the left hind leg with 0.1 ml corn oil containing 25, 100, or 400 µg methylcholanthrene (MCA), and mice were monitored weekly for fibrosarcoma development.

Alternatively, 100 µl acetone containing 10 nmol 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and 3 nmol 7,12-dimethylbenzanthracene (DMBA) was applied twice weekly on the shaved skin of the back to groups of eight C57BL/6 and PKO mice. Skin application was continued during the course of the experiment, and mice were observed twice per week for the development of papillomas (41).

MoMSV. Groups of 5–10 C57BL/6 and PKO (untreated, anti-TNF-treated, CD4- or CD8-depleted) or CD8^-/- mice were injected in the left thigh with 100 µl PBS i.m. containing 10^5 focus forming units of MoMSV complex, as has been described (39). Mice were monitored for tumor growth, and tumor size was determined using a caliper measuring two perpendicular diameters.

Isolation and Culture of MCA-induced Sarcomas

When MCA-induced sarcomas had reached a volume of ~0.5 cm^3, mice were killed and tumors were removed aseptically. Tumors were cut into small pieces and were trypsinized for 5 h at room temperature, clumps were removed, and single cells were cultured in IMDM supplemented with 10% FCS, glutamine, and antibiotics. Cells were split when they reached confluency. All sarcomas could be kept in culture for at least 3 mo. The expression of MHC class I, class II, and Fas was determined by FACS® analysis.

Statistical Analysis

Results were compared statistically using the unpaired Mann-Whitney U test.

Results

Perforin Plays a Role in Surveillance against Most Established Tumor Cell Lines

PKO mice and C57BL/6 mice were injected with syngeneic tumor cell lines of different tissue origins, and tumor growth was monitored.

Tumors of Nonlymphoid Origin. B16 melanoma cells grew unrestricted in both C57BL/6 and PKO mice, even when
Table 2. Control of Syngeneic Tumor Cell Lines by C57BL/6 and PKO Mice

| Tumor       | C57BL/6 | PKO |
|-------------|---------|-----|
|             | Primed* | Unprimed | Primed | Unprimed |
| MC57G       | ND      | 10^6 (4/4) | ND | 10^6 (4/4) |
| MBL-2       | >10^6 (6/6) | 10^6 (6/6) | 10^6 (6/6) | <10^7 (6/6) |
| MBL-2.Fas   | >10^6 (4/4) | 10^6 (6/6) | 10^6 (2/4) | 10^6 (6/6) |
| RMA         | >10^6 (4/4) | 10^4 (7/7) | >10^6 (4/4) | 10^4 (6/6) |
| RMA-S       | ND      | 10^4 (8/8) | ND | 10^4 (8/8) |
| EL-4        | >10^6 (6/6) | 10^5 (6/6) | <10^2 (6/6) | <10^2 (6/6) |
| B16         | ND      | 10^5 (3/3) | ND | 10^5 (3/3) |

The cell numbers in the table represent the lowest number of tumor cells that could not be controlled in vivo. The numbers in brackets are the number of mice with tumors out of the total number tested which were obtained in two to three independent experiments with groups of two to three mice.

*Mice were primed with tumor cells (MBL-2, RMA) by injection of 5 x 10^6 irradiated tumor cells (MBL-2, MBL-2 Fas, RMA) at days -14 and -7 before tumor challenge at day 0 or with viral antigens (VSV, VV-VSV-NP) by injection of 2 x 10^6 PFU at day -14 before tumor (VSV-NP-transfected or mock-transfected EL-4) challenge at day 0.

The cell numbers in the table represent the lowest number of tumor cells that could not be controlled in vivo. The numbers in brackets are the number of mice with tumors out of the total number tested which were obtained in two to three independent experiments with groups of two to three mice.

Figure 1. Adenovirus + ras transformed mouse embryonic cells are controlled by perforin-deficient mice. C57BL/6 (○) and PKO (●) mice were given 5 x 10^6 SR23B cells (expressing Ad5E1 and E7) subcutaneously on day 0, and tumor development was monitored. Each line represents an individual mouse.

Figure 2. Growth of syngeneic EL-4 thymoma cells in primed and unprimed C57BL/6 and perforin-deficient mice. Mice were primed at day 0 with 2 x 10^6 PFU live VSV, 2 x 10^6 PFU live recombinant VV-expressing VSV-NP, or no treatment. Mice were challenged intraperitoneally with 10^6 live VSV-NP-transfected or mock-transfected EL-4 cells. Mice were observed daily for tumor growth by monitoring the weight of the mice. Each point represents one mouse.

Stable Transfection of MBL-2 Lymphoma Cells with Functional Fas

MBL-2 cells were transfected with an expression vector encoding mouse Fas and G418 resistance by electroporation, and G418-resistant MBL-2 cells were subcloned twice. One of the selected subclones (MBL-2.Fas9) displayed a homogeneous expression pattern of Fas (Fig. 3 A) and was killed by FasL + MC57G cells (Fig. 3 B). Spleno-
Aster anti-mouse Fas antibody followed by streptavidin-FITC. MBL-2 cells. 10^6 cells were stained with a blotted monoclonal ham-
deficient effectors. (A) Fas-expresslon of Fas- and mock-transfected
Figure MBL-2 and MBL-2.Fas target cells In a standard 5 h 51Cr release assay.
Enhanced lysls of Fas + MBL-2 cells compared to Fas- MBL-2 cells by
scatter, and were analyzed with a FACScan® cytofluorometer using loga-
peptlde (GP33)-loaded MBL-2 or MBL-2.Fas target cells in a standard
intravenously with 200 PFU LCMV-VE. After 8 d, splenocytes (effec-
tors) were analyzed for ex vivo cytotoxlc acUwty using
The spontaneous release of MBL-2 and MBL-2.Fas target cells was 17
FasL (moi = 2) for 3 h and were used as effectors to kill SlCr-labeled
51Cr release assay. Lysls of MBL-2 and MBL-2.Fas without peptlde was
<5% at the highest E/T ratio, and spontaneous release of targets was
<18%
Figure 3. Lysis of Fas-transfected MBL-2 lymphoma cells by perforin-
deficient effectors. (A) Fas-expression of Fas- and mock-transfected
MBL-2 cells. 10^6 cells were stained with a biotynlated monoclonal ham-
ster anti-mouse Fas antibody (Jo-1) followed by streptavidin-FITC. Viable cells were gated by a combination of forward and 90° side light scatter, and were analyzed with a FACSscan® cytofluorometer using loga-
rithmic scales. Filled curve, MBL-2 cells; open curve, MBL-2 Fas cells. (B) Enhanced lysis of Fas + MBL-2 cells compared to Fas+ MBL-2 cells by Fas-expressing effectors in vitro. MC57G cells were infected with VV-Fas. (moi = 2) for 3 h and were used as effectors to kill ^6Cr-labeled
MBL-2 and MBL-2.Fas target cells in a standard 5 h ^5Cr release assay. The spontaneous release of MBL-2 and MBL-2.Fas target cells was 17 and 14%, respectively, (C) Fas'+ MBL-2 cells are lysed by perforin-defi-
cient effectors in vitro. PKO and control C57BL/6 mice were infected intravenously with 200 PFU LCMV-VE. After 8 d, splenocytes (effectors) were analyzed for ex vivo cytotoxic activity using LCMV-specific peptide (GP33)-loaded MBL-2 or MBL-2.Fas target cells in a standard
51Cr release assay. Lys of MBL-2 and MBL-2.Fas without peptide was <5% at the highest E/T ratio, and spontaneous release of targets was <18%
cytes of PKO and C57BL/6 mice that had been infected with LCMV 8 d previously killed LCMV glycoprotein
(GP)33-loaded MBL-2.Fas cells to a similar extent (Fig. 3 C). As expected, untransfected MBL-2 cells (virtually Fas-, Fig. 3 A) were killed by C57BL/6 effectors, but only to a low extent by perforin-deficient effectors (14); this sug-
gested a major role for the perforin-dependent cytolytic pathway against Fas' or Fas'° targets. Thus, transfection of
MBL-2 cells with Fas generated a cell line with a high, stable, and functional expression of Fas.
We also transfected MC57G and B16 cells with Fas and obtained clones with high expression. In vitro, however, these cells remained insensitive to Fas/FasL-mediated apoptosis (data not shown), probably because of a lack of crucial
intracellular signalling molecules downstream of Fas (43). Therefore, no in vivo experiments were performed with MC57G.Fas and B16.Fas cells.
Resistance against Fas-transfected MBL-2 Cells
To evaluate whether growth characteristics of MBL-2 cells were influenced by Fas, MBL-2 and MBL-2.Fas cells were titrated intraperitoneally in RAG2^-/- mice. In addition, growth kinetics in vitro were compared. We did not observe differences in tumor take or tumor growth between MBL-2 and MBL-2.Fas cells at the doses tested (10^2-10^6, RAG2^-/- mice could not control the tumor) in RAG2^/- mice or of proliferation in vitro (data not shown). The potency of Fas/FasL interactions in tumor surveillance in vivo was investigated in naive or primed mice. Naive C57BL/6 mice controlled 10^5 MBL-2 cells and 10^4 MBL-2.Fas cells, but could not control an inoculum of 10^6 MBL-2 or 10^5 MBL-2.Fas. As little as 10^2 MBL-2 cells grew in an uncontrolled fashion in PKO mice, whereas 10^2 MBL-2.Fas cells were rejected (Fig. 4). This suggests that in an unprimed situation, Fas/FasL interactions may contribute to tumor control.
In a subsequent experiment, mice were primed intraperitoneally with 4 million irradiated MBL-2 cells. Mice were subsequently challenged with different numbers of viable MBL-2 or MBL-2.Fas tumor cells, and tumor growth was monitored. Primed C57BL/6 mice controlled the growth of >10^6 MBL-2 or MBL-2.Fas cells; on the other hand, primed PKO mice exhibited tumor growth after in-
jection of 10^4 MBL-2 cells. Two out of four PKO mice could eliminate 10^5 MBL-2.Fas cells, whereas the other one displayed delayed tumor growth (Table 2). Thus, priming with irradiated MBL-2 cells induced resistance...
Skin papilloma induction by DMBA+TPA: independence of perforin. Groups of eight perforin-deficient and seven control C57BL/6 mice were treated twice weekly with 3 nmol DMBA + 10 nmol TPA in acetone. The time of the appearance of the first papilloma was registered for each mouse.

Figure 6. Skin papilloma induction by DMBA+TPA: independence of perforin. Groups of eight perforin-deficient and seven control C57BL/6 mice were treated twice weekly with 3 nmol DMBA + 10 nmol TPA in acetone. The time of the appearance of the first papilloma was registered for each mouse.

Figure 5. Control of MCA-induced carcinogenesis by perforin. PKO (○), CD8⁻⁻ (△) and control C57BL/6 (●) mice were injected subcutaneously in the left flank with 25, 100, or 400 μg MCA in 0.1 ml corn oil. Mice were observed every 5 d for palpable tumors. If tumors were >15 mm in diameter, mice were killed. Experimental groups consisted of 5 (25 μg, 400 μg) or 10 (100 μg) mice. Differences between PKO and C57BL/6 mice were statistically significant (unpaired Mann-Whitney U test), whereas differences between CD8⁻⁻ and C57BL/6 mice were not.

Tumors Induced by Chemical Carcinogens

Subcutaneous injection of MCA induced fibrosarcomas in a dose-dependent fashion. Injection of 25 μg (Fig. 5, top) induced tumors in 100% of the PKO, but only in 20% of the C57BL/6 mice. Administration of 100 μg MCA (Fig. 5, middle) or 400 μg (Fig. 5, bottom) induced sarcomas in C57BL/6 mice with an incidence of 80 and 100%, respectively. At all doses, a significant accelerated onset of tumor (Fig. 5) and tumor size (data not shown) was observed in PKO mice. Administration of 100 μg MCA to CD8⁻⁻ mice induced sarcomas with comparable kinetics and incidence as those seen in C57BL/6 mice. Experimental groups were statistically compared using the unpaired Mann-Whitney U test, and showed that tumor development in PKO mice and C57BL/6 mice was statistically different after injection of 100 μg MCA (P < 0.0005) or 400 μg MCA (P < 0.009). The difference between PKO and CD8⁻⁻ mice was also statistically significant (P < 0.0008), whereas there was no difference between C57BL/6 and CD8⁻⁻ mice (P = 0.9). Statistical analysis was not performed on groups injected with 25 μg MCA, because only one out of five C57BL/6 mice, but five out of five PKO mice, developed a sarcoma. FACS® analysis performed within 10 d after isolation of different MCA-induced sarcomas showed that the sarcomas were MHC class I low/-, MHC class II-, and Fas- (not shown). The expression of the above-mentioned surface molecules was similar in the three different strains of donor mice.

The first DMBA+TPA-induced skin papillomas developed in C57BL/6 and PKO mice after 18 wk and 21 wk, respectively, and 100% incidence was reached after 31.5 and 34 wk, respectively (Fig. 6). The incidence and kinetics of DMBA+TPA-induced papillomas were similar in PKO and C57BL/6 mice, arguing against a role for perforin-mediated cytotoxicity (CTL and/or NK) in the control of DMBA+TPA-induced papillomas.

Virally Induced Fibrosarcomas

Intramuscular administration of MoMSV to PKO and C57BL/6 mice led to tumor induction in all mice with similar kinetics. In addition, occurrence of regression was not dependent on perforin (Fig. 7). Nevertheless, the tumors were larger on average and regression was retarded in PKO mice, suggesting involvement of perforin in the acute response.
Figure 7. Control of virally induced tumors by PKO mice. PKO and control C57BL/6 mice were given 10³ focus forming units MoMSV intramuscularly in 100 µl PBS in the left hind leg. Mice were monitored regularly for palpable tumors. Each line represents an individual mouse.

Discussion

The perforin dependence of the eradication of several established syngeneic tumor cell lines was investigated. Most cell lines showed a difference in tumorigenic dose between naive PKO and C57BL/6 mice, indicating that even without previous priming, perforin-mediated cytotoxicity is a crucial effector mechanism in tumor control. In primed mice, the difference between PKO and wild-type mice was even more clear. This suggests that priming of CD8⁺ T cells rather than priming of CD4⁺ T cells is responsible for resistance to high tumor loads in C57BL/6 mice.

Two tumor cell lines formed an exception to this rule. RMA cells showed equal growth in both naive (10⁴) and primed (10⁶) PKO and C57BL/6 mice, which may be explained by the fact that control is mediated by perforin-independent mechanisms, probably by CD4⁺ cells using an unidentified effector mechanism (1). Because RMA and MBL-2 are related lymphoma lines and express similarly high levels of MHC class I and no MHC class II, and both cell lines are Fasl⁻, the observation that the control of RMA is not dependent on perforin, whereas the control of MBL-2 cells is, was unexpected. This may be explained by a difference in efficiency of priming CD4⁺ and/or CD8⁺ T cells, or by a different growth rate of RMA vs. MBL-2 cells. The second exception is formed by SR23B cells: this adenovirus + ras-transformed cell line induced larger tumors in C57BL/6 mice than in PKO mice. This unexpected finding may be explained by the fact that PKO mice may have increased basal levels of macrophage activation and/or cytokines such as interferon-γ and TNF-α as compensatory mechanisms, all of which are known to possess antitumor activity.

Stable transfection of MBL-2 cells with Fas rendered them susceptible to Fas/Fasl-dependent apoptosis in vitro. To investigate the contribution of the Fas pathway to in vivo tumor surveillance, control of MBL-2.Fas and MBL-2 cells was compared in naive and primed PKO and C57BL/6 mice. In unprimed PKO and C57BL/6 mice, Fas⁻ cells were controlled 10-fold better compared to Fas⁺ cells. Priming of PKO mice increased the number of tumor cells needed for uncontrolled growth by 100-fold for both MBL-2 (10⁴ cells) and for MBL-2.Fas (10⁵ cells), suggesting priming of perforin-independent mechanisms. Priming of C57BL/6 mice, however, resulted in control of >10⁶ MBL-2 or MBL-2.Fas cells, stressing the need for a collaboration between perforin-dependent and -independent mechanisms for full immune protection against MBL-2 cells. Thus, our experiments failed to detect a major involvement of Fas-dependent cytotoxicity in tumor control, but cannot exclude a minor role.

To investigate the influence of perforin on the induction of primary tumors in vivo, we analyzed different models in which tumors are induced by chemicals (MCA, DMBA + TPA) or virus (MoMSV). The major difference of this approach compared to injection of syngeneic cell lines is that the immune system is not confronted with a considerable tumor load at once, but that immunosurveillance is tested at the very moment a tumor arises. Moreover, in contrast to injection of homogeneous cell lines, each mouse develops its own tumor that may have individually different tumor antigens, even if the same chemical is injected in the same animal (44). Subcutaneous injection of MCA is known to induce sarcomas at the site of injection (44) that usually do not regress once they developed. C57BL/6 and CD8⁻/⁻ mice developed sarcomas with a high incidence but with much slower kinetics than PKO mice. This suggests that NK cells or CD4⁺ T cells may act...
directly or indirectly as important effectors controlling MCA-induced sarcomas. Ex vivo analysis of MHC class I, class II, and Fas expression of MCA-induced sarcomas, however, showed that sarcomas were Fas\(^{+}\) and MHC class II\(^{-}\) and were MHC class I\(^{+/0}\), independent of whether they were isolated from PKO, C57BL/6, or CD8\(^{-/-}\) mice. Although MHC class I expression could be upregulated to high levels by exposure to IFN-\(\gamma\) in vitro (not shown), it remains unclear whether this occurs in vivo. Because four out of four MCA-induced sarcomas tested were sensitive to NK-mediated lysis in vitro, this suggests that NK-mediated control may be an important pathway in vivo. In a second set of experiments in which DMBA+TPA–induced skin papillomas were studied, no differences in incidence, kinetics, or size of papillomas were observed between PKO and C57BL/6 mice, indicating that perforin does not play a key role. Because the latter model is immunologically not characterized (susceptibility of SCID or nu/nu mice is not known), it is not possible at the present time to be more specific about possible effector mechanisms.

Sarcomas induced by inoculation of mice with MoMSV are known to be rejected within 3 wk by immunocompetent mice in which a prominent H-2D\(^{b}\)-restricted CTL response is observed (45–48), whereas lethal tumors develop in nu/nu mice (Ossendorp, F., and C.J.M. Melief, unpublished data). Furthermore, CD4 depletion results in uncontrolled tumor growth (Ossendorp, F., and C.J.M. Melief, unpublished data). CD8 depletion, on the other hand, did not alter the incidence or kinetics of tumor growth, but led to a significant delay in tumor rejection (data not shown). PKO mice displayed a phenotype similar to CD8-depleted mice after inoculation with MoMSV, suggesting a distinct, perforin-dependent role for CD8\(^{+}\) CTLs in tumor control, although the tumor could be rejected without this pathway. It is not known at the present time which effector mechanism is used by CD4\(^{+}\) T cells to reject MoMSV-induced sarcomas, and this question is subject to further studies. Apparently, CD4\(^{+}\) T cells do not eradicate tumors through TNF-\(\alpha\) because treatment with neutralizing anti-TNF antibodies in vivo improved the general well-being of the mice at the peak of the tumor growth, but did not interfere with tumor rejection (data not shown) independent of mouse strain or treatment.

Finally, during the 3 yr we now have bred PKO mice and have kept some (\(\sim 100\)) for >12 mo, we have not observed spontaneous tumors of any kind. This does not necessarily call into question the in vivo relevance of perforin for tumor control because (a) a mouse facility is a relatively protected environment in which mice are not exposed to irradiation, toxins, chemicals, or exogenous oncogenic viruses; and (b) spontaneous tumor development may be too rare for meaningful observation in the few mice that were not used for experiments when young and reached an age of >1 yr in our facilities.

Taken together, our data illustrate that several mechanisms may control tumor growth; perforin-mediated cytotoxicity by CD8\(^{+}\) CTLs (for MHC class I\(^{+}\) tumors) and NK cells (for tumors expressing low levels of MHC class I) are of major importance; Fas/FasL interactions can contribute to tumor control in vivo, albeit to a lesser extent than perforin-dependent lysis, at least in tumor models using several established tumor cell lines or primarily induced types of tumors studied here.

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