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AKR.H-2^b Lymphocytes Inhibit the Secondary In Vitro Cytotoxic T-Lymphocyte Response of Primed Responder Cells to AKR/Gross Murine Leukemia Virus-Induced Tumor Cell Stimulation

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We have previously shown that AKR.H-2^b congenic mice, though carrying the responder *H-2^b* major histocompatibility complex haplotype, are unable to generate secondary cytolytic T-lymphocyte (CTL) responses specific for AKR/Gross murine leukemia virus (MuLV). Our published work has shown that this nonresponsive state is specific and not due to clonal deletion or irreversible functional inactivation of antiviral CTL precursors. In the present study, an alternative mechanism based on the presence of inhibitory AKR.H-2^b cells was examined. Irradiated or mitomycin C-treated AKR.H-2^b spleen cells function as in vitro stimulator cells in the generation of C57BL/6 (B6) anti-AKR/Gross virus CTL, consistent with their expression of viral antigens. In contrast, untreated viable AKR.H-2^b spleen cells functioned very poorly as stimulators in vitro. Viable AKR.H-2^b spleen cells were also able to cause dramatic (up to ≥25-fold) inhibition of antiviral CTL responses stimulated in vitro by standard AKR/Gross MuLV-induced tumor cells. This inhibition was specific: AKR.H-2^b modulator spleen cells did not inhibit allogeneic major histocompatibility complex-specific CTL production, even when a concurrent antiviral CTL response in the same culture well was inhibited by the modulator cells. These results and those of experiments in which either semipermeable membranes were used to separate AKR.H-2^b modulator spleen cells from AKR/Gross MuLV-primed responder cells or the direct transfer of supernatants from wells where inhibition was demonstrated to wells where there was antiviral CTL responsiveness argued against a role for soluble factors as the cause of the inhibition. Rather, the inhibition was dependent on direct contact of AKR.H-2^b cells in a dose-dependent manner with the responder cell population. Inhibition was shown not to be due to the ability of AKR.H-2^b cells to function as unlabeled competitive target cells. Exogenous interleukin-2 added at the onset of the in vitro CTL-generating cultures partially restored the antiviral response that was decreased by AKR.H-2^b spleen cells. Positive and negative cell selection studies and the development of inhibitory cell lines indicated that B lymphocytes and both CD4$^-$ CD8$^+$ and CD4$^+$ CD8$^-$ T lymphocytes from AKR.H-2^b mice could inhibit the generation of AKR/Gross virus-specific CTL in vitro. AKR.H-2^b macrophages were shown not to be required to demonstrate AKR/Gross MuLV-specific inhibition, however, confirming that the inhibition by T-cell (or B-cell)-depleted spleen populations was dependent on the enriched B-cell (T-cell) population per se. The capacity of T-cell- or CD8$^+$ T-cell-enriched AKR.H-2^b cells to modulate inhibition was partially blocked by anti-CD8 monoclonal antibodies.

The major histocompatibility complex (MHC) plays a central role in the outcome of CD8$^+$ cytotoxic T-lymphocyte (CTL) responses to AKR/Gross murine leukemia virus (MuLV). Our studies have shown that mouse strains whose MHC is of the *H-2^b* haplotype, such as C57BL/6 (B6), are high responders, while those strains which are *H-2^k*, as is the AKR strain, are low responders (9). B6 anti-AKR/Gross MuLV CTL are K^b restricted (12) and lyse AKR/Gross MuLV-expressing tumor cells as well as fibroblast target cells infected by most endogenously derived mouse ecotropic retroviruses, including the AKR623 molecular clone (5, 11, 33). Recent studies have shown one virally encoded octameric peptide—peptide 12, KSPWFTTL, located in the p15E region of the envelope protein—to be a dominant epitope for recognition by anti-AKR/Gross MuLV CTL from B6 and other *H-2^b* responder strains (4, 30, 34).

To take advantage of the high-responder phenotype associated with the *H-2^b* haplotype, we have recently used AKR.H-2^b congenic mice in our investigation of immune responses to AKR/Gross virus. The AKR.H-2^b congenic mouse strain retains the full complement of endogenous N-ecotropic proviruses that is found in the parental AKR strain. Also similar to AKR mice, the AKR.H-2^b congenic mice, after immunization and secondary in vitro stimulation with AKR/Gross MuLV-induced syngeneic tumor cells, fails to generate an antiviral CTL response to AKR/Gross MuLV, as measured by in vitro cytolytic assays (31). The lack of a protective immune response to AKR/Gross MuLV is also demonstrated by the inability of AKR.H-2^b mice to survive AKR/Gross MuLV-induced syngeneic tumor challenge (2, 31). AKR.H-2^b mice, however, are able to respond with CTL generation to tumors induced by the essentially non-cross-reactive Friend-Moloney-Rauscher MuLV and are resistant to challenge with Friend-Moloney-Rauscher viral antigen (Ag)-positive tumor cells (31). Thus, any proposed mechanism for CTL nonresponsiveness to AKR/Gross viral Ags in AKR.H-2^b mice must account for the fine specificity of nonresponsiveness observed.

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Given that expression of N-ecotropic proviruses can be detected neonatally if not in utero, in the AKR mice, clonal deletion was a particularly attractive mechanism of preventing responsiveness to AKR/Gross MuLV in the AKR.H-2^b mouse strain. Clonal deletion would manifest itself by an observed decrease in the frequency of precursor CTL (pCTL) specific for AKR/Gross MuLV Ags. We performed limiting-dilution analysis to determine the frequency of AKR/Gross MuLV-specific pCTL in the nonresponder AKR.H-2^b strain and, for comparison, in the responder B6 strain. Surprisingly, for spleen cells from nonimmunized or immunized mice that were further stimulated in vitro with AKR/Gross MuLV-expressing tumor cells, the antiviral pCTL frequencies of nonresponder AKR.H-2^b mice were found to be fairly similar to those of prototypic responder B6 mice (32). Thus, there was no evidence for clonal deletion or irreversible functional inactivation of pCTL as the cause of the specific CTL nonresponsiveness in AKR.H-2^b mice.

In the present study, our focus is turned to the identification of specific inhibitory cells in AKR.H-2^b mice as an alternative basis for the specific antiviral CTL nonresponsiveness. In the related mouse strain AKR.H-2^b:Fv-1^b, which converts from a responder to a nonresponder status for anti-AKR/Gross MuLV CTL with moderate aging (10), inhibitory CD8$^+$ T cells were defined by in vivo adoptive transfer experiments (26). Here, the phenotype of AKR.H-2^b spleen cells which inhibit the generation of AKR/Gross MuLV-specific CTL from primed B6 responder mice and the underlying mechanisms by which these cells modulate inhibition are investigated at the secondary in vitro stimulation stage.

MATERIALS AND METHODS

Mice. B6 mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. AKR.H-2^b (lifelong AKR/Gross virus CTL nonresponders, viremic throughout life) and AKR.H-2^b:Fv-1^b (AKR/Gross virus CTL responders at a young age [5.5 to 7.5 weeks] but progressively nonresponsive with aging, coincident with the development of endogenous MuLV expression) congenic mice were maintained through breeding of brother-sister pairs in the Animal Health Resource facility of Dartmouth Medical School. Breeding pairs were kindly provided by D. Myers (Sloan Kettering Memorial Institute, New York, N.Y.).

Cell lines. The E♂G2 (Gross virus induced and Gross cell surface antigen [GCSA] positive) and E♀K1 (AKR virus induced but GCSA$^-$) tumors are of B6 strain origin and are *H-2^b*. AKR.H-2^b SL1, a spontaneous GCSA$^+$ tumor, was originally derived from the AKR.H-2^b congenic mouse strain. B.GV, a Gross virus-induced GCSA$^+$ tumor, was derived from a BALB.B (*H-2^b*) mouse. These tumor lines have previously been described in detail (9). BALB.B-1K, a tumor line also of BALB.B origin but insusceptible to anti-AKR/Gross MuLV CTL, was the kind gift of Herbert Morse III. P815, a methylcholanthrene-induced tumor line derived from the DBA/2 (*H-2^d*) strain, was provided by the American Type Culture Collection (ATCC). LB 27.4, also provided by the ATCC, is of the *H-2^{d,b}* haplotype. These cell lines were maintained by thrice weekly in vitro passage in RPMI 1640 supplemented with 5% fetal bovine serum, 5×10^{-5} M 2-mercaptoethanol, L-glutamine, and antibiotics (tissue culture medium). CTLL-2 (TIB 214), provided by the ATCC, is interleukin-2 (IL-2) dependent and was maintained in tissue culture medium supplemented with 16 U of recombinant human IL-2 (Cetus Corporation, Emeryville, Calif.) per ml. CTLL.6-1D3, kindly provided by Robert Chervenak and Stephen Jennings at the Louisiana State University School of Medicine, Shreveport, was derived from the CTLL.6 cell line and is highly responsive to IL-4. Recombinant murine IL-4 was kindly provided by Randy Noelle (Dartmouth Medical School) as a means to establish a standard curve for IL-4 quantitation. The AKR/Gross MuLV-specific CTL clone G8 has been previously described (1). Two AKR.H-2^b lines, AKR.H-2^b.BB1 and AKR.H-2^b.KB2, each developed through stimulation of naive AKR.H-2^b splenocytes with concanavalin A (ConA) and IL-2, were shown through flow cytometric analysis to be CD4$^-$ CD8$^+$ T lymphocytes.

Panning, antibody and complement treatments, antibody blocking, macrophage depletions, and flow cytometric analysis. To obtain T-lymphocyte-enriched preparations, splenocytes were negatively selected through two rounds of panning on petri dishes previously coated with goat anti-mouse immunoglobulin (Ig) (Southern Biotechnology Inc., Birmingham, Ala.) for 70 min per round at 4°C. For enrichment of either CD4$^+$ CD8$^-$ or CD4$^-$ CD8$^+$ T lymphocytes, nonadherent, anti-mouse Ig-panned cells were further negatively selected through two rounds of panning on petri dishes coated with 5 μg of an anti-CD8a or anti-L3T4 monoclonal antibody (MAb), respectively, per ml. Alternatively, following an identical depletion of B cells through panning as outlined above, nonadherent cells were incubated with rat IgM 3.155 (anti-Lyt 2 [all alleles] derived from supernatant of TIB 211 hybridoma cells) or rat IgM RL172 (anti-CD4) (kindly provided by R. Noelle, Dartmouth Medical School) for 1 h at 4°C; following washing, 10^7 splenocytes per ml in rabbit complement (Cedarlane) diluted in Cedarlane cytotoxicity medium were incubated for 1 h at 37°C to obtain populations of lymphocytes which were enriched for either CD4$^+$ CD8$^-$ or CD4$^-$ CD8$^+$ T lymphocytes. For positive selection of CD4$^+$ CD8$^-$ lymphocytes, AKR.H-2^b splenocytes were first incubated at a concentration of 1 μg/10^6 cells with a fluorescein isothiocyanate (FITC)-labeled anti-L3T4 MAb (Pharmingen). Then, positively stained cells were selected by fluorescence-activated cell sorting (FACS), using a FACStar cell sorter (Becton Dickinson). As a means to confirm that positive selection had been accomplished, an aliquot of sorted cells was analyzed through flow cytometric analysis. To obtain B-lymphocyte-enriched preparations, splenocytes were negatively selected through two rounds of panning on plates coated with 5 μg each of anti-CD3, anti-L3T4, and anti-CD8a MAbs (Pharmingen, San Diego, Calif.) per ml. Alternatively, 3×10^7 splenocytes were incubated in a cocktail of MAbs 3.155 and RL172 followed by incubation with complement as described above. Alternatively, direct immunofluorescence staining of B lymphocytes was performed by incubation of cells with a 1:20 dilution of FITC-conjugated F(ab′)$_2$ rabbit anti-mouse IgM (μ-chain specific) antiserum (Zymed Laboratories) followed by positive selection through cell sorting as described above. As a means to confirm that positive selection for AKR.H-2^b B lymphocytes had been accomplished, an aliquot of sorted cells was analyzed through flow cytometric analysis. Following negative selection for either T or B lymphocytes, the efficiency of B- or T-cell depletion, respectively, was assessed by measuring [^{3}H]thymidine incorporation following stimulation with lipopolysaccharide (LPS) and ConA. Additionally, as a means to confirm that cell depletions had been accomplished, indirect FACS analysis was used to stain for CD4$^-$ CD8$^+$ or CD4$^+$ CD8$^-$ T lymphocytes through incubation with 10 μg of a rat IgG2a anti-CD8a or anti-L3T4 MAb (Pharmingen), respectively, per ml, followed by incubation with a 1:20 dilution of an FITC-conjugated F(ab′)$_2$ mouse anti-rat IgG, Fc-specific antiserum (Jackson ImmunoResearch Inc., West Grove, Pa.). To detect B lymphocytes, direct immunofluorescence staining was performed with a 1:20 dilution of an FITC-conjugated F(ab′)$_2$ rabbit anti-mouse IgM (μ-chain specific) antiserum (Zymed Laboratories).

For antibody blocking experiments, AKR.H-2^b lymphocytes were first T-cell enriched through panning (as described above) prior to further fractionation of T-lymphocyte subsets (where noted) via incubation for 1 h with either MAb RL172 or MAb 3.155 (described above) or, alternatively, RPMI 1640 supplemented with 5% fetal bovine serum, L-glutamine, and antibiotics (spleen cell culture medium) plus 50 μg of purified rat IgG, anti-L3T4, anti-CD8a, or anti-CD2 MAb (each obtained from Pharmingen) per ml. Then, antibody-coated cells were centrifuged, resuspended to remove excess MAb, and tested for inhibitory function in mixed lymphocyte tumor cell cultures (MLTC) (see below).

Indirect immunofluorescence (FACScan) was used to detect the expression of adhesion and costimulatory molecules, and other cell surface Ags of interest, on splenocytes. Cells were incubated with primary antibodies directed to murine CD3ε, CD4 (L3T4), CD8a (Ly2), α/β T-cell receptor (TcR), γ/δ TcR, CD11a (LFA-1 α chain), CD2 (LFA-2), CD25 (IL-2 receptor, α chain), IL-2 receptor (PC61 5.3), CD44 (pgp-1), CD45 (leukocyte common antigen), B7.1, B7.2, H-2D^b, H-2K^b, and gp70 viral envelope determinants and then incubated with appropriate FITC-labeled secondary antisera.

Depletion of macrophages was accomplished through two rounds of adherence to Sephadex G-10 beads (Pharmacia) at 37°C. FACS analysis was performed with the rat IgG2b MAbs F4/80 (macrophage specific [ATCC HB 198]) and M1/70.15.11.5.HL (anti-MAC-1 α unit [ATCC TIB 128]) followed by a 1:20 dilution of an FITC-conjugated anti-rat IgG antiserum as described above to confirm that this procedure was an efficient means of depleting macrophages. On the other hand, to enrich spleen cell populations for macrophages, 2×10^6 to 4×10^6 AKR.H-2^b spleen cells were sequentially depleted of B and T cells by goat anti-mouse Ig panning followed by treatment with an anti-L3T4 and anti-CD8a antibody cocktail plus complement, respectively, prior to being added to MLTC (see below).

Bulk-cultured CTL and inhibition assays. The protocol for raising polyclonal B6 anti-AKR/Gross virus-specific CTL has been published (9). Briefly, AKR/Gross MuLV-primed spleen cells were obtained 11 to 17 days, except where noted, after in vivo immunization with 10^6 nonsyngeneic *H-2^b*-matched, AKR/Gross MuLV-induced tumor cells. With the primed spleen cells as responder cells and irradiated or mitomycin C-treated *H-2*-compatible tumor cells (8,000 rad) or splenocytes (2,000 rad or mitomycin C treated) as stimulator cells, MLTC cultures were set up. A responder-to-tumor stimulator ratio of 50:1 was achieved by mixing 10^7 responder cells with 2×10^5 irradiated tumor cells. Responder-to-stimulator (inactivated) spleen cell ratios of 2.5:1 to 5:1 were achieved by mixing 10^7 responder cells with 2×10^6 to 4×10^6 spleen cells (except in experiments in which E♂G2 cells or splenic stimulator cells were titrated) in a final volume of 1.5 ml of spleen cell culture medium. In experiments designed to assay inhibition, AKR.H-2^b spleen cells were additionally included in MLTC wells at various cell numbers. These cell mixtures were cultured for 6 days at 37°C in 5% CO_2 before remaining viable cells were harvested and tested for cytolytic activity. In some experiments, 0.45-μm-pore-size semipermeable cell

culture inserts (Falcon) were used as a means to separate inhibitory cells from primed responder cells to assess the requirement for cell-cell contact for inhibition of AKR/Gross MuLV CTL responses. In assessing data on the inhibition of AKR/Gross MuLV cytotoxicity modulated by viable AKR.H-2^b cells, results of only those experiments in which viable AKR.H-2^b cells, in the absence of tumor stimulator cells, stimulated less than 40% lysis (at an effector/target [E:T] ratio of 100:1; see below) of chromium-labeled virus-positive target cells were considered to fall in a range in which inhibition (of tumor-stimulated AKR/Gross MuLV specific CTL) could be quantitated and therefore were included. In some experiments, 8 to 16 U of IL-2 per ml was added to MLTCs to test the effect of exogenous IL-2 on the inhibition of AKR/Gross MuLV responsiveness mediated by AKR.H-2^b cells.

CTL assays. ^{51}Cr release assays were conducted as described previously (12) to measure CTL activity from bulk MLTC cultures. Briefly, 10^4 radiolabeled tumor target cells were mixed with various numbers of effector cells (i.e., several E:T ratios), centrifuged, and incubated for 4 h at 37°C. At the end of this period, the cells were centrifuged again, and an aliquot of cell-free supernatant was removed for gamma counting and data reduction. The percent specific lysis against tumor cells was determined according to the formula $[(X - Y)/Z] \times 100$, in which X is counts per minute released by target cells incubated with effector cells, Y is counts per minute released by target cells incubated alone, and Z is counts per minute released by the freeze-thaw of target cells (approximately 80% of total counts per minute incorporated).

RESULTS

Irradiated or mitomycin C-treated and untreated AKR.H-2^b splenocytes show different capacities to stimulate AKR/Gross retrovirus-specific CTL responses. We have previously shown that among splenocytes of AKR.H-2^b mice, a portion of the cells bear viral Ag on their surface and are recognized by anti-AKR/Gross MuLV-specific CTL (8). One assay used to define such viral expression was the use of inactivated normal AKR.H-2^b spleen cells as stimulators for primed B6 responder cells at the in vitro stimulation phase of CTL generation. Consistent with these findings are the data found in Table 1; in experiments 1 and 2, irradiated or mitomycin C-treated AKR.H-2^b splenocytes, respectively, stimulated substantial AKR/Gross virus-specific CTL responses when mixed in MLTC with either AKR.H-2^b SL1 (experiment 1) or B.GV (experiment 2) tumor-primed B6 responder cells. Lysis of $GCSA^+$ E♂G2 tumor cells exceeded 50% at an E:T ratio of 100:1 in each experiment. In experiment 2, AKR.H-2^b SL1 target cells, also $GCSA^+$, were lysed to essentially the same level as were E♂G2 tumor cell targets, whereas the $GCSA^-$ E♀K1 control target was not lysed in either experiment. In sharp contrast, the same number of untreated viable AKR.H-2^b splenocytes was capable of stimulating only dramatically lower levels of AKR/Gross MuLV-specific CTL activity to E♂G2 (and AKR.H-2^b SL1) target cells. In experiment 1, for example, there was roughly a 25-fold decrease in stimulation of AKR/Gross virus-specific CTL when the AKR.H-2^b splenocytes were viable (30% lysis at an E:T ratio of 100:1) compared with when they were irradiated (32% at an E:T ratio of 4:1). These results were reproducible: the different capacities of viable and inactivated (11 with X irradiation, 7 with mitomycin C) splenocytes derived from AKR.H-2^b mice to stimulate AKR/Gross MuLV responses in vitro were observed in all of 18 experiments. In contrast, when spleen cells from the highly related and moderately aged AKR.H-2^b:Fv-1^b mice (see Materials and Methods) were used as stimulator cells, no difference in stimulatory capacity between treated and untreated spleen cells was noted in six of seven experiments. For example, the capacities of spleen cells from 11- or 24-week-old AKR.H-2^b:Fv-1^b mice (both anti-AKR/Gross CTL nonresponders and viral Ag positive) (10) to stimulate AKR/Gross virus-specific CTL responses, albeit to only low levels, were essentially the same whether the cells used were viable or inactivated (Table 1). Because these two congenic strains differ only at *Fv-1*, with the *Fv-1^n* allele of the AKR.H-2^b strain being permissive for endogenous ecotropic proviral expression, compared with the relative restriction on viral replication imposed by *Fv-1^b*, this finding with AKR.H-2^b:Fv-1^b stimulators controlled for a variety of nonspecific effects. Additionally, as expected, only background levels of stimulation were noted when splenocytes from 8- to 9-week-old B6 mice (experiment 2) were used as a source of stimulator cells. In Table 1, experiment 3, to determine whether viable AKR.H-2^b splenic stimulators have a gross defect in their stimulatory capacity, unprimed BALB/c responder cells were mixed in MLTC with viable and with irradiated AKR.H-2^b stimulator cells. The magnitude of the resulting anti-allogeneic *H-2^b*-specific CTL response was essentially the same whether the AKR.H-2^b splenic stimulator cells were viable or inactivated. When this experiment was repeated, the results were the same. These findings confirmed that there was not a generalized problem with the ability of viable AKR.H-2^b splenocytes to serve as stimulator cells.

Secondary in vitro tumor restimulation of anti-AKR/Gross virus-specific CTL is inhibited in vitro by AKR.H-2^b splenocytes. The substantially reduced ability of viable AKR.H-2^b spleen cells to stimulate primed B6 responder cells in vitro relative to their inactivated counterparts suggested either a passive inability to functionally present viral Ag or an active inhibitory role. To differentiate between these two possibilities, an in vitro cell mixing experiment was designed. AKR.H-2^b spleen cells not only were poorly able to stimulate an antiviral CTL response when viable (relative to irradiated) but also inhibited, when mixed in MLTC with B6 responder cells, the antiviral CTL response to E♂G2 tumor stimulator cells (Table 2). AKR/Gross MuLV-specific lysis of E♂G2 tumor targets at an E:T ratio of 20:1 decreased from 94% when E♂G2 was used alone as a tumor stimulator to only 31% when viable AKR.H-2^b splenocytes were also included. In contrast, addition of viable spleen cells from the closely related AKR.H-2^b:Fv-1^b strain caused much less inhibition of AKR/Gross MuLV responsiveness. Overall, viable AKR.H-2^b splenocytes clearly demonstrated an active inhibitory role in downregulating the AKR/Gross MuLV responsiveness of primed spleen cells to restimulation by E♂G2 tumor cells in vitro in 22 of 23 replicate experiments. To further rule out any possible artifactual effects of simply adding viable, third-party allogeneic spleen cells at the MLTC level, equal numbers of BALB/c and AKR.H-2^b spleen cells were added to parallel MLTC wells containing primed B6 responder cells and E♂G2 stimulator cells. Whereas there was substantial reduction in AKR/Gross MuLV cytotoxicity when AKR.H-2^b cells were used, no such effect was noted where BALB/c spleen cells were used (data not shown). These data thus argue against the possibility that viable AKR.H-2^b splenocytes are merely inefficient in stimulating AKR/Gross MuLV-specific CTL as a result of a passive inability to present viral Ag.

Inhibition by AKR.H-2^b spleen cells is specific for AKR/Gross MuLV-specific CTL responsiveness. To determine whether the effect of viable AKR.H-2^b splenocytes on AKR/Gross MuLV-specific CTL responsiveness was specific, allogeneic CTL responses were additionally monitored. As previously shown, addition of viable, but not irradiated, AKR.H-2^b splenocytes plus E♂G2 stimulators substantially inhibited (by about 25-fold, as judged from E:T ratio comparisons) the AKR/Gross virus-specific CTL response to E♂G2 tumor stimulators (Table 3, experiment 1). In contrast, when these same viable AKR.H-2^b splenocytes were mixed with class I MHC-positive P815 (*H-2^d*) tumor stimulator cells, there was a slight increase, if anything, in the lysis of allogeneic P815 target cells. In experiment 2, these results were confirmed and extended. A marked decrease in AKR/Gross MuLV-specific CTL respon-

TABLE 1. Irradiated but not unirradiated AKR.H-2^b splenocytes efficiently stimulate AKR/Gross retrovirus-specific CTL responses

Exptl protocol[a]	E:T ratio	% Specific lysis		
		Viral		Control
		AKR.H-2^b SL1	E♂G2	E♀K1
B6 anti-AKR/Gross virus CTL response				
Expt 1				
No stimulation	100:1		8	0
Tumor stimulator cells (E♂G2)	100:1		88	NA[b]
	20:1		80	
	4:1		52	
Splenocyte stimulator cells				
3–6 wk AKR.H-2^b				
Not irradiated	100:1		30	2
	20:1		15	1
Irradiated	100:1		87	2
	20:1		74	2
	4:1		32	2
24-wk AKR.H-2^b:Fv-1^b				
Not irradiated	100:1		27	4
	20:1		14	3
Irradiated	100:1		26	2
	20:1		12	1
Expt 2				
No stimulation	100:1	2	2	−2
Tumor stimulator cells (E♂G2)	100:1	73	79	−2
	20:1	53	53	−1
	4:1	21	25	−1
Splenocyte stimulator cells				
8–9-wk AKR.H-2^b				
Untreated	100:1	16	14	1
	20:1	7	6	1
Mitomycin C treated	100:1	49	55	−1
	20:1	25	27	−1
	4:1	9	7	−2
8–9-wk B6				
Untreated	100:1	4	4	0
Mitomycin C treated	100:1	0	3	−1
11-wk AKR.H-2^b:Fv-1^b				
Untreated	100:1	13	14	6
Mitomycin C treated	100:1	18	16	−1
Expt 3, BALB/c anti-H-2^b allogeneic CTL response		AKR.H-2^b SL1 (allogeneic)		E♀K1 (allogeneic)
No stimulation	100:1	2		2
Splenocyte stimulator cells (7-wk AKR.H-2^b)				
Not irradiated	100:1	38		46
	20:1	18		30
	4:1	5		11
Irradiated	100:1	43		46
	20:1	20		28
	4:1	7		10

[a] In experiments 1 and 2, B6 mice were primed with 10^6 AKR.H-2^b SL1 (experiment 1) or B.GV (experiment 2) tumor cells. Eleven days later, 10^7 responder cells were mixed with 2×10^5 tumor stimulator cells or 4×10^6 splenocytes pretreated as indicated. The values for spontaneous release by target cells ranged as follows: experiment 1, 6.5 to 18.6%; experiment 2, 7.6 to 14.5%. In experiment 3, responder splenocytes from unprimed BALB/c mice (*H-2^d*) were mixed with untreated or irradiated AKR.H-2^b splenic stimulator cells as indicated. Six days later, effector cells were assayed for the generation of an anti-H-2^b allogeneic CTL response. The values for spontaneous release by target cells ranged from 4.6 to 6.8%.

[b] NA, not applicable.

siveness was again noted when viable AKR.H-2^b splenocytes were included with E♂G2 tumor stimulators compared with stimulation with E♂G2 plus irradiated AKR.H-2^b splenocytes (or E♂G2 stimulation alone; data not shown). In contrast, lysis of allogeneic P815 target cells was again essentially the same following in vitro stimulation with LB27.4 (*H-2$^{d/b}$*, class I and II MHC positive) tumor cells plus either viable or irradiated AKR.H-2^b splenocytes. It was possible that (following its generation in an Ag-specific fashion) a soluble, nonspecific inhibitory factor was the cause of the observed inhibition of antiviral CTL production. To test this possibility, both viral Ag-positive and allogeneic tumor stimulator cells were added to viable AKR.H-2^b splenocytes and primed B6 responder cells in the same MLTC well. Although AKR/Gross MuLV-specific CTL generation was significantly inhibited (compared with stimulation with irradiated E♂G2 plus LB27.4 tumor cells with or without irradiated AKR.H-2^b splenocytes), allogeneic CTL activity to P815 targets was essentially unaffected. Confinement

TABLE 2. Secondary in vitro tumor restimulation of anti-AKR/Gross retrovirus-specific CTL is inhibited in vitro by AKR.H-2^b splenocytes[a]

Secondary in vitro stimulation	E:T ratio	% Specific lysis	
		Viral	Control
		E♂G2	E♀K1
None	100:1	10	−1
	20:1	2	−1
	4:1	0	0
AKR.H-2^b splenocytes	100:1	25	NA[b]
	20:1	24	
	4:1	6	
Irradiated AKR.H-2^b splenocytes	100:1	83	2
	20:1	64	−1
	4:1	21	−1
E♂G2 tumor cells	100:1	93	0
	20:1	94	0
	4:1	61	−1
E♂G2 plus AKR.H-2^b splenocytes	100:1	41	NA
	20:1	31	
	4:1	8	
E♂G2 plus AKR.H-2^b:Fv-1^b splenocytes	100:1	82	NA
	20:1	74	
	4:1	33	

[a] B6 mice were primed with 10^6 AKR.H-2^b SL1 tumor cells. Eleven days later, 10^7 responder cells were mixed with 2×10^5 irradiated E♂G2 tumor cells and/or 2×10^6 splenocytes, the latter either with or without having received γ irradiation, as indicated. The splenocytes used were from 6-week-old AKR.H-2^b mice and were those not adherent to normal goat Ig-coated panning plates. The AKR.H-2^b:Fv-1^b splenocytes were obtained from 30-week-old donor mice. The values for spontaneous release by target cells were 6.9 and 7.7% for E♂G2 and E♀K1, respectively.

[b] NA, not applicable.

of inhibition to AKR/Gross MuLV-specific, not allogeneic cell-specific, CTL responses was confirmed in all of seven experiments. Similarly, in an experiment not shown, viral Ag-positive, irradiated B.GV tumor cells (derived from a BALB.B mouse) were mixed with B.GV-primed B6 responder cells to generate both MHC-restricted anti-AKR/Gross MuLV and anti-BALB minor histocompatibility antigen-specific CTL responses. Although lysis of AKR.H-2^b SL1 and E♂G2 viral targets was inhibited substantially (compared with restimulation with irradiated B.GV cells without AKR.H-2^b spleen cells), lysis of viral Ag-negative BALB.B-1K minor H target cells was unaffected by addition of the AKR.H-2^b cells to the MLTC. These data underscored the presence of inhibitory cells specific for AKR/Gross MuLV-specific CTL responses and argued against a nonspecific, soluble inhibitory factor.

Results from three additional types of experiments further argued against the involvement of soluble inhibitory factors and demonstrated the need for cell-to-cell interaction (not shown). In the first type of these experiments, a semipermeable membrane insert was used to separate viable AKR.H-2^b splenocytes from B6 responder cells and irradiated E♂G2 stimulator cells. No inhibition of the generation of AKR/Gross MuLV-specific CTL was observed when the AKR.H-2^b modulator cells were separated from, rather than cocultured with, the responder and stimulator cells. This lack of inhibition across the semipermeable membrane was observed even when primed responder cells plus E♂G2 tumor stimulator cells (which were contained within one chamber and resulted in the generation of a vigorous antiviral response) were separated from the combination of primed responder cells, AKR.H-2^b modulator cells, and E♂G2 stimulator cells (contained in the second chamber and resulting in inhibition of the antiviral CTL response in that chamber). Thus, the negative effect of viable AKR.H-2^b spleen cells on the generation of AKR/Gross MuLV-specific CTL does not appear to be mediated by a nonspecific inhibitory factor(s). In the second type of experiment, supernatant derived from MLTC wells containing primed B6 responder cells, E♂G2 tumor stimulator cells, and AKR.H-2^b inhibitory cells, added at various time points during the 6-day MLTC to wells containing primed B6 responder cells and E♂G2 stimulatory cells only, did not inhibit the generation of AKR/Gross MuLV CTL. It was further demonstrated that supernatant derived from wells in which AKR/Gross MuLV-specific CTL were successfully generated was not able to increase the development of antiviral cytotoxic activity when added to MLTC wells containing inhibitory cells. In the third type of experiment, supernatants taken from MLTC wells were assayed for the ability to promote the proliferation of CTLL-2 (IL-2 responsive) and CTLL.6-1D3 (IL-4 responsive) cells to assess the possibility that AKR.H-2^b inhibitory cells shifted the balance between Th1- and Th2-type lymphokines and thereby caused a decreased antiviral CTL response. It was found that the addition of AKR.H-2^b inhibitory cells to wells in which there was either an antiviral or anti-allogeneic CTL response did not significantly change the concentration of IL-2 (in the range of 0.3 to 0.5 U/ml), whereas IL-4 levels were not detectable in the presence or absence of inhibitory cells.

Characterization of the inhibition of AKR/Gross MuLV-specific CTL generation by viable AKR.H-2^b splenocytes. To further characterize the mechanism of specific inhibition by AKR.H-2^b spleen cells, a dose-response experiment was conducted. Figure 1 clearly demonstrates that decreasing the numbers of added AKR.H-2^b cells allows restoration of the capacity of a constant number of cocultured E♂G2 tumor cells to stimulate primed B6 responder cells to generate AKR/Gross MuLV-specific CTL in MLTC. At $\geq 10^6$ AKR.H-2^b splenocytes, there was nearly total loss of AKR/Gross MuLV CTL lysis of E♂G2 tumor cells. As the number of splenocytes was decreased, the percent lysis of E♂G2 target cells increased in a titratable and proportional fashion. Interestingly, in a parallel titration, as the number of viable AKR.H-2^b splenocytes used directly as stimulator splenocytes (without addition of E♂G2 cells) decreased, a small but significant increase in their ability to stimulate the production of AKR/Gross MuLV CTL was apparent. This pattern of stimulation was in contrast to the expected titration of antiviral CTL stimulation observed when mitomycin C-treated AKR.H-2^b splenic stimulator cells were used: decreased stimulation with decreased stimulator cell numbers. When 2×10^6 to 4×10^6 mitomycin C-treated AKR.H-2^b splenocytes were added, the level of AKR/Gross MuLV CTL stimulation approached that observed with E♂G2 tumor stimulator cells. Thus, although it is clear that viable AKR.H-2^b splenocytes can modulate specific inhibition, this cell population may also serve as a source of stimulation, although not nearly as effectively as when irradiated or mitomycin C treated. This allows for the possibility that subpopulations of AKR.H-2^b splenocytes are either inhibitory or stimulatory.

To show that inhibition of the generation of antiviral cytotoxicity was not a simple consequence of a limitation of tumor stimulator cells such that nonstimulating AKR.H-2^b spleen cells successfully competed for the primed responder cells, E♂G2 stimulator cells were added at various numbers to MLTC wells containing a constant number of responder and AKR.H-2^b inhibitory cells (not shown). Increasing the number of E♂G2 stimulator cells by as much as fivefold, however, did not decrease the degree of inhibition of AKR/Gross MuLV-

TABLE 3. Viable AKR.H-2^b splenocytes do not inhibit in vitro stimulation of anti-allogeneic CTL responses[a]

Secondary in vitro stimulation	Splenocyte irradiation	E:T ratio	% Specific lysis	
			Viral	Allogeneic
Expt 1			AKR.H-2^{b}SL1	P815 (*H-2^d*)
None		100:1	2	0
E♂G2	NA[b]	100:1	70	NA
		20:1	69	
		4:1	43	
E♂G2 plus AKR.H-2^b splenocytes	−	100:1	40	NA
		20:1	32	
		4:1	7	
P815 (*H-2^d*)	NA	100:1	NA	98
		20:1		85
		4:1		36
P815 plus AKR.H-2^b splenocytes	−	100:1	NA	102
		20:1		103
		4:1		59
Expt 2			E♂G2	P815
None		20:1	0	0
E♂G2 plus AKR.H-2^b splenocytes	−	20:1	16	NA
		4:1	3	
E♂G2 plus AKR.H-2^b splenocytes	+	20:1	75	ND
		4:1	47	
LB 27.4 (*H-2$^{d/b}$*) plus AKR.H-2^b splenocytes	−	20:1	ND	60
		4:1		21
LB 27.4 plus AKR.H-2^b splenocytes	+	20:1	ND	51
		4:1		14
E♂G2 plus LB27.4	NA	20:1	79	60
		4:1	40	19
E♂G2 plus LB27.4	−	20:1	25	73
plus AKR.H-2^b splenocytes		4:1	7	23
E♂G2 plus LB27.4	+	20:1	72	73
plus AKR.H-2^b splenocytes		4:1	32	20

[a] B6 mice were primed with AKR.H-2^b SL1 or 10^6 B.GV tumor cells in experiments 1 and 2, respectively. Eleven days later, responder cells were mixed with tumor cells or tumor cells plus splenocytes as indicated; 2×10^6 (experiment 1) or 4×10^6 (experiment 2) splenocytes were mixed per 10^7 B6 responder cells. Tumor stimulator cells were always used at 2×10^5 cells per 10^7 B6 responder cells. In experiment 1, splenocytes used were those not adherent to normal goat Ig-coated panning plates. The values for spontaneous release by tumor target cells were as follows: experiment 1, 8.1 and 10.8% for AKR.H-2^b SL1 and P815, respectively; experiment 2, 9.9 and 15.4% for E♂G2 and P815, respectively.

[b] NA, not applicable.

specific CTL, arguing against a passive competition between AKR.H-2^b spleen cells and limiting numbers of E♂G2 stimulator cells as the basis for the inhibition of the generation of AKR/Gross MuLV-specific CTL.

Because AKR.H-2^b splenocyte populations express virus associated Ags on their surface (8), a somewhat trivial explanation for their inhibitory effects was cold-target (unlabeled-target) competition. Thus, viable, but not inactivated, AKR.H-2^b splenocytes might be carried over from the 5- to 6-day MLTC to the cytolytic assay wells and thereby specifically compete as unlabeled target cells for AKR/Gross MuLV-specific, but not alloantigen-specific, CTL. The experiment shown in Fig. 2 addresses this possibility. With viable AKR.H-2^b modulator splenocytes (plus E♂G2 stimulator cells), there was nearly total loss of the generation of AKR/Gross MuLV CTL when the modulator cells were added at the initiation of the MLTC (compared with AKR/Gross virus-specific CTL generation after stimulation with E♂G2 tumor cells alone). In contrast, when the same number of AKR.H-2^b, or control B6, spleen cells were first cultured for 6 days with or without E♂G2 tumor stimulator cells (but separately from responder cells) and then mixed with an aliquot of anti-AKR/Gross MuLV CTL just prior to the ^{51}Cr release assay, no inhibition of AKR/Gross MuLV-specific cytotoxicity was observed. Variations of this test for unlabeled-target competition, including one in which fresh uncultured AKR.H-2^b spleen cells were mixed with pregenerated anti-AKR/Gross MuLV-specific CTL, were performed, with the same result of a lack of inhibition. In total, three experiments clearly demonstrated that the decrease in AKR/Gross MuLV-specific lysis of GCSA$^+$ tumor target cells does not result from unlabeled-target competition by viable AKR.H-2^b splenocytes.

Exogenous IL-2 restores AKR/Gross MuLV CTL generation inhibited by viable AKR.H-2^b splenocytes. To test whether the in vitro inhibition of AKR/Gross MuLV-specific CTL generation might be overcome by increasing the concentration of IL-2, as is often the case when anergy is the mechanism of nonresponsiveness, exogenous IL-2 was added at the onset of MLTC. It should be noted that the medium routinely used in MLTC was not supplemented with exogenous IL-2. Viable AKR.H-2^b spleen cells used alone as in vitro stimulator cells failed to stimulate high levels of lysis of virus-positive tumor target cells as described above. Addition of 8 or 16 U (Table 4, experiments 1 and 2, respectively) of exogenous IL-2 per ml significantly increased the capacity of these cells to stimulate AKR/Gross MuLV-specific CTL responses. Additionally, in both experiments, the inhibition of E♂G2-stimulated AKR/Gross MuLV CTL generation mediated by viable AKR.H-2^b spleen cells was partially overcome through addition of exogenous IL-2. These findings were reproduced in five of six experiments, although the restoration of antiviral lysis was not as dramatic in each case as that shown in Table 4, experiment 1.

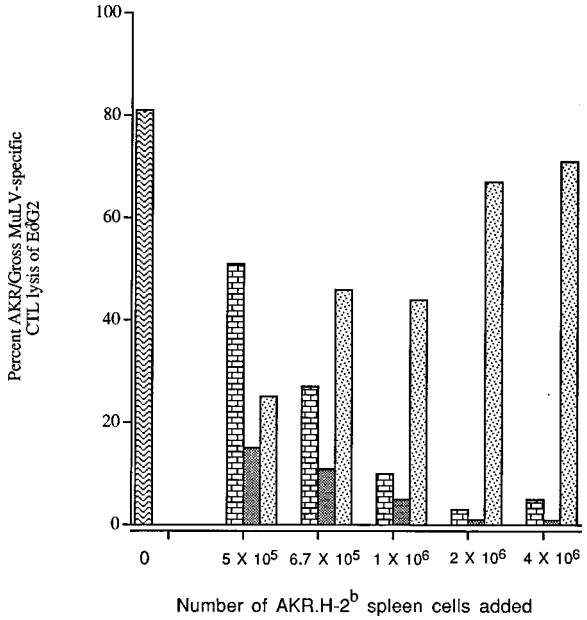

FIG. 1. AKR.H-2^b spleen cells, cocultured with E♂G2 tumor stimulator cells, inhibit the generation of AKR/Gross MuLV-specific CTL in vitro. B6 mice were immunized with 10^6 AKR.H-2^b SL1 tumor cells. Eleven days later, primed responder cells were mixed with either 2×10^5 mitomycin C-treated E♂G2 cells or 2×10^5 treated E♂G2 cells plus untreated AKR.H-2^b splenocytes at various responder cell-to-AKR.H-2^b modulator cell ratios in MLTC. Additionally, untreated or mitomycin C-treated AKR.H-2^b splenocytes were used in vitro for the stimulation of AKR/Gross virus-specific CTL at various numbers in the absence of E♂G2 stimulator cells as shown. Six days later, effector cells were harvested and assayed in a ^{51}Cr release assay for AKR/Gross virus-specific CTL lysis of E♂G2 tumor cells at various E:T ratios. Shown here are data resulting when the E:T ratio was 100:1. The spontaneous release of E♂G2 target cells was 10.3%. In vitro stimulator or modulator cells added: ▨, E♂G2 cells only; ⊞, nontreated AKR.H-2^b cells plus E♂G2 cells; ▩, nontreated AKR.H-2^b cells; ⊡, mitomycin C-treated AKR.H-2^b cells.

In experiment 2 and in a second experiment (not shown), the natural killer cell-sensitive YAC-1 target was additionally used to show that the increased lysis of virus positive tumor targets was not due to an increase in natural killer cell lytic activity.

The finding that exogenous IL-2 partially restored the generation of AKR/Gross MuLV-specific CTL suggests that the inhibition mediated by AKR.H-2^b cells may be an early event, perhaps directed against pCTL before their full proliferation and differentiation into mature CTL in the MLTC. These experiments suggested that IL-2 may cause the target cell for inhibition to differentiate to a point at which it is no longer susceptible to inhibition. To test whether AKR.H-2^b cells could mediate inhibition of fully differentiated AKR/Gross MuLV-specific CTL, experiments were conducted by culturing the CD8$^+$ G8 CTL clone of similar antiviral specificity (1), in place of primed bulk B6 responder cells, for 6 days with tumor stimulator cells with or without AKR.H-2^b inhibitory cells. It was found in both of two experiments (not shown) that this CTL clone was able to lyse AKR/Gross MuLV-positive tumor target cells to equivalent levels regardless of whether AKR.H-2^b modulator cells had been included in the culture, supporting the possibility that the inhibitory event(s) occurs early in CTL development. Alternatively, it is possible that if the AKR.H-2^b inhibitory cells express virus-encoded CTL determinants, the G8 CTL clone simply eliminated these inhibitory cells before they could inhibit this AKR/Gross MuLV-specific CTL clone.

Inhibition might be merely a passive consequence of endogenous IL-2 being bound to IL-2 receptors that appear on viable AKR.H-2^b cells that became activated in the MLTC, such that sufficient IL-2 was not available to antiviral CTL. To test this possibility, viable CTLL-2 (IL-2-dependent, IL-2 receptor-positive) cells were used. At a cell concentration at which AKR.H-2^b splenocytes repeatedly inhibited virus-specific CTL dramatically, there was only a slight decrease in the generation of antiviral lytic activity when CTLL-2 cells were instead added to MLTC wells containing primed B6 responder cells and E♂G2 stimulator cells (not shown).

AKR.H-2^b CD4$^-$ CD8$^+$ and CD4$^+$ CD8$^-$ T lymphocytes and AKR.H-2^b B cells inhibit the generation of AKR/Gross MuLV CTL in vitro. To identify the AKR.H-2^b cell types which could mediate inhibition of AKR/Gross MuLV-specific CTL responsiveness, depletion of either B lymphocytes, T lymphocytes, or B lymphocytes followed by CD4$^+$ CD8$^-$ or CD4$^-$ CD8$^+$ T lymphocytes (see Materials and Methods) was undertaken prior to their addition to MLTC. As shown in Table 5, experiments 1 and 2, AKR/Gross MuLV-specific CTL lysis of E♂G2 tumor target cells exceeded 50% at an E:T ratio of 100:1 following standard coculture of primed B6 responder cells with irradiated E♂G2 cells. In contrast, in experiment 1, the addition of enriched populations of AKR.H-2^b T lymphocytes, or either the CD4$^-$ CD8$^+$ or CD4$^+$ CD8$^-$ T-lymphocyte subset, could inhibit the generation of E♂G2-stimulated AKR/Gross MuLV-specific CTL. Addition of AKR.H-2^b T cells or the T-cell subsets led to the generation of lytic activity to E♂G2 target cells that ranged between only 13 and 23% (E:T ratio of 100:1), which was comparable to the decreased lysis (22%) resulting when complement-only treated AKR.H-2^b cells were

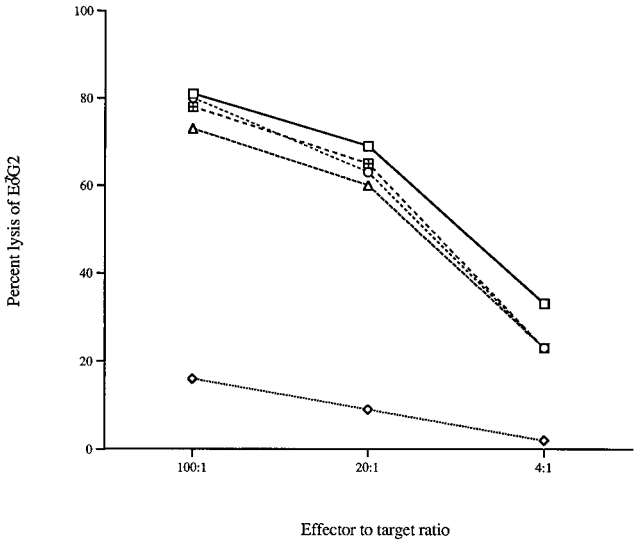

FIG. 2. Inhibition of AKR/Gross MuLV-specific CTL by AKR.H-2^b spleen cells is not due to unlabeled-target competition. B6 mice were immunized with 10^6 B.GV tumor cells. Eleven days later, responder cells were placed in MLTC with 2×10^5 E♂G2 tumor cells (no modulator cells) or 2×10^5 E♂G2 cells plus 4×10^6 viable AKR.H-2^b splenocytes added at the onset of MLTC. To test for unlabeled-target competition, 4×10^6 AKR.H-2^b cells were placed in culture without or with 2×10^5 E♂G2 cells but without any responder cells as shown. Immediately prior to assaying for AKR/Gross MuLV-specific cytotoxicity against ^{51}Cr-labeled E♂G2 target cells, an aliquot of control effector cells generated by in vitro stimulation with E♂G2 cells was harvested, counted, and mixed at a responder-to-modulator cell ratio of 2.5:1 with AKR.H-2^b splenic modulator cells or, as a control, B6 spleen cells, cultured as described above. Spontaneous release by E♂G2 target cells was 8.5%. Modulator spleen cells added: □, none; ◇, AKR.H-2^b cells added at onset of MLTC; ○, cultured AKR.H-2^b cells added to CTL assay; △, cultured (with E♂G2 stimulator cells) AKR.H-2^b cells added to CTL assay; ⊞, cultured B6 cells added to CTL assay.

TABLE 4. Exogenous IL-2 partially restores inhibition of anti-AKR/Gross retrovirus-specific CTL by AKR.H-2^b splenocytes[a]

Secondary in vitro stimulation	IL-2	E:T ratio	% Specific lysis		
			Viral	Control	Natural killer cell
			E♂G2	E♀K1	YAC-1
Expt 1					
None	−	100:1	2	−1	
	+	100:1	13	8	
E♂G2	−	100:1	83	1	
		20:1	84	0	
		4:1	50	−2	
	+	100:1	73	3	
		20:1	78	3	
		4:1	48	1	
AKR.H-2^b splenocytes	−	100:1	12	0	
	+	100:1	48	7	
		20:1	30	5	
		4:1	10	1	
E♂G2 plus AKR.H-2^b splenocytes	−	100:1	18	3	
	+	100:1	72	8	
		20:1	65	6	
		4:1	22	1	
Expt 2					
None	−	100:1	8	6	10
	+	100:1	7	8	5
E♂G2	−	100:1	88	7	5
		20:1	71	3	1
		4:1	24	−1	0
	+	100:1	91	7	5
		20:1	78	3	2
		4:1	42	0	0
AKR.H-2^b splenocytes	−	100:1	19	8	11
	+	100:1	36	10	14
		20:1	19	6	6
E♂G2 plus AKR.H-2^b splenocytes	−	100:1	36	9	6
		20:1	21	5	2
		4:1	4	1	1
	+	100:1	65	12	10
		20:1	35	8	4
		4:1	11	1	1

[a] B6 mice were primed with 10^6 B.GV tumor cells. Eleven days later, 10^7 responder cells were mixed with 2×10^5 mitomycin-treated E♂G2 stimulator cells and/or 1.2×10^6 (experiment 1) and/or 2×10^6 (experiment 2) splenocytes with or without 8 (experiment 1) or 16 (experiment 2) U of IL-2 per ml, as indicated. Six days later, cells were assayed for the ability to lyse ^{51}Cr-labeled target cells. The values for spontaneous release by target cells ranged from 4.8 to 12.3%.

used as inhibitory cells. Purified AKR.H-2^b T cells mediated inhibition in six of seven experiments, whereas AKR.H-2^b CD4$^-$ CD8$^+$ and CD4$^+$ CD8$^-$ T lymphocytes each did so in three of three and five of six experiments, respectively.

To determine whether inhibition of AKR/Gross MuLV-specific CTL could also be mediated by AKR.H-2^b B lymphocytes, spleen cell populations were depleted of T lymphocytes, and the remaining cells were assayed for inhibitory function (Table 6). It was found that AKR.H-2^b B lymphocytes mediated a substantial decrease in AKR/Gross MuLV-specific CTL lysis of E♂G2 target cells. In experiment 1, lysis of E♂G2 target cells following in vitro stimulation with E♂G2 cells was 27% at an E:T ratio of 20:1 in the absence of AKR.H-2^b B lymphocytes and 27% at an E:T ratio of 100:1, approximately a fivefold reduction, when AKR.H-2^b B lymphocytes were added to the MLTC wells. This result was reproducible in three additional experiments, although the degree of inhibition varied such that in some cases AKR.H-2^b B cells were as inhibitory as AKR.H-2^b T cells.

To rule out small numbers of contaminating B cells as being the mediators of inhibition in experiments in which enriched populations of T lymphocytes were assayed for inhibitory function, various numbers of B cells (approximating the number of possible contaminating B cells, judged from the efficiency of cell separation; see Materials and Methods) were mixed in MLTC with E♂G2 stimulator cells and primed B6 responder cells. Little if any, inhibition of antiviral cytotoxicity could be mediated by such contaminating numbers of B cells. Likewise, to rule out contaminating T cells as being the mediators of inhibition in experiment 2, in which B cells were assayed for inhibitory function, the addition of possible contaminating numbers of T cells resulted in only slight, if any, levels of inhibition of AKR/Gross MuLV-specific cytotoxicity.

To further verify the phenotype of the inhibitory cells and rule out the possible activity of small numbers of contaminating cells present after the negative selection approach, positive cell selection of inhibitory AKR.H-2^b CD4$^+$ T or B lymphocytes was also performed. As shown in experiments 2 of Tables 5 and 6, respectively, both AKR.H-2^b CD4$^+$ T and AKR.H-2^b B lymphocytes, obtained by direct immunofluorescence staining followed by cell sorting, dramatically inhibited the generation of antiviral CTL. The levels of inhibition mediated by positively selected cells were comparable with the levels mediated by their negatively selected counterparts. Although negatively selected AKR.H-2^b CD4$^-$ CD8$^+$ T lymphocytes were also inhibitory (Table 5, experiment 1), positive selection of

TABLE 5. $CD4^-$ $CD8^+$ and $CD4^+$ $CD8^-$ T lymphocytes inhibit AKR/Gross retrovirus-specific CTL responses in vitro[a]

Secondary in vitro stimulation	E:T ratio	% Specific lysis	
		Viral	Control
		E♂G2	E♀K1
Expt 1			
None	100:1	4	−1
E♂G2	100:1	72	0
	20:1	42	−1
	4:1	13	−1
E♂G2 plus complement-treated AKR.H-2^b splenocytes	100:1	22	0
	20:1	12	0
E♂G2 plus negatively selected AKR.H-2^b T lymphocytes	100:1	18	0
	20:1	6	0
E♂G2 plus negatively selected $CD4^-$ $CD8^+$ AKR.H-2^b T lymphocytes	100:1	13	2
	20:1	5	2
E♂G2 plus negatively selected $CD4^+$ $CD8^-$ AKR.H-2^b T lymphocytes	100:1	23	0
	20:1	9	1
Expt 2			
None	20:1	4	0
E♂G2	20:1	79	−1
	4:1	41	−1
E♂G2 plus AKR.H-2^b splenocytes	20:1	9	1
	4:1	3	−1
E♂G2 plus control sorted AKR.H-2^b T lymphocytes[b]	20:1	7	−1
	4:1	3	−1
E♂G2 plus positively selected $CD4^+$ AKR.H-2^b lymphocytes	20:1	30	0
	4:1	10	−1

[a] B6 mice were primed with 10^6 B.GV tumor cells. Seventeen days later, 10^7 responder cells were mixed with 2×10^5 E♂G2 tumor stimulator cells without or with addition of 2×10^6 AKR.H-2^b inhibitory cells as follows: experiment 1, complement-only-treated, negatively selected T-cell-enriched $CD4^-$ $CD8^+$ or $CD4^+$ $CD8^-$ AKR.H-2^b T lymphocytes; experiment 2, positively selected (FITC-conjugated anti-CD4 sorted) $CD4^+$ $CD8^-$ AKR.H-2^b T lymphocytes. See Materials and Methods for details. In experiment 1, as a means to assess the efficacy B-cell depletion, representative portions of unfractionated AKR.H-2^b and T-cell-enriched AKR.H-2^b lymphocytes were monitored by measuring [^{3}H]thymidine incorporation expressed, following subtraction of counts per minute resulting from stimulation with medium alone, as a percentage of unfractionated cell stimulation [(delta experimental/delta unfractionated cell stimulation) × 100] as follows: LPS, 3%; ConA, 105%. In experiment 2, a representative portion of positively selected cells was analyzed and shown to be 99% pure $CD4^+$ $CD8^-$ AKR.H-2^b T lymphocytes. The values for spontaneous release by E♂G2 and E♀K1 target cells ranged from 6.7 to 13.7%.

[b] AKR.H-2^b lymphocytes (unstained) were sham sorted and assayed for inhibitory function.

this subset through cell sorting was not a reasonable approach in that anti-CD8 MAbs were shown to block the inhibitory function of AKR.H-2^b $CD8^+$ cells (see below). However, independent verification of the inhibitory ability of AKR.H-2^b $CD8^+$ T cells was obtained by isolation of two $CD4^-$ $CD8^+$ AKR.H-2^b lines, AKR.H-2^b.BB1 and AKR.H-2^b.KB2. As shown in Table 7, AKR.H-2^b.BB1 and AKR.H-2^b.KB2 cells dramatically inhibited the generation of AKR/Gross MuLV CTL when added to MLTC wells containing primed responder and E♂G2 stimulator cells. In experiment 2, the observed inhibition was again shown to be specific for AKR/Gross viral CTL as lysis of P815 (*H-2*^{d}) cells by allospecific CTL was not decreased through addition of AKR.H-2^b.BB1 cells to MLTC wells containing LB27.4 (*H-2*$^{d/b}$) stimulator cells.

Because anergy often involves an inappropriate interaction of responder cells with antigen-presenting cells such as macrophages, it seemed reasonable to test whether AKR.H-2^b macrophages present in the modulator cell preparations are involved in the inhibitory process. Adherence to Sephadex G-10 beads (two sequential passages at 37°C) was used to deplete macrophages from AKR.H-2^b splenocyte preparations (see Materials and Methods for details). In both of two experiments, depletion of macrophages (confirmed by flow cytometric analysis using MAb F4/80) prior to mixing with primed B6 responders and E♂G2 tumor stimulator cells in MLTC did not relieve the inhibition of AKR/Gross MuLV CTL production (not shown). The degrees of inhibition of the response were similar whether or not the macrophages were depleted. These data thus suggested that the presence of macrophages in the modulator cell preparation was not required for the specific inhibition mediated by AKR.H-2^b spleen cells. To determine if macrophages, although not required for, might be capable of inhibition of AKR/Gross MuLV-specific CTL responsiveness, 2×10^6 to 4×10^6 AKR.H-2^b splenocytes were sequentially depleted of both B and T lymphocytes. Then, all remaining cells, including macrophages, were placed in MLTC with B6 responder cells and E♂G2 stimulator cells. In both of two experiments (not shown), it was found that the generation of lytic activity to E♂G2 targets was essentially the same as when AKR.H-2^b cells were not added, thereby demonstrating that macrophages, at these cell numbers, are not an inhibitory cell type in this system in the absence of AKR.H-2^b T or B lymphocytes. Representative portions of these cell preparations stained with the macrophage-specific and -reactive MAbs F4/80 and anti-MAC-1, respectively, for FACS analyses showed that sequential depletion of B and T lymphocytes represented a highly efficient means of enriching splenic AKR.H-2^b macrophages, with enrichment ranging up to 25- to 30-fold for the anti-MAC-1 MAb.

TABLE 6. AKR.H-H-2^b B lymphocytes inhibit AKR/Gross retrovirus-specific CTL responses in vitro[a]

Secondary in vitro stimulation	E:T ratio	% Specific lysis	
		Viral	Control
		E♂G2	E♀K1
Expt 1			
None	100:1	0	0
E♂G2	100:1	53	0
	20:1	27	1
	4:1	6	1
E♂G2 plus negatively selected AKR.H-2^b B lymphocytes	100:1	27	9
	20:1	12	3
Expt 2			
None	100:1	13	5
E♂G2	100:1	91	8
	20:1	87	6
	4:1	62	6
E♂G2 plus complement-treated AKR.H-2^b splenocytes	100:1	31	9
	20:1	14	7
E♂G2 plus negatively selected AKR.H-2^b B lymphocytes	100:1	22	9
	20:1	9	5
E♂G2 plus positively selected AKR.H-2^b B lymphocytes	100:1	40	8
	20:1	26	5

[a] B6 mice were primed with 10^6 B.GV tumor cells. Eleven days later, 10^7 responder cells were mixed with 2×10^5 E♂G2 tumor stimulator cells without or with addition of AKR.H-2^b inhibitory cells as follows: experiment 1, 3.5×10^6 negatively selected B lymphocytes; experiment 2, 2×10^6 negatively or positively selected [FITC-conjugated $F(ab')_2$ rabbit anti-mouse IgM (μ-chain specific) sorted] B lymphocytes as listed. In experiment 1, as a means to assess the efficacy of T-cell depletion, representative portions of unfractionated AKR.H-2^b and B-cell-enriched AKR.H-2^b lymphocytes were was monitored by measuring [^{3}H]thymidine incorporation expressed, following subtraction of counts per minute resulting from stimulation with medium alone, as a percentage of unfractionated cell stimulation [(delta experimental/delta unfractionated cell stimulation) × 100] as follows: LPS, 181%; ConA, 2%. In experiment 2, a representative portion of positively selected cells was tested by flow cytometric analysis and shown to be 98% pure AKR.H-2^b B lymphocytes. The values for spontaneous release by E♂G2 and E♀K1 target cells ranged from 6.1 to 11.8%.

TABLE 7. AKR.H-2^b CD4$^-$ CD8$^+$ T-cell lines inhibit the generation of AKR/Gross MuLV-specific CTL in vitro[a]

Secondary in vitro stimulation	E:T ratio	% Specific lysis		
		Viral	Control	Allogeneic
		E♂G2	E♀K1	P815
Expt 1				
None	100:1	2	0	
E♂G2	100:1	76	0	
	20:1	55	0	
	4:1	20	0	
E♂G2 plus AKR.H-2^b splenocytes	100:1	15	7	
	20:1	6	2	
E♂G2 plus AKR.H-2^b.KB2 (T-cell line 1)	100:1	20	ND[b]	
	20:1	9		
Expt 2				
None	100:1	−1	−2	2
E♂G2	100:1	74	−1	NA[c]
	20:1	53	−1	
	4:1	13	−2	
E♂G2 plus AKR.H-2^b splenocytes	100:1	20	2	NA
	20:1	4	0	
E♂G2 plus AKR.H-2^b.BB1 (T-cell line 2)	100:1	19	−2	NA
	20:1	4	−1	
LB27.4	100:1	NA	−2	59
	20:1		0	22
LB27.4 plus AKR.H-2^b splenocytes	100:1	NA	4	81
	20:1		2	35
LB27.4 plus AKR.H-2^b.BB1	100:1	NA	2	73
	20:1		−2	34

[a] B6 mice were primed with 10^6 B.GV tumor cells. Twelve days later, 10^7 responder cells were mixed with 2×10^5 E♂G2 or LB27.4 stimulator cells, as indicated. The numbers of inhibitory cells used were as follows; experiment 1, 2×10^6 unfractionated AKR.H-2^b or 3.5×10^4 AKR.H-2^b.KB2; experiment 2, 1.5×10^6 unfractionated AKR.H-2^b or 1.5×10^6 AKR.H-2^b.BB1. The values for spontaneous release by tumor target cells were as follows: experiment 1, 5.2 to 11.5%; experiment 2, 6.7 to 12.7%.
[b] ND, not determined.
[c] NA, not applicable.

Inhibition by AKR.H-2^b CD4$^-$ CD8$^+$ T lymphocytes is blocked with an anti-CD8 antibody. As one means to help establish what cell surface Ags expressed on inhibitory AKR.H-2^b T lymphocytes might serve as ligands (in binding inhibitory T cells to pCTL or CTL or to other cells in the responder cell population) or actually play a functional role in triggering inhibition, we performed experiments whereby inhibitory cells were pretreated with antibody prior to being used in MLTC with primed B6 responder cells and tumor stimulator cells. Isotype-matched negative control antibodies and/or B6 modulator spleen cells, having received parallel antibody treatment, were used to assess control levels of lysis. In some experiments, inhibition mediated by AKR.H-2^b T lymphocytes (obtained through negative selection by goat anti-mouse Ig panning as described above) was partially reversed through preincubation of inhibitory cells with an anti-CD8a, but not isotype-matched or anti-CD2, MAb (data not shown). In other experiments, the anti-CD8a MAb did not show significant abrogation of the inhibition caused by AKR.H-2^b splenic T cells, presumably because the CD4$^+$ T-cell subset is also inhibitory. To avoid this complication, in further experiments AKR.H-2^b T cells were depleted with either an anti-CD4 or anti-CD8a MAb plus complement, and the resulting CD8$^+$- or CD4$^+$-enriched populations were then incubated with either an anti-CD8a or anti-CD4 MAb, respectively, before being added to the MLTC. In the experiment shown in Fig. 3, both CD4$^+$ and CD8$^+$ AKR.H-2^b T lymphocytes could inhibit the generation of lytic activity to AKR.H-2^b SL1 target cells to essentially the same levels, 18 and 17% specific lysis at an E:T ratio of 20:1 (not shown), compared with 54% at the same E:T ratio when AKR.H-2^b SL1 tumor stimulatory cells were used in the absence of inhibitory cells. The lysis of AKR.H-2^b SL1 target cells following inclusion of anti-CD8a antibody-treated CD8$^+$ T lymphocytes approached the level of lysis observed when AKR.H-2^b SL1 tumor stimulator cells were used in the absence of inhibitory cells. In sharp contrast, the generation of anti-AKR/Gross MuLV CTL to AKR.H-2^b SL1 target cells was essentially completely inhibited by either unfractionated AKR.H-2^b spleen cells or anti-CD4-treated CD4$^+$ AKR.H-2^b T lymphocytes. In all, treatment with either of two anti-CD8a MAbs substantially blocked the inhibition mediated by AKR.H-2^b CD8$^+$ T lymphocytes in three of four experiments. It should be noted that the one experiment in which the anti-CD8a MAb did not relieve inhibition was the only experiment in which the CD8$^+$ AKR.H-2^b lymphocytes inhibited the antiviral CTL response totally. Conversely, alternative pretreatment of purified CD4$^+$ AKR.H-2^b lymphocytes with either of two anti-CD4 MAbs did not block their ability to inhibit antiviral CTL generation in five of six experiments. Thus, only the inhibition mediated by CD8$^+$ AKR.H-2^b T cells could be reversed by the MAb to the respective T-subset-specific cell surface marker, indicating the involvement of CD8 in the inhibitory process.

In addition to performing these antibody blocking experiments, we assessed the possible differential expression of adhesion and costimulatory molecules, and other cell surface molecules of interest, on inhibitory AKR.H-2^b cells and non-inhibitory spleen cell preparations from B6 and AKR.H-2^b:Fv-1^b mice by indirect immunofluorescence. Cell surface antigens stained for included CD3ε, CD4, CD8a, α/β TcR, γ/δ TcR, CD11a, CD2, CD25, CD44 (pgp-1), CD45, B7.1, B7.2, H-2D^b, and H-2K^b. There was no striking difference in the expression of these determinants on the cell surface of spleen

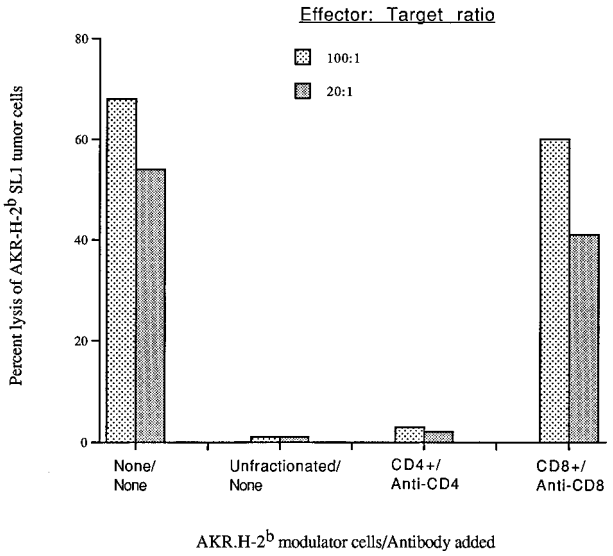

FIG. 3. Inhibition of AKR/Gross MuLV-specific CTL by AKR.H-2^b $CD4^-$ $CD8^+$ T lymphocytes is reversible by treatment with an anti-CD8a antibody. B6 mice were immunized with 10^6 B.GV tumor cells. Eighteen days later, 10^7 responder cells were mixed with 2×10^5 AKR.H-2^b SL1 tumor stimulator cells without or with 2×10^6 AKR.H-2^b modulator cells. AKR.H-2^b $CD4^+$ $CD8^-$ and $CD4^-$ $CD8^+$ modulator cells were obtained first by negative selection on petri dishes coated with goat anti-mouse Ig and then by further depletion with anti-CD8a and anti-CD4 MAbs plus complement, respectively. Efficiencies of B-cell depletion, prior to further depletion of AKR.H-2^b T lymphocyte subsets, as assessed by measuring ^3H incorporation following mitogenic stimulation with LPS or ConA as described in footnote *a* to Table 5, were as follows: LPS, 1%; ConA, 56%. To determine the efficiency of subsequent depletions of either $CD4^-$ $CD8^+$ or $CD4^+$ $CD8^-$ T lymphocytes, flow cytometric analysis was performed as described in Materials and Methods. Because the levels of staining of $CD4^-$ $CD8^+$ cells in $CD4^+$ $CD8^-$-enriched populations (and vice versa) were at near background levels, it was deduced that the enriched populations of T lymphocytes were essentially pure. Enriched $CD4^+$ $CD8^-$ or $CD4^-$ $CD8^+$ T lymphocytes were then incubated with an anti-CD4 or anti-CD8a MAb, respectively, prior to testing for inhibitory function in MLTC as described in Materials and Methods. The value for spontaneous release by AKR.H-2^b SL1 tumor cells was 14.5%.

cells originating from AKR.H-2^b mice and from B6 or AKR.H-2^b:Fv-1^b mice.

DISCUSSION

Because the frequency of pCTL specific for endogenous AKR/Gross MuLV Ags in AKR.H-2^b mice was found to be comparable to the frequency found in prototypic B6 responder mice (32), AKR/Gross MuLV nonresponsiveness could not be attributed to tolerance through elimination of self-reactive T cells in the thymus. Apart from clonal deletion, mechanisms which may inhibit the host's ability to develop CTL-mediated immune surveillance to clear cells expressing retroviral Ag include veto cell function, whereby MHC molecules expressed on the inhibitory veto cell surface present peptide Ag such that the complex is recognized by the TcR of target CTL, the result being either the elimination or functional deletion of that CTL; anergy, inactivation as a result of specific Ag presentation in the absence of adequate costimulation in the peripheral lymphoid organs; or T suppressor (T_s) function.

As we have previously shown (8), both T- and B-cell-enriched, irradiated, normal AKR.H-2^b lymphocyte populations were able to serve as in vitro stimulatory cells in the generation of anti-AKR/Gross MuLV CTL. This stimulatory ability is a consequence of endogenous retroviral Ag expression by multiple AKR.H-2^b cell types, particularly envelope proteins, and the availability of these Ags for processing and presentation by class I MHC. In the present study, FACS analyses performed on enriched AKR.H-2^b $CD4^+$ $CD8^-$ or $CD4^-$ $CD8^+$ T lymphocytes, or B lymphocytes, directly demonstrated the expression of cell surface viral Ag expression through staining with the MAb to the f antibody neutralization epitope of gp70 (not shown). We have previously shown the immunodominant peptide recognized by K^b-restricted anti-AKR/Gross MuLV CTL is the octameric peptide KSPWFTTL, located in the p15E region of the AKR/Gross MuLV envelope protein (4, 34). Therefore, a rationale is clearly provided for either the elimination or inactivation of AKR/Gross MuLV-specific pCTL or CTL in order to avert widespread autoimmune disease in AKR.H-2^b mice. The fact that viable AKR.H-2^b T and B lymphocytes are AKR/Gross MuLV Ag positive and modulate inhibition rather than stimulation of an antiviral CTL response makes plausible the possibility that inhibition is due to a veto cell mechanism(s). Fink et al. (7) have suggested that in theory, any cell type expressing Ag might serve as a veto cell when the expressed Ag is nonimmunogenic and accessible to other cells for inhibitory interactions. Along these lines, although $CD4^-$ $CD8^+$ T cells have been regarded as particularly efficient veto cells, other cells have been shown to be capable of veto activity (16, 20–22). In this case, it is possible that viral Ag peptides (presumably the immunodominant p15E epitope presented by class I MHC K^b molecules for direct veto of CTL) on the inhibitory veto cell surface would interact with the TcR of either the AKR/Gross MuLV-specific pCTL and CTL or, alternatively, with class II-restricted TcR of required $CD4^+$ T helper cells. The veto cell would then function to either eliminate or inactivate the antiviral T cell.

Although overt lysis or elimination by veto cells dominated early thinking, it has been proposed that inhibitory $CD4^-$ $CD8^+$ veto cells may also inhibit by a nonlytic mechanism whereby only CD8 expression (in the absence of other adhesion or costimulatory molecules) is crucial and delivers a downregulatory signal leading to pCTL or CTL inactivation (13, 14, 18, 28, 29). Although the specific mechanism through which this downregulator signal is delivered remains somewhat obscure, it is generally agreed that CD8 plays an important role in increasing cell-to-cell adhesion in a variety of immune interactions and may also be involved in signalling (13, 24, 36). Although AKR.H-2^b $CD4^+$ T and B lymphocytes were also clearly specifically inhibitory in the present system, it is reasonable to postulate that the AKR.H-2^b $CD8^+$ splenic T lymphocytes, and particularly the AKR.H-2^b $CD8^+$ T-cell line which was used at very low cell numbers, were particularly efficient in inhibitory function because of their concomitant expression of CD8 and viral Ag. Indeed, we have found that the generation of anti-AKR/Gross MuLV CTL was mostly restored by pretreatment of inhibitory AKR.H-2^b $CD8^+$ T lymphocytes with an anti-CD8a antibody. In other in vitro systems, it has been shown that antibodies to CD8 can reverse the inhibition mediated by these cells (6, 29).

It has been reported that competition between anergic T cells and T cells specific for the same (limiting numbers of) antigen-presenting cells may lead to inhibition as a consequence of a lack of costimulation and a limitation on locally produced IL-2 (19). We have found that increasing the number of E♂G2 stimulator cells in MLTC wells by as much as fivefold did not restore the production of AKR/Gross MuLV-specific CTL in the presence of AKR.H-2^b spleen cells. These data also argued against a passive competition between AKR.H-2^b spleen cells and E♂G2 stimulator cells as the basis for the inhibition in the generation of AKR/Gross MuLV-specific CTL. Additionally, depleting AKR.H-2^b spleen cell popula-

tions of macrophages prior to testing in MLTC for inhibitory function did not alter the observed AKR/Gross MuLV inhibition, arguing against an inappropriate interaction between responder cells and macrophage antigen-presenting cells as the underlying mechanism of inhibition. On the other hand, addition of relatively high concentrations of exogenous IL-2 (approximately 16- to 50-fold higher than the low endogenous levels; see below) significantly increased the generation of AKR/Gross MuLV CTL when AKR.H-2^b inhibitory cells were present. Because exogenous IL-2 initiated increased proliferation and differentiation of at least a portion of the pCTL to mature CTL, these data could be taken to suggest that an anergic mechanism is partially responsible for the inhibition. The finding that AKR.H-2^b spleen cells were unable to inhibit the fully differentiated AKR/Gross MuLV-specific CTL clone G8 when used in place of primed polyclonal responder cells in MLTC further supported the possibility that the inhibitory event(s) occurs early in CTL development.

A growing literature has emphasized nonresponsiveness or diminished states of certain T-cell-mediated immune reactivity as due to an imbalance in the production of cytokines, with inhibitory lymphokines such as IL-4 and/or IL-10 predominating (15, 17, 23, 25, 27, 35) relative to positive-acting cytokines such as IL-2, gamma interferon, and IL-12 (3, 15, 25, 27, 35). Our data, which have demonstrated a fine specificity of AKR.H-2^b inhibitory cells for AKR/Gross viral pCTL or CTL, but not anti-minor H or anti-allospecific CTL, argue strongly against soluble Th2-type lymphokines as the mediators of the observed inhibition. Experiments using semipermeable membranes to separate inhibitory AKR.H-2^b cells from responder cells, as well as other experiments in which supernatant transferred from wells in which inhibition was demonstrated was unable to inhibit the generation of antiviral CTL in wells containing primed responder cells and tumor stimulator cells, further argued against a role for soluble inhibitory lymphokines. In addition, the concentration of soluble IL-2 in supernatants derived from MLTC wells which contained primed responder and tumor stimulator cells plus AKR.H-2^b inhibitory cells did not differ substantially from the IL-2 levels found in supernatants in which a vigorous antiviral CTL response was noted (no addition of inhibitory cells was used). Conversely, addition of inhibitory cells did not augment IL-4 to detectable levels. Similar results were obtained when supernatants were derived from MLTC wells in which allogeneic responses were generated in the presence or absence of AKR.H-2^b spleen cells. Whether cell-bound and/or antigen-specific cytokines or other factors play a role in the inhibitory process will require further investigation.

Additionally, in particular because a subset of inhibitory AKR.H-2^b cells are CD4$^-$ CD8$^+$ T lymphocytes, it is also possible that some or all of these inhibitory cells function as T_s cells. Although the existence and mechanism of action of Ag-specific T_s cells remain controversial, two commonly proposed mechanisms, involving specificity either for Ag or for the TcR of the T cell that is the target for suppression, serve to distinguish T_s from veto cells. Alternatively, both veto and T_s cells (whether CD8 or not) may be cellular players in AKR/Gross MuLV-specific CTL nonresponsiveness in AKR.H-2^b mice. Indeed, although different mechanisms are involved, T_s and veto cells may target the same or different cell types in the nonresponder AKR.H-2^b model: either pCTL and CTL or AKR/Gross MuLV-specific T_h cells.

This report illustrates the complexity of the interactions of retroviruses with the immune system, with multiple possible mechanisms of inhibition of the CTL response. AKR.H-2^b mice provide an excellent animal model with which to study negative modulation of CTL responses by retroviral infection. Further experiments probing the precise mechanisms through which AKR.H-2^b mice are rendered nonresponsive may prove to be insightful in furthering a general understanding of how retroviruses escape immune elimination.

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