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Advances in Brief

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

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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 periodical 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[3] recognized by CTL (3), which would allow the use of specific CTL for antitumor immmunotherapy. 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 that are constitutively expressed on APC and interact with their natural ligands CD28 and CTLA-4 on T cells (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 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-2^b 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-2^b B16F1 and the human M14 melanoma cell lines, the H-2^d 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-2^b) 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 3×10^4 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 $\times 10^6$ NWSC were mixed to 3×10^6 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 lympholyte 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, 4×10^5 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 ^{51}Cr-release cytotoxicity assay (see

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[3] The abbreviations used are: Ag, antigen; SC, spleen cells; IL-2, human recombinant interleukin 2; APC, antigen-presenting cells; NWSC, nylon wool spleen cells; mit-c, mitomycin-C; mAb, monoclonal antibody; PHA, phytohemagglutinin; E:T, effector: target.

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below). Blasts stimulated with RMA/B7.1 cells were restimulated with an equal number of RMA/B7.1 at days 10 and 20. PHA blasts, to be used as a target in the cytotoxicity assay, were obtained from B6 SC by stimulation with PHA (10 μg/ml) for 3 days and further expanded with IL-2.

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:

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

^{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 μg/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^b), although highly tumorigenic (minimal tumorigenic dose, 1×10^3 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. 1*A*) or RMA cells (Fig. 1*B*) 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).

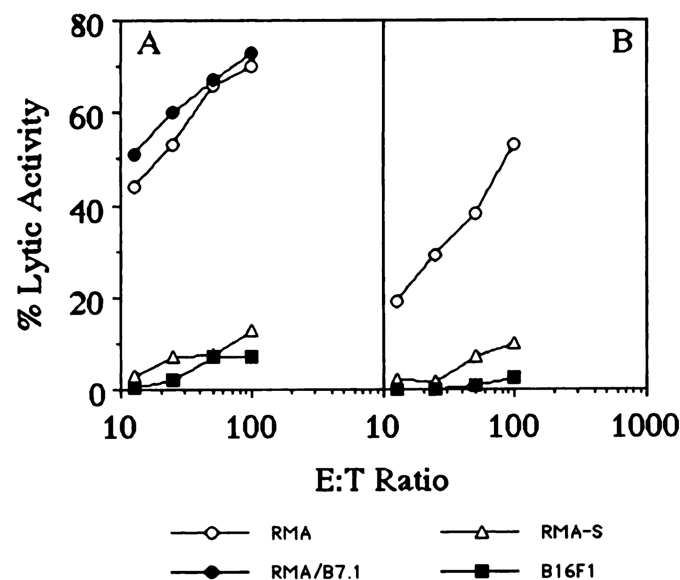

Fig. 1. Comparison of CTL activity generated from NWSC of mice immunized with RMA or RMA/B7.1 cells. NWSC from mice injected with viable RMA/B7.1 (*A*) or RMA (*B*) cells were restimulated *in vitro* with mit-c-treated RMA cells and tested for their cytolytic activity in a standard ^{51}Cr-release assay. As targets, RMA (○), RMA/B7.1 (●), RMA-S (△), and B16F1 (■) cells were used. Each panel represents one of at least three independent experiments. Values are expressed as a percentage of specific ^{51}Cr release at E:T ratios ranging from 100:1 to 12:1.

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. 1*A*), 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

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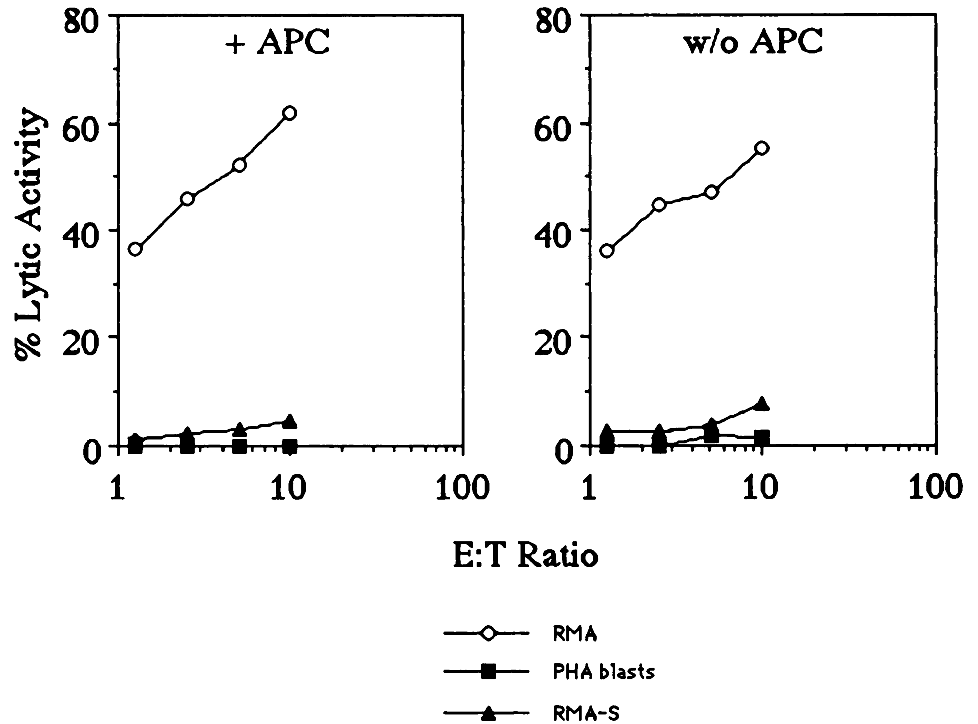

Fig. 2. Stimulation of anti-RMA CD8$^+$ blasts with RMA/B7.1 cells in the absence of professional APC does not alter their lytic potential. 7-4-94 cells were cultured in the presence of mit-c-treated RMA/B7.1 cells with (*left panel*) or without (*right panel*) irradiated syngenic professional APC and tested for their cytolytic activity in a standard ^{51}Cr-release assay. Target cells were RMA cells (○), RMA-S cells (▲), and syngenic PHA blasts (■). Values are expressed as a percentage of specific ^{51}Cr release at E:T ratios ranging from 10:1 to 1:1.

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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. 3*A*) and 10-3-95 cells (after 5 cycles of restimulation; Fig. 3*B*), 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. 1*A*) or with RMA cells (Fig. 1*B*), 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 ^{51}Cr-release assay (Fig. 3*A*). 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 used as targets 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^b 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^b 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^b and not K^b mAbs (data not shown).

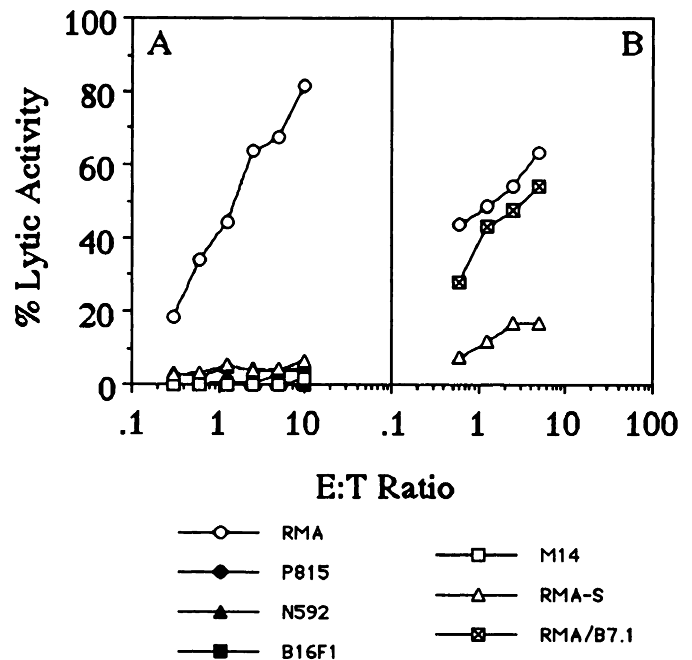
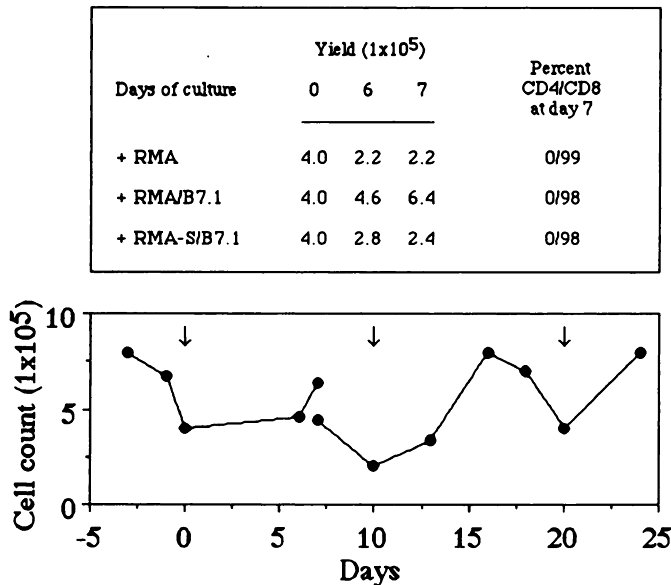


Fig. 3. Anti-RMA CTL are highly specific and enriched during the *in vitro* culture. Anti-RMA 7-4-94 (CD8$^+$: 96%; *A*) and 10-3-95 (CD8$^+$: 95%, *B*) lines were tested in a standard ^{51}Cr-release assay after 6 or 5 cycles, respectively, of restimulation. Target cells were RMA (○), P815 (●), N592 (▲), B16F1 (■), M14 (□), RMA-S (△), and RMA/B7.1 (⊠) cells. Values are expressed as percentage of specific ^{51}Cr release at E:T ratios ranging from 10:1 to 0.3:1 for 7-4-94 cells and from 5:1 to 0.6:1 for 10-3-95 cells.



Days of culture	Yield (1×10^5) 0	6	7	Percent CD4/CD8 at day 7
+ RMA	4.0	2.2	2.2	0/99
+ RMA/B7.1	4.0	4.6	6.4	0/98
+ RMA-S/B7.1	4.0	2.8	2.4	0/98

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, 2.2×10^5 cells were subtracted to be used for cytotoxicity assay and flow cytometry analysis. See "Materials and Methods" for experimental details.

To verify whether the cyclic *in vitro* stimulation of anti-RMA CTL with RMA/B7.1 cells has modified the killing activity against B7.1-positive cells, 10-3-95 cells were tested in a standard cytotoxicity assay against both RMA and RMA/B7.1 cells. CD8+ blasts killed both targets equally well (Fig. 3*B*), therefore demonstrating that the periodical contact with RMA/B7.1 cells did not alter the specificity of the lytic potential of anti-RMA CTL.

Both Signals 1 and 2 Are Required on Tumor Cells for *in Vitro* Stimulation of Anti-RMA CD8+ Cells. It is quite clear that signal 1 is not sufficient for activation of T lymphocytes, and a second co-stimulatory signal is needed (5, 6). The key costimulatory signal seems to be given by the interaction between the CD28 molecule on T cells and its ligands on the surface of APC (7, 8). The intracellular signals delivered by this contact are not identical in T cells at rest or blasts (8). It is well known that *in vitro* activation of naive T cells can be obtained by signal 1 in the presence of anti-CD28 mAbs, which supply the costimulatory signal in the absence of professional APC (7, 8). 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 T-cell blasts do not require signal 1 for activation. Reports from different laboratories have shown that anti-CD28 mAb alone can stimulate mitogen-activated T-cell blasts, in contrast to T cells at rest (7, 8, 27). Our model seems to be ideal to give an answer to this question because we can rely on a well-characterized population of antigen-specific CD8+ cells (the 7-4-94 line) and a set of potential APC, which possess signal 1 only (RMA cells), or both signals 1 and 2 (RMA/B7.1 cells). Furthermore, we devised a system to deliver signal 2 only by transfecting B7.1 into RMA-S cells (RMA-S/B7.1; Ref. 13).

Resting 7-4-94 cells were cultured in the presence of equal numbers of mit-c-treated RMA, RMA/B7.1, or RMA-S/B7.1 cells (day 0). At day 6, blasts stimulated with RMA/B7.1 cells were actively proliferating, reaching the maximal expansion on day 7 (Fig. 4, *upper panel*). On the other hand, the number of 7-4-94 cells cultured with RMA or RMA-S/B7.1 cells decreased rapidly. We can, therefore, exclude, at least in our model, that either signal 1 or signal 2 alone is able to restimulate CD8+ blasts. In Fig. 4 (*lower panel*), the cell yield is reported starting from day −3, when T-cell blasts were still actively proliferating. The other two cycles of restimulation with RMA/B7.1 cells are reported to give a sample of cell yield during the *in vitro* culture of the 7-4-94 line.

A standard cytotoxicity assay was also preformed on day 7, which showed no significantly different cytolytic activity for the three groups of 7-4-94 cells (data not shown). Otten and Germain (28) demonstrated that the engagement of the T-cell receptor of CD8+ cells without signal 2 prevents the subsequent production of IL-2 and proliferation but does not alter the cytotoxic function. Our data confirm those previous findings. It is noteworthy that our results also suggest a similar behavior for CD8+ cells reached by signal 2 in the absence of signal 1.

Huang *et al.* (23) proposed recently that the presentation of tumor-associated Ag to the reactive T cells *in vivo* is primarily due to professional APC of the host ("indirect presentation") and plays the major role in the induction of antitumor immune response (23). The model reported herein is the first demonstration of an efficient *in vitro* activation of Ag-specific CD8+ blasts in the absence of professional APC. We can exclude the possibility of a presentation of RMA-associated Ag by CD8+ blasts, as it has been reported for CD4+ cells (29), because 7-4-94 cells cultured with RMA or RMA-S/B7.1 cells, from which CD8+ cells could have taken up antigenic material, did not proliferate. These data are in accordance with the ones reporting of activation of purified naive T cells by different B7.1-transfected cells (10, 13, 15–18, 25) and sustain a direct role of B7.1-positive tumor cells ("direct presentation") in the activation of a specific CTL response.

In conclusion, we demonstrate that long term antitumor-specific CD8+ cell lines can be efficiently maintained and propagated *in vitro* by repeated cycles of stimulation with tumor cells transfected with the costimulatory molecule B7.1. However, at least two considerations may be taken into account before using this approach for therapy. An efficient strategy for *in vitro* generation of tumor lines 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 transfectable. 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.

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