TUMORS INDUCED BY MURINE SARCOMA VIRUS CONTAIN PRECURSOR CELLS CAPABLE OF GENERATING TUMOR-SPECIFIC CYTOLYTIC T LYMPHOCYTES*

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The relevance of the immune response in the development of a tumor has been studied by analyzing the leukocyte infiltration within the tumor mass. Human tumors (1), as well as a number of experimental tumor systems in mice and rats, have been analyzed in this manner (1–8). Monocytes and activated macrophages possessing cytotoxic and/or cytostatic activities (1–3, 5, 7) and natural killer (NK) cells (4) have been identified among the infiltrating thymus-independent (i.e., non-T) immune-reactive cells which might play a role in tumor rejection.

In addition to non-T immune cells, cytolytic thymus-derived lymphocytes (CTL) have also been identified among the leukocyte infiltrate of solid tumors (3, 4, 8, 9) in mice. The characterization of CTL, extracted by mechanical and enzymatic procedures from murine sarcoma virus (MSV)-induced tumors, indicated that these cytolytic cells consisted mainly of small lymphocytes (3) specific for MSV-associated antigens (3, 4). Furthermore, intra-tumoral CTL were effective only against tumor target cells of the same *H-2* haplotype as the effector cells (4). In this report we present studies showing that, in addition to active CTL generated in vivo, the T-lymphocyte fraction extracted from MSV-induced tumors in C57BL/6 (B6) mice also contains precursor cells capable of differentiating into active CTL when restimulated in vitro. These CTL showed a high degree of specificity for virus-associated antigens and their cytolytic activity was restricted to tumor target cells of the $H-2^b$ haplotype. The identification of active CTL and of CTL precursor cells within MSV-induced tumors points to the existence of a dynamic state involving T-cell differentiation within the tumor mass before the onset of regression.

Materials and Methods

Tumors. MSV tumors were induced in 4-wk-old inbred C57BL/6 (B6, $H-2^b$) mice bred in our own animal colony. Each mouse received a 0.2-ml injection, intramuscularly into the thigh, of a cell-free homogenate containing Moloney murine sarcoma and leukemia virus complex (4, 10).

RBL-5 lymphoma cells, induced by Rauscher leukemia virus in B6 mice ($H-2^b$, 4, 11) were used both as syngeneic target cells in the ^{51}Cr-release assay and as stimulator cells in secondary mixed lymphocyte tumor cell cultures (MLTC). Friend erythroleukemia virus-induced HFL/b cells (12) and Gross leukemia virus-induced BɑGV cells (13), both derived from tumors of BALB.B mice, were also used as $H-2^b$ targets in some cytotoxicity assays. Cultured LSTRA

tumor cells, induced by Moloney leukemia virus in BALB/c mice (H-2^d, 14) were used as allogeneic tumor target cells. Friend, Moloney, and Rauscher leukemia viruses induce strongly cross-reacting cellular antigens, referred to collectively as FMR, which are, however, quite distinct from those induced by Gross leukemia virus (15). These tumor cell lines were all maintained as stationary suspension cultures.

Lymphoid Cells. The spleen, the inguinal lymph node regional to the MSV-induced tumor (RLN) and the mesenteric lymph node (MLN) were removed and cell suspensions were prepared as described elsewhere (16). Leukocytes were extracted from MSV-induced tumors by a combination of mechanical and enzymatic treatment (4), with an average yield of 2×10^6 leukocytes per tumor.

Lymphocyte Cultures. Syngeneic MLTC were established in 16-mm microwell plates (Costar, Data Packaging, Cambridge, Mass.) using 5×10^6 leukocytes and 0.5×10^6 X-irradiated tumor cells (5,000 rads) as stimulators in 2 ml RPMI-1640 culture medium (Grand Island Biological Co., Grand Island, N. Y.) containing 5% fetal calf serum (FCS) and 5×10^{-5} M 2-mercaptoethanol (17). Cells were harvested 6 d later and assayed for cytotoxicity.

Elimination of T Cells. Cell suspensions were depleted of T cells by incubation with an antiserum prepared by immunizing AKR mice with C3H normal thymus cells (anti-Thy-1.2 serum) and rabbit complement (4).

^{51}Cr-Release Cell-Mediated Cytotoxicity Assay. Cell-mediated anti-tumor cytolytic activity was detected using a modification of the method of Brunner et al. (16). For primary cytotoxic cells generated in vivo, varied numbers of lymphoid cells were mixed with 3×10^3 ^{51}Cr-labeled tumor target cells in a final volume 0.4 ml minimal essential medium containing 10% FCS and 10 mM Hepes in 0.5-ml microcentrifuge tubes. After a 19-h incubation at 37°C, the tubes were centrifuged at 11,500 g for 30 s, and 0.2 ml of the supernatant medium was harvested. The radioactivity released from the target cells was measured with a well-type gamma scintillation counter.

Lymphocytes harvested from MLTC cultures were assayed for cytolytic activity by incubation with 10^4 ^{51}Cr-labeled target cells at various lymphocyte to target cell ratios in a 0.2-ml total volume in round-bottomed multi-well plates (Linbro Chemical Co., Hamden, Conn.). After a 6-h incubation at 37°C, the supernates were harvested and measured for radioactivity. In all cases, percent specific ^{51}Cr release was calculated by the formula:

$$\frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release (in 1 N HCl)} - \text{spontaneous release}} \times 100.$$

Results and Discussion

Adult B6 mice were highly efficient in rejecting tumors induced by MSV. Under our experimental conditions, tumors became apparent 3-4 d after virus injection and reached diameters as large as 2.5 cm on day 9. Tumor rejection occurred rapidly because the tumor mass had completely disappeared by day 15. Previous studies of the CTL response in these mice showed that lymphoid tissues including spleen, peripheral blood, and RLN contained peak CTL numbers on days 8–10, coincidentally with maximum tumor size (4). Studies of the leukocyte fraction extracted by mechanical and enzymatic procedures from the tumors of the same mice revealed the presence of large numbers of active CTL between days 8 and 10; however, maximum CTL numbers were detected on day 12, concurrent with the initiation of tumor regression (4, 9).

The experiments described in the present communication were designed to detect precursor cells capable of differentiating into MSV tumor-specific CTL. B6 mice were sacrificed on day 8 or 9 after virus inoculation when they bore large tumors, and leukocyte suspensions were prepared from spleen, RLN, MLN, and tumor mass. Each leukocyte suspension was assayed in a 19-h ^{51}Cr-release assay for the presence of primary CTL generated in vivo after tumor induction; other portions of the same
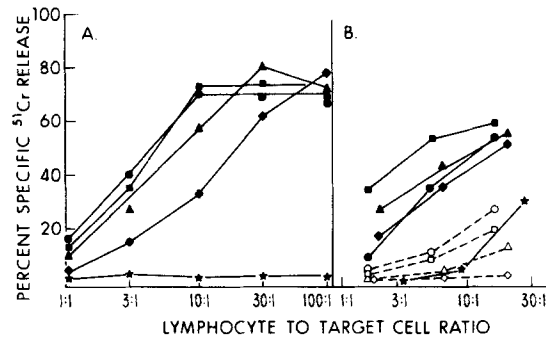
Fig. 1. Lysis of ⁵¹Cr-labeled RBL-5 target cells by lymphocytes from B6 mice taken 8 d after MSV inoculation. Activity of leukocytes extracted from MSV-induced tumors (●), immune spleen (■), RLN (▲), MLN (◆), and from normal B6 spleens (★), as detected in a 19-h cytotoxicity assay. (B) Cytolytic activity generated in vitro after restimulation of the same leukocyte populations with RBL-5 cells (■, ▲, ●, ◆, ★) or normal B6 spleen cells (○, □, △, ◇), as detected in a 6-h cytotoxicity assays.

leukocyte suspensions were restimulated in culture with syngeneic RBL-5 cells, which share tumor antigens in common with MSV-induced tumor cells (10, 11, 17).

Fig. 1 A shows that, in agreement with previous results (4), leukocytes extracted from the tumor mass contained primary cytolytic cells generated in vivo and capable of destroying syngeneic RBL-5 target cells to a degree comparable to that of leukocytes from the spleen or RLN of the same mice. Under the same experimental conditions, spleen cells from normal B6 mice did not show detectable levels of spontaneous cytotoxicity attributable to NK cells. Previous reports (4, 10) have indicated that the high degree of cytolytic activity of leukocytes from MSV-immune mice on day 9 is mainly a result of thymus-derived effector cells (i.e., CTL).

The same leukocyte suspensions which yielded high numbers of primary CTL also contained precursor cells capable of differentiating into cytolytic effector cells in MLTC. The data summarized in Fig. 1 B indicated that the leukocytes, recovered from MLTC after a 6-d cultivation, contained secondary cytolytic effector cells detectable in a 6-h ⁵¹Cr-release assay. Leukocytes extracted from the tumor mass were capable of generating cytolytic cells comparable in activity to those generated by leukocytes from RLN or MLN and about three times less active than effector cells generated from spleen cell suspensions. This fact pointed to the existence among these leukocyte suspensions of primed MSV-specific CTL precursor cells sensitive to secondary stimulation by tumor antigen, because parallel incubation of normal B6 spleen cells with RBL-5 stimulator cells for 6 d yielded 7-times less CTL than cultures established with intratumoral leukocytes, and ≅16 times less CTL than analogous cultures of MSV-immune spleen cells (Fig. 1 B). Furthermore, incubation of MSV-immune lymphocytes in the presence of x-irradiated B6 normal spleen cells (instead of RBL-5 cells) revealed a residual cytolytic activity on ⁵¹Cr-labeled RBL-5 target cells (Fig. 1 B). This residual cytotoxicity was 3- to 10-fold lower than the activity generated in secondary MLTC with the same lymphocyte suspensions incubated with RBL-5-stimulating cells. The latter observations recall data reported by Gillespie et al. (19) in the same tumor system indicating the maturation in vitro of MSV-immune CTL precursor cells in the absence of stimulating tumor antigen.

Cytolytic cells generated in secondary MLTC were specific for antigens induced by
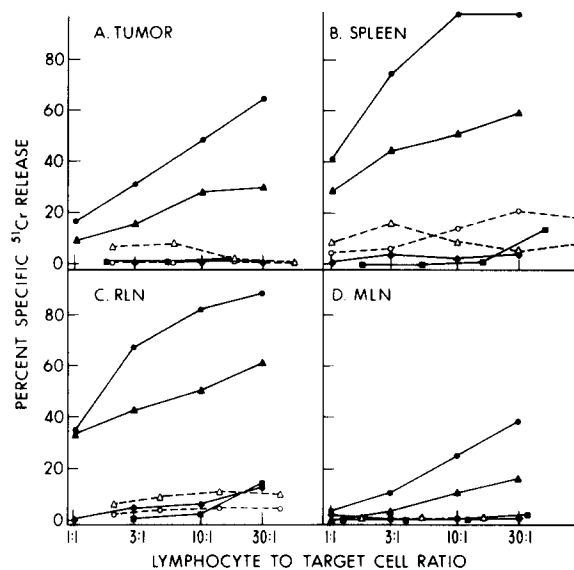
Fig. 2.   Specificity of effector cells generated in vitro by restimulation with RBL-5. Leukocytes from (A) the tumor mass, (B) spleen, (C) RLN, and (D) MLN were extracted, restimulated in secondary MLTC, and assayed after 6 d for cytolytic activity on ^{51}Cr-labeled RBL-5 (●), LSTRA (■), HFL/b (▲), and BḍGV (◆). Aliquots of each effector cell suspension were also pretreated with AKR anti-Thy-1.2 serum and complement (△, ○), then tested on the same target cells as the untreated suspensions in a 6-h ^{51}Cr release assay.

leukemia viruses belonging to the FMR group (Fig. 2). Friend virus-induced HFL/b (H-2^b) cells and Rauscher virus-induced RBL-5 cells were lysed efficiently, whether MLTC were initiated with leukocytes from MSV tumors, spleen, RLN, or MLN. However, the same CTL did not lyse Gross virus-induced BḍGV (H-2^b) cells and allogeneic Moloney leukemia virus-induced LSTRA (H-2^d) target cells. Other studies (18) have shown that LSTRA cells are highly susceptible to lysis by BALB/c (H-2^d) MVS-immune CTL, and BḍGV cells to lysis by Gross virus-immune BALB.B CTL (13). Thus, B6 cytolytic cells generated in syngeneic secondary MLTC with leukocytes from the MSV tumor, the spleen, RLN, or MLN were specific for H-2^b FMR-positive tumor target cells. In addition, Fig. 2 shows that the cytolytic activities detected on the various tumor target cells were a result of T lymphocytes. Incubation of the effector cell suspensions with AKR anti-Thy-1.2 serum and rabbit complement yielded viable cell suspensions depleted of T cells and showing very low to undetectable target cell lysis under the same assay conditions.

Our data indicate that MSV-induced tumors contain CTL precursor cells, as well as active CTL generated in vivo during tumor growth. Whether generated in vivo or in vitro, these T-killer cells had a high degree of virus specificity and were H-2 restricted. These findings point to a possible migration of MSV-specific CTL precursor cells between the lymphoid organs and the tumor mass. A similar dynamic flow of T cells has been described by Sprent and Miller (20, 21) in another experimental system involving allograft rejection by H-2-activated T lymphocytes from CBA mice.

Although CTL precursor cells may migrate in mice undergoing tumor rejection, it is not known if this is true in mice bearing progressively growing tumors. Gillespie and collaborators (9) showed that CTL could not be recovered from progressively

growing MSV-induced tumors, suggesting perhaps that the CTL precursor traffic had been radically altered. In agreement with this hypothesis, preliminary experiments in our laboratory have indicated that the leukocyte fraction extracted from progressively growing tumors not only was deficient in active CTL generated in vivo, but also appeared to lack CTL precursor cells.

Summary

Leukocyte fractions extracted from the tumor mass and the lymphoid organs of C57BL/6 (B6) mice carrying murine sarcoma virus-induced tumors contained primed cytolytic T-lymphocyte (CTL) precursor cells, in addition to active cytotoxic T cells. These leukocyte fractions gave a secondary response when stimulated in vitro with syngeneic tumor cells, generating large numbers of specific CTL. The activity of these CTL ($H-2^b$) was apparently $H-2$-restricted, because it was ineffective on tumor targets bearing strongly cross-reacting tumor-specific antigens but with the $H-2^d$ haplotype. Furthermore, only $H-2^b$ cells bearing the Friend, Moloney, Rauscher-associated antigen, such as Rauscher leukemia virus-induced RBL-5 cells and Friend leukemia virus-induced HFL/b cells, were lysed efficiently. BδGV cells ($H-2^b$ cells induced by Gross leukemia virus) were not affected by the same CTL. We propose the existence of a dynamic state involving the migration of primed CTL precursor cells between the lymphoid organs and the tumor mass, as well as the differentiation of these precursor cells within the tumor mass into highly specific CTL.

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