ABSTRACT

Cells required for the in vitro generation of syngeneic cytotoxic T-lymphocytes (CTL) against the P815 mastocytoma in the DBA/2 mouse strain were investigated. For both immune and tumor-bearing host spleen cells, CTL effector cells were eliminated by treatment with anti-Thy1.2, anti-Lyt1.1, or anti-Lyt2.1 and C^, but were resistant to anti-L3T4 (GK1.5). Thus, CTL effectors (and their precursors) were Lyt1^2\*\+ \L3T4^+ .

However, P815-specific CTL could not be generated in the absence of L3T4^+ cells, whose function could be replaced with exogenous interleukin-2 (IL-2). When monoclonal antibodies against L3T4 were added to mixed leukocyte tumor cultures, CTL generation was markedly inhibited. Deposition of accessory cells also led to a marked reduction in CTL generation, which could be restored to control levels by adding adherent cells from normal spleens or with exogenous IL-2, but not with IL-1. Thus, accessory cells are apparently required to present the tumor antigens of this in vitro model to T-helper cells.

INTRODUCTION

Several studies have demonstrated that adoptive transfer of specifically sensitized lymphocytes, either with or without in vitro resensitization, can cause tumor regression in the adoptive host (1-3). In the course of trying to develop strategies for adoptive immunotherapy of tumors using autologous lymphoid cells, a number of T-cell subpopulations have been defined with different antitumor functions. As numerous animal models and in vitro correlates of antitumor immunity have been developed, a number of apparent conflicts have arisen regarding the phenotypes and/or functions of the T-cells which can mediate host resistance to tumors. Previous studies of antitumor effector cells have relied on the use of lymphocytes from immunized tumor-resistant animals, but experiments to delineate the details of the in vitro responses of cells from animals bearing progressive tumors have not been described. In order to exploit the immune response to cancers clinically, the responses of lymphocytes from TBHs^ must be understood.

In some models, development of Lyt1^2\+ CTL appears to be the critical factor in tumor destruction (4-9), while for other tumors Lyt1^2\- cells, which act as helper cells or produce delayed-type hypersensitivity responses apparently mediate host resistance (10-13). Recently, Greenberg (14) showed that in the Friend leukemia (FBL-3) model some CTL are Lyt1^2\+ . Adoptive transfer of CTL could cure animals with disseminated tumor, but only if IL-2 was given to support their proliferation in vivo (14). In the same system it was shown that adoptive transfer of noncytotoxic Lyt1^2\+ cells from immune mice also could induce complete remissions without adding IL-2 and without generation of Lyt2^+ CTL (11). Shu and Rosenberg (15) have described a sarcoma model in which cytotoxic effector cells from immune mice boosted in vitro could mediate tumor regression if IL-2 was provided, whereas adoptive transfer of fresh, noncultured Lyt1^2\+ immune lymphocytes, which were not cytotoxic, induced tumor regression without added IL-2.

Recently, we have described an in vitro model of syngeneic CTL generation from tumor antigen-primed splenocytes against the P815 mastocytoma and showed that this response is regulated by Ts cells from "late" TBH (16). In the course of characterizing the mechanisms of this suppression, we noted that the in vitro CTL response of immune spleen cells was more susceptible to suppression than the response of "early" TBH spleen cells. As a step towards determining the reasons for this difference and working out the detailed mechanisms of Ts function, we set out to determine the cell phenotypes and interactions involved in the in vitro CTL response to syngeneic tumor for both TBH and immune spleen cells. In this report, we describe the details of this response and the cells involved. This represents the first description of such data for the in vitro response to the P815 tumor, or for any tumor using lymphocytes from syngeneic TBH mice.

MATERIALS AND METHODS

Virus-free DBA/2NCrBR mice were obtained from Charles River Laboratories, Wilmington, MA. Mice were housed in a virus-free environment, given food and water ad libitum, and screened regularly for pathogenic viruses using standard serological tests. Sprague-Dawley rats were also obtained from Charles River. Care of animals was in compliance with institutional and NIH guidelines.

Tumors. The P815 mastocytoma (H-2^), was obtained from Dr. Vincent J. Merluzzi at the Sloan Kettering Institute, Rye, NY. P815 was passaged as ascites in syngeneic DBA/2 mice. All tumor lines were also cryopreserved in RPMI 1640 (Hazelton Research Products, Denver, PA) plus 20% fetal calf serum and 10% dimethyl sulfoxide over liquid N. For each experiment, frozen P815 tumor cells were thawed, cultured in vitro for 24-48 h, washed, and resuspended in HBSS. Five million cells were then injected i.p. into DBA/2 mice, and after one i.p. passage, tumor ascites was harvested 1 wk later for use to inject TBHs, to immunize mice, or for in vitro experiments.

Monoclonal Antibodies. Monoclonal anti-Lyt-1.1 antibody (18-2.3 ascites) was kindly provided by Dr. F. W. Shen, Memorial Sloan-Kettering Cancer Center, New York, NY and was used at a dilution of 1:1000. Hybridoma cell lines secreting monoclonal anti-Thy 1.2 (30.H12, ATCC TIB107), anti-Lyt2.1 (116-13.1, ATCC HIB129), anti-L3T4 (GK1.5, ATCC TIB207), anti-I-A^ (MK-D6, ATCC HB3) were obtained from the American Type Culture Collection (Rockville, MD). Ascites containing monoclonal rat IgG antibody against human C3
cytes were lysed by a 5-min incubation in Gey's hemolytic medium volume of 0.05 ml. 

T. parvum (Burroughs Wellcome, Research Triangle, NC) in a total cells/mouse in 0.05 ml for TBHs. For immunization, 10^6 P815 cells were then treated with monoclonal anti-I-Ad and C'. For these experiments, P815 stimulator cells from ascites were also depleted of adherent cells before mitomycin C treatment. As a source of adherent accessory cells in reconstitution experiments, 1.5 x 10^7 normal spleen cells were incubated at 37°C for 2 h in each well of the 24-well plates in which the MLTC were to be set up. Nonadherent cells were removed by vigorous washing, and then the accessory cell-depleted responder cells and stimulator cells were added to these wells.

Interleukin Preparations and Assays. Rat T-cell growth factor-containing supernatants were prepared by incubating Sprague-Dawley rat spleen cells with 2 μg/ml Con A (Calbiochem-Behring, San Diego, CA) and harvesting supernatants at 48 h. These were stored at -80°C, and residual Con A was neutralized before use by addition of 10 mg/ml methyl-β-mannoside (Sigma, St. Louis, MO). This material contained 80 units/ml of IL-2 activity, when a unit is defined by 50% maximal stimulation of CTL-2 proliferation (20). IL-2 levels were assayed by stimulation of the IL-2-dependent cell line CTLL-2, as previously described (20). Briefly, after washing in HBSS, 5-10 x 10^5 CTL-2 cells in 100 μl were plated in each well of a 96-well plate, in which serial dilutions of materials to be assayed were already present in 100 μl (assayed in triplicate). Plates were incubated for 24 h at 37°C in 5% CO2 and humidified air. Each well was then pulsed with 1 μCi [methyl-3H] thymidine, incubated for 6 h, and harvested onto glass-fiber filter paper for counting in a liquid scintillation counter. SEs for triplicate assays were 10% or less and are not shown. Human recombinant IL-2 was obtained from Cetus Corporation, Emeryville, CA. Amounts of this material used are expressed in terms of the manufacturer's units, which we found to be the equivalent of approximately 2 units in the CTLL assay.

IL-1 was produced from lipopolysaccharide-stimulated macrophages by the method of Hoffmann et al. (21). Briefly, peritoneal exudate cells were collected from AKR mice, injected 7 days previously with 1 mg C. parvum i.p., and incubated at 37°C in 24-well tissue culture plates at 2.5 x 10^4 cells/ml, 1 ml/well, in tissue culture medium containing 10 μg/ml lipopolysaccharide (Escherichia coli 055:B5; Difco Laboratories, Detroit, MI). Nonadherent cells were removed after 6 h by washing wells with warm HBSS. Adherent cells were lysed by the addition of 1 ml/well of distilled, deionized water and a cycle of freezing and thawing. Cell lysates were filtered (pore size, 0.45μm) and stored at -20°C.

IL-1 activity was determined by stimulation of the IL-1 responsive D10.G4.1 helper T-cell line (22) (ATCC TIB224). Twenty thousand D10.G4.1 cells were cultured in flat-bottomed microtiter plates in tissue culture medium containing 2.5 g/ml Con A and serial dilutions of the IL-1 preparation for 72 h at 37°C. Cultures were pulsed with 1 μCi of [3H] thymidine for the last 6 h of incubation, then harvested and processed as described above for the IL-2 assay. The IL-1 preparation contained 450 units/ml of activity (20).
tumor cell inoculation (Days 1–21) could spleen cells from either immune or TBH mice be shown to have any cytotoxic activity against P815 when tested directly after removal from the animal. In vitro restimulation with tumor cells was required for CTL to be activated. Spleen cells from normal mice did not produce any CTL activity in vitro, whether stimulated with mitomycin C-treated P815 cells or not.

Phenotype of the CTL Effectors. Treatment of cells derived from early TBH spleens after a 5-day MLTC with anti-Thy1.2, anti-Lyt-1, or anti-Lyt-2 antibody and C' eliminated their cytotoxic activity (Fig. 2). Initial experiments had suggested that CTL effectors were predominantly Lyt1^−2^ and that a minor population of Lyt1^+2^ CTL might also be present. By using 2 cycles of depletion and removal of dead cells between treatments, however, all of the anti-P815 CTL effectors could be depleted with either anti-Lyt1 or anti-Lyt2 antibody. Moreover, this response could not be reconstituted by mixing Lyt1^−2^ cells.

The inability to restore the response by mixing Lyt1^−^ and Lyt2^−^ cells indicates that both markers are present on the same cells, and thus the antitumor CTL in our system are Lyt1^+2^, Treatment of CTL with anti-L3T4 and C', on the other hand, had no significant effect on CTL activity (Fig. 2). The results of similar experiments were identical for spleen cells from immune mice (data not shown).

Phenotype(s) of Cells Required for in Vitro CTL Response. When L3T4^+^ cells were depleted on Day 0 rather than Day 5, before establishing the MLTC, the CTL responses of both immune and early TBH cells were essentially eliminated (Table 1). Anti-Lyt-1 or anti-Lyt-2 antibody and C' treatment also abrogated the response, and remixing Lyt1^−^ and Lyt2^−^ cells did not restore it (data not shown). In contrast, when L3T4^+^ and Lyt2^−^ cells were combined, normal levels of CTL could be generated. Thus, it appears that L3T4^+^, Lyt2^−^, and L3T4^+^ CTL precursors and Lyt2^−^, L3T4^+^ (T_H) cells interact in the secondary in vitro response to syngeneic P815 tumor cells for both immunized and TBH mice. The importance of L3T4^+^ T_H cells in this response was further demonstrated by the addition of anti-L3T4 monoclonal antibody to the MLTC (Table 2). This has been shown by others to block the activation of major histocompatibility complex class II-dependent T_H cells (23, 24), and was able to inhibit P815-induced CTL generation in vitro. Results shown are for TBH spleen cells, and were similar for immune spleen cells.

Role of Interleukin-2. Unresponsive L3T4-depleted spleen cells could be made responsive by the addition of 10–20 units/ml of exogenous IL-2, as either crude supernatant or purified recombinant material (Table 3). These doses were comparable to the levels we have been able to measure in MLTC supernatants and did not induce significant killing activity by normal spleen cells nor by immune cells in the absence of stimulating tumor cells (data not shown). Moreover, treatment of responding cells on Day 0 with anti-L3T4 and C' completely abrogated measurable IL-2 production in MLTCs (Fig. 3); C' alone or anti-Lyt2.1 and C' had no effect.

Role of Accessory Cells. Since L3T4^+^ cells apparently recog-
were harvested on Days 1 or 2 of culture, filtered, and assayed for IL-2 activity. Wells. PSISmc, mitomycin C-treated P815 cells; N.S., normal spleen. Results are for Day 2 and were similar for Day 1. Background incorporation of [3H]thymidine (medium alone) was 3372 cpm.

and the remaining viable cells were then set up in MLTCs. Supernatant fluids were harvested and assayed for cytotoxicity against P815 target cells. IL-1 was added to MLTC medium on Day 0. After 5 days in culture, cells were harvested and assayed for cytotoxicity against P815 target cells. Early TBH spleen cells were depleted of accessory cells by incubation on nylon wool columns and plastic dishes. Nonadherent cells were then treated with monoclonal anti-I-A^d antibody (MK-D6) and C'. The remaining viable cells were then plated in MLTC at numbers equal to untreated cells.

DISCUSSION

In the experiments reported here, details of the cell interactions involved in the in vitro generation of tumor-specific CTL from syngeneic immune or TBH animals against the P815 tumor have been delineated. While similar experiments have been reported in other syngeneic tumor systems using lymphocytes from immune, tumor-resistant animals, they have not been reported for the P815 tumor, nor for any tumor-specific response by lymphocytes from TBHs with progressively growing tumors.

Although no active CTL could be detected in the spleens of early TBH or immune animals, in vitro stimulation of splenocytes from either source with specific tumor antigen resulted in marked CTL activity. We have shown that primed CTL precursors from early TBH and immune animals and the cytotoxic effectors generated in MLTC are all Lyt1^t2, L3T4^T cells and that CTL activation requires the presence of Lyt2^t2, L3T4^+ cells. The function of the latter T-cells could be replaced with crude or recombinant IL-2, and CTL development could be inhibited by antibody against L3T4.

Thus, the secondary in vitro CTL response to the P815 mastocytoma involves Lyt1^t2, L3T4^+ CTL precursors and Lyt2^, L3T4^+ TH cells. The fact that L3T4^+ cells could be replaced by recombinant IL-2 suggests that other TH cell factors (e.g., CTL-differentiation factor) (26, 27) may not be required in this system. We cannot rule out, however, that L3T4-depleted cells might produce such factors in the presence of IL-2. Accessory cells were also required for the CTL response, as might be predicted from the Class II restriction on antigen recognition by L3T4^+ cells (23, 24), the fact that P815 cells are Ia^− (25), and the ability to block the anti-P815 MLTC with anti-L3T4 antibody. Removal of accessory cells markedly reduced or eliminated the CTL response, and the response could be restored to control levels by addition of plastic-adherent cells from normal spleens or with exogenous recombinant IL-2. IL-1, on the other hand, could not bypass the requirement for accessory cells. These results suggest that accessory cells are required to process tumor antigen and present it to L3T4^+ cells.

Anti-P815 CTL effectors generated in vitro and their precursors were found by us to be Lyt1^t2, L3T4^+. This agrees with the findings in several other tumor models (7, 8, 28-30). Although other investigators have found some or all CTL effectors specific for syngeneic tumors to be Lyt1^t2 (6, 14, 15, 31), similar to the descriptions of "classical" allospecific CTL (32, 33), most, if not all, mature functional T-lymphocytes have not been shown to bear some Lyt1 determinants (14, 34, 35).
Thus, Lyt1 phenotype is actually a quantitative rather than a qualitative feature of T-cells. Our finding that 2 treatments with anti-Lyt1.1 antibody were required to eliminate CTL suggests a low density of Lyt1 on these cells and may explain why some of the studies cited above suggested that CTL were Lyt1-2.

We found that L3T4+ T\textsubscript{H} cells are required for the syngeneic anti-P815 CTL response, apparently to produce IL-2. Whether or not there are Lyt2*, L3T4-, Class I-restricted T\textsubscript{H} cells which respond to the P815 tumor, as has been shown for alloantigen (36-38), is not known. However, if they do exist, they are insufficient for the secondary in vitro CTL response of spleen cells to this syngeneic tumor, since only background levels of CTL responsiveness and no IL-2 production were seen in the absence of L3T4+ cells. In contrast to these results, Kern et al. (39) found that for the FBL-3 leukemia, treatment of responder cells with anti-L3T4 and C- only partially inhibited CTL generation, suggesting that L3T4+, Lyt2* T\textsubscript{H} cell may provide some help in that response. This difference between our results and those of Kern et al. (39) may be simply methodological, perhaps resulting from our use of 2 cycles of treatment with antibody and C- . Alternatively, it may be that the cellular interactions involved in the response to a virally induced tumor (FBL-3) are different from the response to a chemically induced tumor (P815). There is precedent for differential requirements in responses to cellular antigens. For example, Mizuochi et al. (37) have shown that the primary CTL response to Class I alloantigen involves both L3T4+ and Lyt2* T\textsubscript{H} cells, which both produce IL-2. In contrast, only L3T4+ T\textsubscript{H} cells participate in the response to trinitrophenylated-self, and no involvement of Lyt2* T\textsubscript{H} cells could be demonstrated (38). Thus, it may be that FBL-3 tumor antigen(s) is analogous to Class I alloantigen, while the P815 tumor antigen(s) is analogous to trinitrophenylated-self. Aside from our findings suggesting that help for the CTL response to the syngeneic P815 tumor is provided exclusively by L3T4+ cells, our results with TBH and immune cells are otherwise in agreement with those in the FBL-3-immune model (39). For both tumors CTL are Lyt2*, and to some extent Lyt1*; T\textsubscript{H} cells, IL-2, and macrophages are required for the CTL response to both tumors. The relationship of these in vitro observations to in vivo antitumor effects is unclear. Our results are consistent with the observation that in the FBL-3 model exogenous IL-2 must be given along with CTL in vitro to induce tumor regression (14). In fact, it is interesting to note that the finding that Lyt2* cells alone could not mediate FBL-3 tumor regression in vivo conflicts somewhat with the suggestion that Lyt2* T\textsubscript{H} cells may play a role in the in vitro CTL response to FBL-3 tumor cells (14). In contrast, the finding by North's group that adoptive transfer of Lyt1-2* precursors of cytotoxic cells alone is sufficient for the destruction of several tumors (including P815) in vitro in T-cell deficient hosts (4, 5, 40) raises some question as to whether, in fact, T\textsubscript{H} cells are always required for in vivo CTL generation. As we have shown, however, primed T\textsubscript{H} cells which support CTL generation in vitro are present in early TBHs. Therefore, when primed CTL precursors are transferred to TBHs treated with irradiation or cyclophosphamide to inactivate suppressor cells, resistant T\textsubscript{H} cells may already be present in the adoptive host.

Further delineation of the functions of cells required for in vitro responses and active in adoptive immunotherapy for different tumor models is needed. CTL precursors and effectors in the DBA/2 anti-P815 MLTC are shown here to be Lyt1 2*. Generation of CTL activity in this syngeneic mouse-tumor combination also requires L3T4+ T\textsubscript{H} cells, adherent accessory cells, and IL-2 (the product of T\textsubscript{H} cells). We have also shown that the CTL response of TBH lymphocytes is similar to that of cells from immune mice. Now that the components of this in vitro, tumor-specific CTL response have been delineated, we are proceeding with experiments to determine which of these are the targets of Ts cells and which may be defective in late TBHs.

REFERENCES


Phenotype of Syngeneic Tumor-specific Cytotoxic T-Lymphocytes and Requirements for Their *in Vitro* Generation from Tumor-bearing Host and Immune Spleens
