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Precursor CTL Specific for Retrovirus-Induced T Lymphoma Are Found at High Frequency in Unprimed Syngeneic Mice

MICHEL P. MOUTSCHEN, HUA SHAN, BRUCE J. GIANTONIO, AND MICHAEL A. BOOKMAN

Department of Medical Oncology, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

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The retrovirus-induced RBL5 lymphoma can be rejected by adoptive transfer of noncytolytic $CD4^+$ Th1 lymphocytes in normal hosts, without a requirement for transfer of specific $CD8^+$ CTL. Therefore, we hypothesized that host precursor CTL (pCTL) might cooperate with transferred $CD4^+$ Th1 cells to mediate tumor rejection. To evaluate this hypothesis, lymphocytes from non-immunized mice were analyzed for cytolytic activity after short-term bulk lymphocyte tumor culture (BLTC) with rIL-2 (5 U/ml). BLTC induced the differentiation of anti-RBL5 CTL distinct from non-MHC-restricted LAK. These effectors were $CD8^+$, TCR α/β^+, and utilized the CD3–TCR complex for MHC class I-restricted lysis. The majority of pCTL were found within the CD44/PgP-1^{hi} population of memory/activated lymphocytes. However, there was no serologic evidence for prior exposure to RBL5-related tumor or viral Ags. CTL activity was susceptible to partial blockade with mAbs directed against CD8 and MHC class I, suggesting a relatively low-affinity Ag–TCR interaction. These data are most consistent with the recruitment of a population of Ag-specific, but cross-reactive, pCTL during BLTC.

INTRODUCTION

Experimental murine tumors that elicit an immune response in syngeneic mice have been studied as models of immunotherapy. Inoculation of irradiated tumor cells promotes T lymphocyte clonal expansion, differentiation, and an efficient memory response capable of preventing tumor growth after secondary challenge (1). T lymphocytes from immunized animals can also eradicate established tumors after adoptive transfer to tumor-bearing hosts.

RBL5 is a Rauscher retrovirus-induced T cell leukemia-lymphoma of C57Bl/6 mice, sharing features with other Friend–Moloney–Rauscher (FMR) group lymphoid tumors (2). The predominant tumor-associated Ag are of viral origin, although nonviral Ag have been described (3–5). In a model of adoptive immunotherapy, we demonstrated that transfer of clonal noncytolytic $CD4^+$ Th1 cells derived from immunized animals was sufficient to cure a proportion of mice with established RBL5 tumors (6, 7). Although the therapeutic efficacy of noncytolytic $CD4^+$ Th cells has been confirmed in other models (8–10), the mechanisms ultimately involved in tumor elimination remain unclear.

Tumor rejection has usually been attributed to transfer of the tumor-specific cells, without a defined role for preexisting host lymphocytes. For example, FBL3 leukemia can be eradicated by adoptive transfer of $CD4^+$ Th cells without apparent recruitment

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of host $CD8^+$ CTL (11). However, there is considerable evidence for MHC class I-restricted $CD8^+$ CTL responding against MuLV-induced tumors (12, 13), including specific recognition of a Friend MuLV *gag* gene product by $CD8^+$ CTL (14).

We hypothesized that anti-RBL5 $CD4^+$ Th cells might recruit and activate a population of CTL precursors present in nonimmunized or tumor-bearing mice. Existence of such a mechanism would imply the presence of a high frequency of anti-RBL5 precursor CTL in nonimmunized animals. In the present study, we evaluated the generation of CTL following bulk lymphocyte tumor cultures (BLTC) using lymph node (LN) lymphocytes from naive (i.e., nonimmunized) animals, irradiated RBL5 tumor cells, and rIL-2 at low concentration (5 U/ml).

MATERIALS AND METHODS

Mice. Male C57Bl/6NIcr, 8–12 weeks old, were obtained from the barrier colony of the Laboratory Animal Facility (LAF) of the Fox Chase Cancer Center. Lymphoid cell suspensions were prepared from mice sacrificed by CO_2 asphyxiation followed by aseptic removal of LN and spleen. Single-cell suspensions were prepared by teasing with 20-gauge needles, nylon straining, and washing.

Tumors. RBL5 (Rauscher retrovirus-induced) and EL-4 (benzpyrene-induced, Rauscher Ag negative) are T cell lymphomas from C57Bl/6 ($H\text{-}2^b$) mice that were maintained by serial ip transplantation in syngeneic animals or short-term tissue culture. YAC-1 is a Moloney virus-induced T leukemia derived from A/J mice (K^kD^d) used as a prototype NK-sensitive target and maintained *in vitro.* All tumor lines were free of detectable mycoplasma by immunofluorescence and *in situ* hybridization screening (performed by S. Howard, Cell Culture Facility, Fox Chase Cancer Center).

Reagents. FITC-conjugated anti-CD8 (Rat IgG_{2a} clone 53-6.7) and anti-Thy1.2 (Rat IgG_{2b} clone 30-H12) were purchased (Becton–Dickinson, Mountain View, CA). Anti-CD3 (hamster IgG clone 145-2C11) was provided by Dr. J. A. Bluestone (University of Chicago, Chicago, IL). Anti-IL4 ascites (clone 11B11) was a kind gift of Dr. K. Hayakawa (Fox Chase Cancer Center, Philadelphia, PA). Biotinylated anti-mouse CD44 (rat IgG_{2b} clone IM7) was purchased from Pharmingen. Anti-TCRβ hybridoma (hamster IgG clone H57-597) was obtained from the American Type Culture Collection (Rockville, MD). Anti-K^bD^b ascites (mouse IgG_{2a} clone 28.8.6) and anti-I-A^b ascites (clone 25.9.17) were kindly provided by A. Kruisbeek (NIH). Recombinant human IL-2 was kindly provided by Chiron Corporation (Emeryville, CA).

Bulk lymphocyte tumor culture. Cultures were established in 24-well flat-bottom tissue culture plates with 5×10^6 LN cells, 5×10^6 irradiated spleen cells (20 Gy), and 1×10^5 irradiated tumor cells (80 Gy) in the presence of 5 U/ml of rIL2 in 2 ml complete medium (CM) per well. CM was composed of RPMI 1640 with 10% heat-inactivated fetal bovine serum, 2 m*M* L-glutamine, 100 U/ml penicillin, 50 μg/ml gentamicin, and 5×10^{-5} *M* 2-mercaptoethanol. Plates were incubated at 37.5°C in a moist atmosphere with 5% CO_2. Viable cells were isolated on Day 4 or 5 by Ficoll–Hypaque density gradient centrifugation.

Cytotoxicity assay. Cytolytic activity was measured in a ^{51}Cr-release assay after 4 hr at 37°C. Effector cells were washed and resuspended in CM. Target cells were labeled with ^{51}Cr (100 μCi/10^6 cells) and added as triplicates of 5×10^3 cells/well in plastic round-bottom plates in a final volume of 200 μl. Plates were centrifuged at

1000 rpm for 5 min before incubation. Maximal release was determined by detergent lysis. Data were expressed as mean specific cytotoxicity using a standard formula.

Cold-target inhibition. CTL specificity was evaluated by adding unlabeled competing targets to the ^{51}Cr-release assay. Varying ratios of cold targets were mixed with ^{51}Cr-labeled targets (5×10^3 cells/well) in 100 μl CM. Effector lymphocytes (5×10^4 cells/well) were then added and the plates incubated for 4 hr at 37°C. The percentage inhibition of cytotoxicity was calculated according to the formula (1 − (expt/control)) × 100.

Limiting dilution culture of CTL. After immunoselection, lymphocytes were serially diluted and added as responder cells in 24-microwell replicates together with irradiated RBL5 (80 Gy; 5×10^3 cells/well), irradiated spleen (20 Gy; 5×10^5 cells/well), and rIL-2 (5 U/ml). After 6 days, 100 μl of cell suspension was removed from each well and individually assayed against 2×10^3 ^{51}Cr-labeled RBL5 targets. Individual wells were scored positive for lytic activity if the total cpm released was >3 SD above the mean cpm released by control wells. The fraction of nonresponding wells was plotted (y axis, log) vs the number of input lymphocytes (x axis, linear). A straight line was fit using least-squares analysis, and the precursor frequency was defined as $1/x$, where x was the calculated lymphocyte number corresponding to a 37% nonresponse rate (15).

ELISA. Microplates were coated with serial dilutions of band centrifugation-purified Friend virus from tissue culture (kindly provided by S. Ruscetti, NIH) in pH 9.6 bicarbonate buffer. Sera from RBL5-immunized and nonimmunized mice were diluted with PBS + 0.05% Tween 20. Coated plates were incubated with sera in duplicate, washed, reacted with horseradish peroxidase-conjugated goat anti-mouse IgG, and developed with ABTS substrate, followed by reading at 490 nm on a plate spectrophotometer (performed by A. Lero, Laboratory Animal Facility, Fox Chase Cancer Center).

FACS. Immunoselections were performed with a FacStar (Becton–Dickinson, Mountain View, CA) in the Flow Cytometry Facility of Fox Chase Cancer Center according to standard procedures. Depletions were performed either at the effector stage (i.e., postactivation) or at the precursor stage as indicated. After staining, cells were washed twice, kept on ice, and analyzed with gating for viable cells according to forward and side scatter histograms and propidium iodide uptake. Sorted populations were washed three times in CM to eliminate sodium azide prior to assay or culture.

Dual-chamber culture system. Dual-chamber culture was carried out in 24-well Transwell plates with a 6.5-mm porous cell culture insert and 8.0-μm pore polycarbonate membrane. In order to prevent cell migration, the upper chamber membrane was coated with agarose 1% prior to addition of cells. BLTC was established in the lower chamber as described above in a total volume of 1.5 ml, with the exception that no exogenous IL-2 was added. The membrane of the upper chamber was completely immersed in the medium of the lower chamber. Clone C8 (anti-gp70, CD4$^+$, Th1) was used as a source of activation-associated lymphokines, including IL-2 and IFN-γ, and has been previously described (6). C8 cells were maintained with cyclic Ag stimulation followed by expansion in CM with rIL-2 (50 U/ml) and used in resting phase, at a minimum of 1 week following Ag stimulation. After washing twice to eliminate exogeneous rIL-2, they were plated in the upper chamber at 5×10^4 cells with 5×10^5 irradiated spleen cells and 1×10^3 irradiated RBL5 or EL4 in a total volume of 300 μl.

RESULTS

Anti-RBL5 CTL are generated following BLTC with irradiated RBL5 and rIL-2. Fresh LN lymphocytes had no cytolytic or proliferative response against RBL5 (data not shown). However, lymphocytes cultured 4 days in the presence of irradiated RBL5 and rIL-2 (5 U/ml) developed lytic activity which was uniformly and significantly greater than that achieved following culture with rIL-2 alone (Fig. 1). Cytolytic activity against the NK-sensitive allogeneic target YAC-1 was also induced by rIL-2, but with only minimal effects from addition of irradiated RBL5. The effect of irradiated RBL5 could not be duplicated by cell-free RBL5 culture supernatant, RBL5 soluble total glycoprotein mixture, or purified Friend virus (data not shown). Fixation of RBL5 with 1% paraformaldehyde abolished their ability to stimulate effector cells (Fig. 2). However, the presence of paraformaldehyde-fixed RBL5 did not prevent activation when nonfixed RBL5 were also added (data not shown).

Initial results suggested that BLTC induced a combination of Ag-specific CTL and nonspecific LAK activity. In order to elucidate if a single effector population could be responsible for both components of the response, reciprocal cold-target inhibition assays were performed. Importantly, the NK-sensitive YAC-1 could not efficiently block the killing of RBL5 and, likewise, RBL5 could not block the lysis of YAC-1 (Fig. 3). Thus, the RBL5-specific effectors induced during BLTC were distinct from IL-2 induced LAK or activated NK cells with non-MHC-restricted cytolytic activity.

Effector cells activated during BLTC are CD8$^+$, TCR α/β^+, with TCR-dependent and MHC class I-restricted cytolytic activity. To further identify the nature of RBL5-

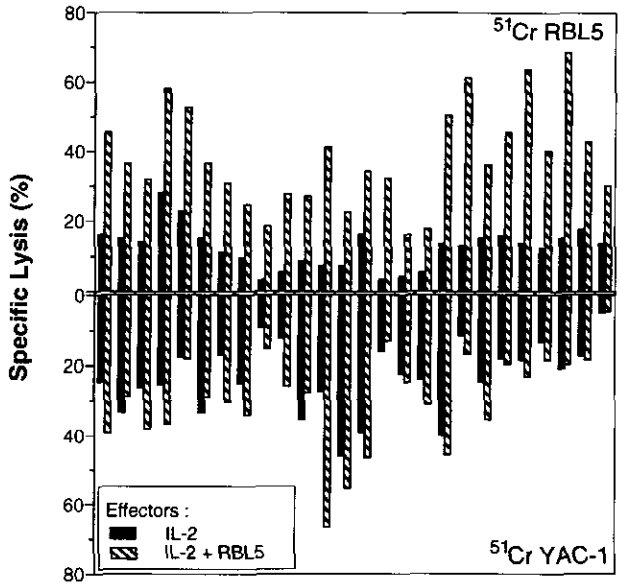

FIG. 1. Lymphocytes from nonimmunized mice can be consistently activated *in vitro* to specifically lyse RBL5. Results shown from 26 independent 4-hr ^{51}Cr-release experiments at E:T 20:1 performed over 4 months using effectors activated with rIL-2 (5 U/ml) or rIL-2 and irradiated RBL5. Targets for each experiment included RBL5 (upper bars) and NK-sensitive YAC-1 (lower bars). Addition of RBL5 increased mean lysis of RBL5 from 12.4 ± 5.9 to 38.1 ± 14.4% ($P < 0.001$) and marginally increased lysis of YAC1 from 23.2 ± 10.0 to 29.3 ± 13.8% ($P = 0.002$).

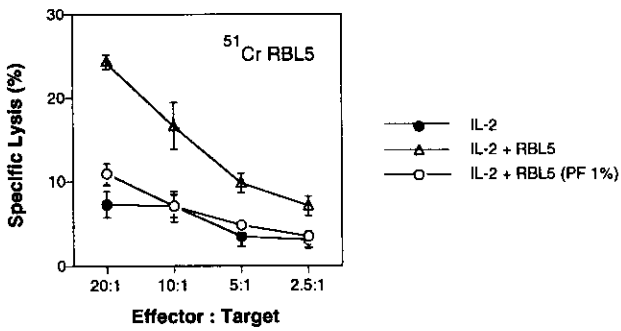

FIG. 2. Fixation of irradiated RBL5 stimulator cells with 1% paraformaldehyde abolishes their ability to induce RBL5-specific effector cells. Mean ± SD specific lysis shown from a representative 4-hr ^{51}Cr-release assay.

specific CTL, negative selection by FACS was performed after BLTC. Depletion of $CD8^+$ lymphocytes greatly reduced the lysis of RBL5 but not YAC-1 (Fig. 4). All $CD8^+$ lymphocytes generated in culture were found by flow cytometry to be $Thy1.2^+$ and $CD3^+$, demonstrating that $CD8^+$ CTL were responsible for the majority of anti-RBL5 activity. TCR utilization was evaluated by addition of anti-CD3 (145-2C11) and anti-TCR α/β (H57-597) mAb to cytotoxicity assays at saturating concentrations. Anti-CD3 strongly inhibited lysis of RBL5 without effect against YAC-1. Addition of anti-TCR α/β inhibited lysis of the $CD3^-$ subline RBL5-2B7 to a similar extent as anti-CD3, again without significant effects against YAC-1 (Fig. 5A). In contrast, addition of anti-CD8 or anti-K^bD^b mAb achieved only partial inhibition of RBL5 lysis, suggesting that the TCR interaction with RBL5 was not uniformly of high affinity (Fig. 5B). Overall, these results demonstrated that the $CD8^+$ CTL generated in primary BLTC lysed RBL5 through an MHC class I-restricted, TCR-dependent pathway. However, there was also a variable degree of non-MHC-restricted LAK activity generated in each experiment, which presumably accounted for residual lysis of RBL5-2B7 in the presence of blocking mAbs.

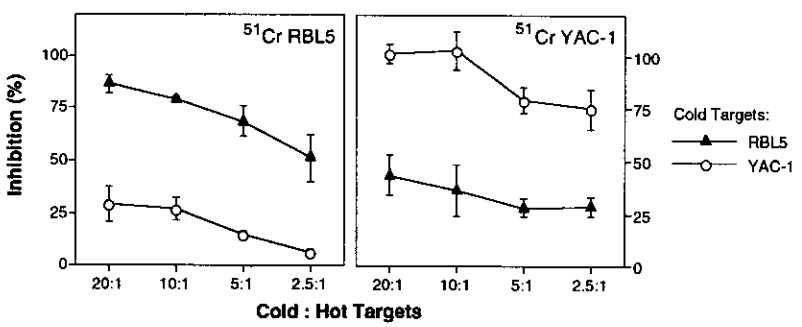

FIG. 3. Reciprocal cold-target inhibition distinguishes anti-RBL5 from anti-YAC-1 cytolytic activity. Effector cells were activated by BLTC with irradiated RBL5 and rIL-2 for 96 hr followed by assay at E:T 10:1 with addition of competing unlabeled target cells as indicated. Results expressed as mean inhibition of specific lysis.

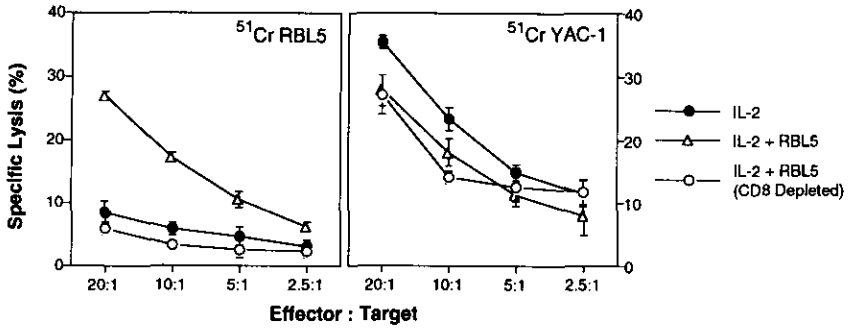

FIG. 4. Depletion of $CD8^+$ lymphocytes distinguishes anti-RBL5 from anti-YAC-1 cytolytic activity. Following BLTC with RBL5, activated lymphocytes were stained with anti-CD8 (83.12.9), depleted by FACS, and evaluated as effector cells in a 4-hr ^{51}Cr-release assay.

Nonimmune $CD4^+$ Th cells are not required for generation of anti-RBL5 CTL in the presence of exogenous IL-2. In view of the dependence on IL-2, we investigated whether $CD4^+$ Th cells were required for development of the anti-RBL5 response during BLTC. Addition of anti-CD4 mAb (GK1.5) at the start of BLTC inhibited the activation of $CD4^+$ Th cells, which were undetectable by FACS in treated cultures by Day 4 (data not shown). However, Th cell inhibition had no effect on generation of RBL5-specific CTL, provided that exogenous rIL-2 (5 U/ml) was present. Similarly, blockade of $CD4^+$ MHC class II-restricted responses with anti-I-A^b mAb (25.9.17) also failed to inhibit generation of an anti-RBL5 response in the presence of rIL-2

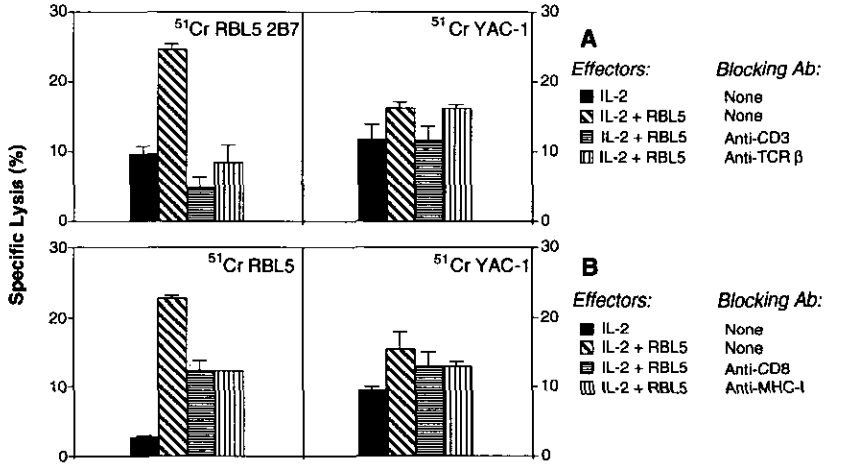

FIG. 5. Inhibition of cytolytic activity with anti-CD3, anti-TCR, anti-CD8, and anti-K^bD^b mAbs. Lymphocytes were activated by BLTC and evaluated as effectors at E:T of 10:1 in a 4-hr ^{51}Cr-release assay with and without addition of mAb. Each blocking mAb was added at a final concentration of 1 μg/ml, which achieved maximal inhibition of cytotoxicity without nonspecific toxicity. Data from effectors activated using rIL-2 without RBL5 (solid bars) are included to illustrate non-MHC-restricted cytotoxicity. (A) Nearly complete inhibition of specific anti-RBL5 cytotoxicity with anti-CD3 (145-2C11) and anti-TCR (H57-597) mAbs. (B) Partial inhibition of specific anti-RBL5 cytotoxicity with anti-CD8 (83.12.9) and anti-K^bD^b (28.8.6) mAbs.

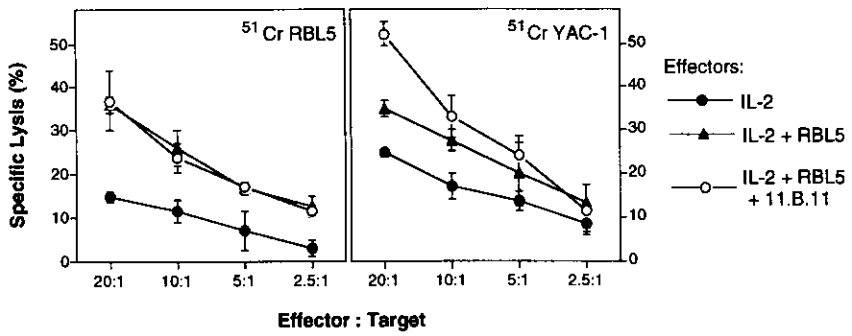

FIG. 6. Blockade of IL-4 during BLTC. Anti-IL-4 blocking mAb (11.B.11) was included during BLTC with RBL5 and rIL-2. After 96 hr, effector activity was evaluated in a 4-hr ^{51}Cr-release assay.

(data not shown). Furthermore, the anti-RBL5 response was not dependent on IL-4, as addition of anti-IL-4 mAb (11.B.11) during BLTC failed to block generation of anti-RBL5 activity, while enhancing non-MHC-restricted activity against YAC-1 (Fig. 6).

Soluble factors released after Ag-specific activation of a CD4$^+$ Th1 clone can substitute for exogenous IL-2 in the generation of anti-RBL5 CTL. A dual-chamber culture system was employed to determine if endogenous IL-2 production by Th1 cells would be sufficient to mimic the effect of exogenous IL-2 during BLTC. When lymphocytes from nonimmunized animals were plated with RBL5 in the lower chamber and anti-gp70 CD4$^+$ cells (clone C8) were plated alone in the upper chamber, no CTL response could be demonstrated from the lower chamber. However, if irradiated RBL5 (gp70$^+$) were also added to the upper chamber, significant anti-RBL5 activity developed in the lower chamber (Fig. 7). Irradiated EL4 (gp70$^-$) in the upper chamber could not replace RBL5. Furthermore, the effect was also dependent on the presence of RBL5 cells in the lower chamber.

CTL precursors recruited during BLTC from unprimed animals have a memory phenotype. Allospecific CTL generated from unprimed animals during primary *in*

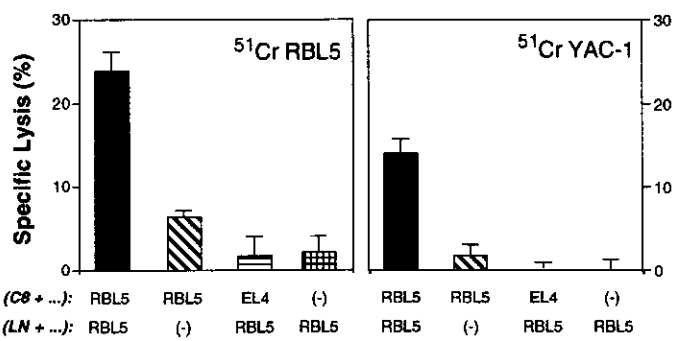

FIG. 7. BLTC with a dual-chamber culture system. An anti-gp70 CD4$^+$ Th1 clonal cell line (C8) was plated with irradiated spleen cells in the upper chamber (UC), with or without addition of irradiated RBL5 (gp70$^+$) or EL4 (gp70$^-$) as a source of activating Ag. Responder LN lymphocytes were plated without exogenous IL-2 in the lower chamber (LC) with or without irradiated RBL5 for 96 hr, followed by analysis of cytolytic effector activity in a 4-hr ^{51}Cr-release assay.

vitro mixed lymphocyte cultures arise largely from naive CTL precursors. Anti-RBL5 CTL generated during BLTC may be derived similarly, or possibly from recruitment of cross-reactive CTL precursors presensitized *in vivo* by environmental Ag. In order to distinguish between a naive and memory origin for anti-RBL5 precursor CTL, we evaluated the anti-RBL5 response from LN populations depleted of memory T cells prior to establishing BLTC. Murine memory T lymphocytes can be identified phenotypically by high-level surface expression of Pgp-1 (CD44) following antigen-mediated activation *in vitro* (16) and *in vivo* (17). Depletion of the Pgp-1^{hi} subset before BLTC was associated with a dramatic reduction of the anti-RBL5 response (Fig. 8). Activity against YAC-1 was not modified by depletion, this fact argues against spurious effects of the depletion procedure or reagents. The presence of $CD8^+$/Pgp-1^{lo} cells postdepletion was confirmed by flow cytometry, although their antigen-specific cytolytic capability was not assessed. These results were extended by limiting dilution analysis to estimate the precursor CTL frequency in both the naive and memory $CD8^+$ lymphocyte populations from unprimed animals. Precursor frequency was 50-fold higher among $CD8^+$ cells that were Pgp-1^{hi} compared to $CD8^+$ cells that were Pgp-1^{lo} (1/1503 vs 1/51229 with $P < 0.0001$).

The finding that precursor anti-RBL5 CTL exhibited a memory phenotype was of interest, as the mice had no history of FMR retrovirus infection. In order to further exclude prior exposure to RBL5-associated Ag, RBL5-immunized and nonimmunized mice were tested for seropositivity by ELISA using Friend virus-coated plates. Mice immunized with one subcutaneous injection of irradiated RBL5 without adjuvant promptly demonstrated antibodies against Friend retroviral Ag by ELISA (0.62 AU at 1:30 serum dilution), while there was an absence of antibodies in nonimmunized animals (0.02 AU at 1:30 serum dilution).

DISCUSSION

Development of successful strategies for adoptive immunotherapy of cancer using Ag-specific lymphocytes holds many challenges (reviewed in 18). Murine models with syngeneic tumors permit a careful analysis to be made of the specificity and regulation of the anti-tumor immune response, including the role of distinct lymphocyte subpopulations. Our previous experiments demonstrated that adoptive transfer of a noncytolytic $CD4^+$ Th1 clonal line was sufficient to eliminate established tumors without a requirement for transfer of $CD8^+$ CTL (6). We hypothesized that preexisting CTL

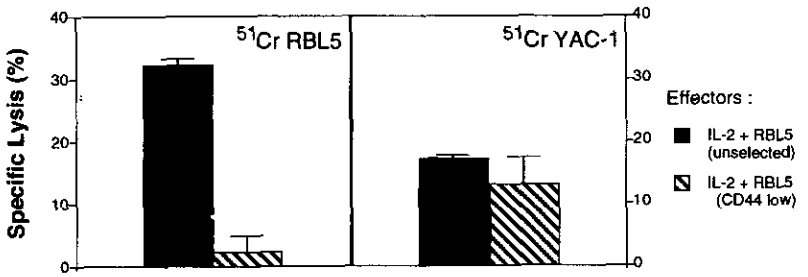

FIG. 8. Depletion of CD44/PgP-1^{hi} cells prior to BLTC. Lymphocytes were stained and sorted according to expression of CD44 (PgP-1), followed by BLTC with irradiated RBL5 for 96 hr, and evaluation in a 4-hr ^{51}Cr-release assay.

precursors in tumor-bearing mice could cooperate with transferred $CD4^+$ Th cells to mediate tumor rejection. We began evaluation of this hypothesis through examination of lymphocytes from nonimmunized (i.e., naive) mice to determine the extent and specificity of anti-tumor CTL activity that could be elicited.

Incubation of lymphocytes from naive mice with irradiated RBL5 or rIL-2 alone was not sufficient to generate RBL5-specific CTL. However, incubation with the combination of irradiated RBL5 and a low concentration of rIL-2 for 96 hr consistently generated anti-RBL5 CTL with activity in a 4-hr assay. Reciprocal cold-target inhibition experiments with YAC-1 demonstrated that the anti-RBL5 CTL were distinct from MHC nonrestricted LAK or NK cells. CTL were further characterized as $CD8^+$, class I MHC restricted, and $TCR(\alpha/\beta)^+$; a profile resembling conventional CTL obtained following tumor immunization.

We identified no evidence of prior exposure to RBL5 Ag based on the young age of the mice, reproducibility of CTL induction from different litters, absence of illness, and absence of anti-viral antibodies. Naive $CD4^+$ lymphocytes also showed no anti-RBL5 response, which can be readily demonstrated against viral gp70 after tumor or viral immunization (4). Finally, older mice (>1 year) retained the ability to respond *in vitro;* this fact argues against a transient maturational phenomenon (data not shown). Thus, we felt that occult exposure to infectious virus or viral Ag would not explain the primary anti-RBL5 CTL response.

We considered three alternative hypotheses to explain the appearance of primary RBL5-specific CTL following BTLC using lymphocytes from nonimmunized mice. Historically, only MHC-different (i.e., allogeneic) stimulators (19, 20) or hapten-modified syngeneic cells (21) have generated measurable primary responses *in vitro.*

The recent description of viral or bacterial superantigens suggested that RBL5 may express a retroviral gene product capable of activating a broad range of Ag-specific CTL. Published experience with superantigens is more extensive with regard to $CD4^+$ class II MHC-restricted Th, rather than $CD8^+$ class I MHC-restricted CTL. Nonetheless, we hypothesized that if a superantigen was contributing to the anti-RBL5 response, it might be possible to detect preferential utilization of specific TCR variable gene segments. Screening by quantitative flow cytometry, FACS depletion, and blocking experiments was performed with a number of anti-$V\beta$ mAbs (provided by P. Nakajima, Hybridoma Facility, Fox Chase Cancer Center). In no instance was there evidence for either preferential utilization or exclusion of specific $V\beta$ subunits (data not shown). These data are not sufficient to exclude a superantigen response, and direct analysis of TCR gene utilization in BLTC-generated CTL would be of interest.

Alternatively, minor histocompatibility Ag can be encoded by retroviruses or have their expression controlled by retroviral sequences (22–24). The primary effectors directed against these Ag are class I MHC-restricted $CD8^+$ CTL (25). Therefore, the primary CTL response observed in our model could be due to the presence of new minor histocompatibility Ag on retrovirus-induced tumors. Although measurable responses against minor histocompatibility Ag *in vitro* usually require priming *in vivo,* definite primary responses have been observed after culture with low doses of rIL-2 and tumor, similar to those of BLTC (26), suggesting that this could be a plausible mechanism.

Aberrant H-2 class I-like Ag have also been described on several murine tumors (reviewed in 27). To further evaluate the possibility of a response against non-self-MHC Ag, the frequency of anti-RBL5 precursor CTL was evaluated in both the mem-

ory (CD44/PgP-1^{hi}) and the naive (CD44/PgP-1^{lo}) subpopulations separated by FACS. The relative absence of precursor CTL in the naive population was taken as evidence against an allogeneic response, which should have been capable of activating a broad spectrum of lymphocytes from either population (28–30). The expected distribution of precursor CTL for minor histocompatability Ag or superantigens remains less established.

Identification of precursor CTL within the memory population suggests that Ag are expressed by RBL5 that are cross-reactive with endogenous or environmental Ag to which the mice have been previously exposed. The ability to partially block RBL5 lysis with soluble anti-CD8 and anti-K^bD^b mAb indicates the presence of CTL with relatively low-affinity TCR–Ag–MHC interactions, which might be expected from recruitment of cross-reactive populations (27). Presumably, some unspecified characteristic of the *in vitro* BLTC model (i.e., high cell density, exogenous rIL-2, high density of RBL5 MHC–Ag complexes) could promote these cross-reactive responses.

The finding that lymphocytes from nonimmunized mice can be activated to lyse RBL5 has implications for the design and interpretation of adoptive immunotherapy experiments. For example, a proportion of mice with established RBL5 peritoneal tumors can be cured by adoptive transfer of noncytolytic IL-2-producing CD4$^+$ Th1 clonal lines without a requirement for transfer of CTL (6), suggesting that cooperation may occur with host precursor CTL. However, administration of exogenous rIL-2 alone was not sufficient to eliminate peritoneal RBL5 tumors *in vivo*. Thus, the complex regulatory relationship *in situ* between CD4$^+$ Th cells, CD8$^+$ host CTL, cytokines, and tumor antigen may not be readily reproduced by the unfocused administration of individual cytokines.

Successful murine models of Ag-specific adoptive immunotherapy have not yet translated into successful human clinical trials. Continuing research efforts are indicated to establish the cellular requirements for efficient tumor rejection and evaluate potential clinical applications.

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