B7.1 Expression on Tumor Cells Circumvents the Need of Professional Antigen Presentation for in Vitro Propagation of Cytotoxic T Cell Lines

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Abstract

In vitro propagation of tumor-specific CTLs, to be used for identification of tumor antigens (Ag) and/or adoptive immunotherapy, is hampered by the need of large amounts of professional antigen-presenting cells (APC) used for periodic cycles of restimulation. We evaluated whether RMA T lymphoma cells, stably transfected with the cDNA encoding for the B7.1 costimulatory molecule, provided the activation signals to CD8+ T lymphocytes in the absence of professional APC and CD4+ helper cells. We demonstrate here that long-term CD8+ cell lines can be efficiently propagated in vitro by repeated cycles of stimulation with tumor cells stably expressing B7.1. Professional APC and CD4+ helper cells are not required as far as interleukin 2 is exogenously provided. Furthermore, CD8+ blasts needed both signal 1 (Ag in the contest of the MHC molecule) and signal 2 (interaction of costimulatory molecules) for restimulation. T cell blasts in the presence of signal 1 or 2 only still retained their effector potential but did not undergo clonal expansion. These results are very promising for further applications of specific immunotherapies in humans.

Introduction

CTLs play a major role in the rejection of immunogenic tumors (1, 2). In the last years, a great deal of attention has been focused on the identification of tumor-specific Ag recognized by CTL (3), which would allow the use of specific CTL for antitumor immunotherapy. Antitumor-specific CTL can be generated in vitro from cancer patients and tumor-bearing mice by culturing peripheral blood mononuclear cells, tumor-infiltrating lymphocytes, SC, or lymph node cells in the presence of irradiated tumor cells or professional APC pre-pulsed with tumor Ag (2—4). However, CTL propagation in vitro requires frequent cycles of restimulation with professional APC. This technique, even if quite efficient in terms of specificity, is hampered by the difficulty to obtain large amounts of APC from cancer patients. It is, therefore, necessary to define more feasible strategies for induction and propagation of highly specific CTL, without the need of autologous professional APC.

For optimal activation of T lymphocytes, both signals 1 and 2 are required (5, 6). Signal 1 is supplied by the formation of the trimolecular complex T-cell receptor-peptide-MHC between T cells and APC. Signal 2 is delivered through the interaction of costimulatory molecules (5). The B7 family represents a group of costimulatory molecules (7). The interaction between B7 and its counter-receptors determines an increased stability of mRNA for several lymphokines, including IL-2 (7, 8). Tumor cells do not usually express the B7 molecules and, therefore, are not able to directly activate tumor-specific CTL (9). Transfection of the B7.1 or B7.2 gene into tumor lines can lead to the induction of antitumor CTL both in vivo and in vitro (9—18). Moreover, a short-term, tumor-specific CTL line has been generated against the murine T lymphoma cell line EL-4 by repeated in vitro stimulation with B7.1-transfected or wild-type EL-4 cells in the presence of irradiated syngenic SC (11). We asked whether transfection of B7.1 onto RMA T lymphoma cells (RMA/B7.1) was necessary and sufficient for induction and in vitro propagation of antitumor CTL, in the absence of any other APC or accessory cell.

Materials and Methods

Tumor Cell Lines. The H-2b T-cell lymphoma lines RMA and RMA-S were kindly provided by Vincenzo Cerundolo (John Radcliff Hospital, Oxford, United Kingdom). The RMA/B7.1 cells (clone 6) and the RMA-S/B7.1 cells (clone 3A1) were generated as described elsewhere (13, 16). The H-2b B16F1 and the human M14 melanoma cell lines, the H-2b P815 mastocytoma line, and the human lung carcinoma N592 were purchased from American Type Culture Collection (Rockville, MD). The cells were cultured in RPMI 1640 supplemented with penicillin-streptomycin and 10% FCS.

Mice and Immunization Procedures. C57BL/6 (B6; H-2b) female mice, 8—10 weeks old, were purchased from Charles River (Calco, Italy), housed in a specific pathogen-free animal facility, and treated in accordance with the European Community guidelines. The animals were immunized by a single s.c. injection of 3 X 106 viable RMA or RMA/B7.1 cells.

In Vitro CTL Induction. SC were isolated and fractionated from naive or tumor-challenged mice by a passage on a nylon wool column (19). The eluted nonadherent cells (NWSC) were resuspended in RPMI 1640 containing 10% heat-inactivated FCS, 50 μM 2-mercaptoethanol, 2 mm l-glutamine, 10 mm HEPES, 1 mm sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin (culture medium). Thirty X 106 NWSC were mixed to 3 X 106 mit-c-treated (50 μg/ml in RPMI 1640 at 37°C for 1 h) RMA cells in 10 ml culture medium. Five days later, blasts were isolated on a lymphocyte gradient (Lympholyte-M; Cedarlane, Hornby, Ontario, Canada) and either analyzed by flow cytometry on a FACStar plus (Becton Dickinson) using anti-CD4-PE, anti-CD8-FITC, anti-NK-PE, and antimouse immunoglobulin-FITC mAb (PharMingen) or expanded in culture medium supplemented with 10—20 IU/ml IL-2. Cells were checked every other day, and medium was changed when needed. CTL lines were generated from the blasts obtained from the NWSC of mice challenged with RMA (10-3-95 line) or RMA/B7.1 (7-4-94 line) by repeated cycles of restimulation and expansion in 20 IU/ml IL-2. For the first 14 cycles of restimulation, 7-4-94 blasts were cultured in 24-well plates with equivalent numbers of mit-c-treated RMA or RMA/B7.1 cells and irradiated syngenic SC. For the following cycles of restimulation of the 7-4-94 CTL line and for restimulation of the 10-3-95 CTL line, blasts were cultured in the presence of RMA/B7.1 cells only. For the experiment indicated in Fig. 4, 4 X 104 blasts were cocultured with equivalent numbers of mit-c-treated RMA, RMA/B7.1, or RMA-S/B7.1 cells (day 0). At day 4, blasts were isolated on a Lympholyte-M gradient and at day 6 and 7 were counted by trypan blue exclusion in a blind test by two individuals. At day 7, blasts were analyzed by flow cytometry and tested in a standard 51Cr-release cytotoxicity assay (see...
Cytotoxicity Assays. Blasts were tested for cytolytic activity in a standard 4-h $^{51}$Cr-release assay as described (13). The percentage-specific $^{51}$Cr release of triplicates was calculated as:

$$\% \text{specific }^{51}\text{Cr release} = \frac{\text{Average experimental cpm} - \text{average spontaneous cpm}}{\text{Average maximum cpm} - \text{average spontaneous cpm}} \times 100$$

$^{51}$Cr release of target cells alone (spontaneous release) was always <25% of maximal $^{51}$Cr release (target cells in 0.25 M HCl). In inhibition experiments using mAb, target and effector cells were preincubated at 37°C for 15 min with anti-K$^b$, anti-D$^b$, anti-CD8, or anti-CD4 mAb, respectively, before addition in the cytotoxicity assay. The final mAb concentration ranged between 0.05 and 5 $\mu$g/ml.

Results and Discussion

Anti-RMA CTL Can Be Generated by in Vivo Immunization with RMA or RMA/B7.1 Cells. The Rausher virus-induced T-lymphoma RMA (H-2$k$), although highly tumorigenic (minimal tumorigenic dose, $1 \times 10^3$ cells; Ref. 20), is immunogenic. In fact, a single injection of irradiated tumor cells completely protects syngenic mice from further challenge with RMA cells (11).

Toward the induction of anti-RMA CTL, NWSC from virgin B6 mice were cultured in vitro for 5 days with mit-c-treated RMA cells. Blasts were tested for cytolytic activity on day 6 in a standard $^{51}$Cr-release assay. In vitro priming of naive NWSC with RMA cells did not elicit antitumor CTL (data not shown), confirming our previous results (16). Therefore, we inoculated s.c. B6 mice with RMA or RMA/B7.1 cells. Two weeks later, NWSC from both groups of mice were cocultured in vitro with mit-c-treated RMA cells. NWSC from mice challenged with RMA/B7.1 cells (Fig. 1A) or RMA cells (Fig. 1B) highly efficiently killed RMA cells. No lytic activity was detectable against the syngenic B16F1 melanoma (Fig. 1). The specificity of anti-RMA CTL was also tested against the NK target RMA-S line, a RMA mutant, which is defective in Ag presentation (21) and maintains unaltered the main surface marker profile of the parental cell line, except for the almost undetectable expression of K$^b$ and D$^b$ MHC-I molecules (22). At the highest E:T ratio, the killing activity against RMA-S was <20% (Fig. 1).

As reported previously in other models (11), CTL obtained from mice injected with RMA/B7.1 cells killed the target cells more efficiently than NWSC from mice immunized with wild-type tumor cells (Fig. 1), despite the equivalent percentage of CD8$^+$ cells obtained and used for the cytotoxicity assay (37 and 42%, respectively). This is probably due to a higher efficiency in Ag-specific CTL recruitment by B7.1-positive tumor cells.

Our data suggest that for in vitro generation of anti-RMA CTL in vivo, priming is necessary and that the "bonus" effect of B7.1 expression on tumor cells (see above) is not essential. Upon injection with RMA cells, it could either be that MHC-I-associated tumor antigens are transferred to the professional APC of the host (23), or anti-RMA CTL receive the costimulatory signal by bystander APC (24).

An open question is whether the B7.1 molecule acts as an accessory molecule also in the effector phase of the antitumor immune response. Different groups reported that CTL induction by B7.1-transfected tumor cells lysed transfected and wild-type target cells with similar efficiency (14, 25). Ramarathinam et al. (12), on the other hand, demonstrated that freshly isolated tumor-infiltrating lymphocytes preferentially lysed B7.1-transfected tumor cells. In our model, NWSC from mice injected with RMA/B7.1 cells and restimulated in vitro with RMA cells killed both RMA and RMA/B7.1 cells with equal efficiency (Fig. 1A), confirming the findings of Chen et al. (14) and Harding and Allison (25). The difference in the findings between our report and the one by Ramarathinam et al. (12) might be due to the different source of effector cells used, i.e., SC restimulated in vitro in our case and for Chen et al. (14) and Townsend et al. (25) and freshly isolated tumor-infiltrating lymphocytes in the case of Ramarathinam et al. (12).

Long-Term Anti-RMA Cell Lines Can Be Propagated in Vitro by Stimulation with RMA/B7.1 Cells in the Absence of Professional APC and CD4$^+$ Cells. Anti-RMA CTL obtained from NWSC of mice injected with RMA/B7.1 cells (hereinafter referred to as 7-4-94 cells) were expanded in IL-2 and enriched by cyclic stimulation with mit-c-treated RMA or RMA/B7.1 cells and irradiated syngenic SC. We asked whether it was possible to obtain an efficient in vitro stimulation of the 7-4-94 cells by culturing them in the presence of RMA/B7.1 cells only. To this aim, 7-4-94 cells were cultured for 48 h in the presence of equivalent numbers of mit-c-treated RMA/ B7.1 cells, with or without irradiated syngenic SC, as a source of professional APC. Blasts were then isolated by centrifugation on a Lympholyte-M gradient, counted, and analyzed by flow cytometry. The cell yield was similar (data not shown), and the percentage of CD8$^+$ cells was 89 and 92%, respectively, for 7-4-94 cells cultured with or without irradiated APC. The cytotoxic activity of anti-RMA blasts obtained was also tested in a standard $^{51}$Cr-release assay. As shown in Fig. 2, RMA cells were strongly lysed by 7-4-94 cells, despite the different condition of restimulation. No significant killing of RMA-S cells was detectable in either effector populations. We can, therefore, conclude that irradiated syngenic SC are not required for in vitro optimal restimulation of anti-RMA CTL and that RMA/B7.1 cells are able to supply both signals 1 and 2 to antitumor-specific CTL.

In several models, it has been demonstrated that optimal activation and differentiation of CTL can be achieved in the absence of T-helper cells (13, 25). In our model, we can exclude the need for CD4$^+$ cells for the in vitro maintenance of anti-RMA CTL because no CD4$^+$ cells were ever added to the culture of the 7-4-94 cell line. We can also rule out that soluble factors, other than IL-2, released by CD4$^+$ cells are...
necessary for CTL induction and maintenance because 7-4-94 cells were always cultured in the presence of IL-2 only.

We also generated a line from NWSC of mice injected with RMA cells by cycles of restimulation with RMA/B7.1 cells following the procedure set for the 7-4-94 line. This line, named 10-3-95, and the 7-4-94 line have been successfully kept in culture for over 1 year and 4 months, respectively, by alternate cycles of expansion in IL-2 and restimulation with RMA/B7.1 cells. Hence, the possibility to maintain antitumor CTL lines in vitro by cycles of restimulation with RMA/B7.1 cells is not an occasional phenomenon, and it does not depend on the type of in vivo priming.

Anti-RMA CTL Are Highly Specific and Enriched during the in Vitro Culture. The anti-RMA 7-4-94 and 10-3-95 CTL lines were tested repeatedly for their lytic potential during the in vitro culture. The lytic activity against RMA increased progressively, reaching a plateau after approximately 5 cycles of stimulation. As it is shown in Fig. 3 for both 7-4-94 cells (after 6 cycles of restimulation; Fig. 3A) and 10-3-95 cells (after 5 cycles of restimulation; Fig. 3B), the lytic activity against RMA cells was significantly increased if compared with the lytic activity of NWSC from mice immunized with RMA/B7.1 cells (Fig. 1A) or with RMA cells (Fig. 1B), suggesting a progressive enrichment in anti-RMA CD8+ cells.

To verify the specificity of the 7-4-94 line, a panel of syngenic (B16F1), allogenic (P815), and xenogenic (N592 and M14) tumor cells were used as targets in a standard 51Cr-release assay (Fig. 3A). 7-4-94 cells did not lyse any of the targets used, therefore demonstrating the high specificity of anti-RMA CTL. To rule out an autoreactive activity of 7-4-94 cells, PHA blasts from syngenic SC were also tested in cytotoxicity assays. In Fig. 2, two experiments are reported where PHA blasts were not lysed by 7-4-94 blasts.

It has been reported that the cytotoxic activity of anti-RMA CD8+ cells is H-2-D^d restricted (26). 7-4-94 and 10-3-95 cell lines were tested for their cytolytic potential against RMA-S cells. The absence of a significant lysis of RMA-S cells by 7-4-94 and 10-3-95 cells (Fig. 3) suggests an MHC-I restriction of the anti-RMA activity of these lines. The cytotoxic activity of 7-4-94 and 10-3-95 CD8+ cells was also tested in the presence of anti-CD4 or anti-CD8 and anti-K^b or anti-D^d mAbs. As expected for a population homogeneously represented by CD8+ cells (>95% for both T-cell lines), RMA killing was significantly inhibited by increasing concentrations of anti-CD8 and not anti-CD4 mAbs (data not shown). A significant inhibition of specific killing was also found when target cells were incubated with increasing concentrations of D^d and not K^b mAbs (data not shown).
Fig. 4. Both signals 1 and 2 are required for in vitro stimulation of antigen-specific CD8+ blasts. In the upper panel, a representative of at least two experiments is reported where 7-4-94 cells were cocultured with equivalent numbers of mit-c-treated RMA, RMA/B7.1, or RMA-S/B7.1 cells. Viable cells were counted by trypan blue exclusion and analyzed by flow cytometry for CD4 and CD8 expression. In the lower panel, a period of culture of 7-4-94 cells is reported, expressed as cell yield related to the days of culture. Arrows, the day of stimulation with RMA/B7.1 cells. At day 7, 2.2 X 10^5 cells were obtained by signal 1 in the presence of anti-CD28 mAbs, which supply the costimulatory signal in the absence of professional APC. The same results can be achieved by the use of cells transfected with B7.1 or B7.2 (10, 13, 15—18). On the other hand, it appears that efficient strategy for in vitro generation of tumor cells from surgical specimens is still not available. It has been reported that antitumor CTL may be obtained in vitro by culturing human peripheral blood mononuclear cells in the presence of allogeneic tumor lines sharing at least one HLA allele (30, 31). We speculate that the creation of a bank of human tumor lines stably expressing costimulatory molecules may be possible. Additionally, many tumors do not express the B7 family of molecules (9), and not all tumors are easily transflectable. Much work still needs to be done in finding a suitable and safe vector for transfection of B7 molecules into tumors. Nonetheless, the results reported herein are very promising for future application of specific immunotherapies in humans.

Acknowledgments

We thank Silvia Heltai and Giuseppe Consogni for excellent technical assistance, Paolo Dellabona for discussions and criticism, Angelo A. Manfredi and Marina Ferrarini for critical reading of the manuscript, and Vincenzo Cerundolo for RMA and RMA-S lines.

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