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CAPACITY OF DIFFERENT CELL TYPES TO STIMULATE CYTOTOXIC T LYMPHOCYTE PRECURSOR CELLS IN THE PRESENCE OF INTERLEUKIN 2

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Plastic-adherent cells enriched for dendritic cells (AC) were found to be among the most potent stimulator cells for the activation of cytotoxic T lymphocytes (CTL) *in vitro* in the presence of interleukin 2 (IL 2) and a constant second set of allogeneic stimulator cells. Concanavalin A-activated nylon wool-nonadherent spleen cells (CNWT), concanavalin A-activated unfractionated spleen cells (Cspl), and some variants of the ESb T lymphoma line were equally effective as stimulator cells, however, and provoked a substantial cytotoxic response at concentrations of 10^4 cells per culture or less. In contrast, nonactivated nylon wool-nonadherent spleen cells (NWT) or unfractionated spleen cells (Spl) and cells of the P815 mastocytoma, the Meth A fibrosarcoma, and the T cell lymphomas Ly 5178 Eb and ESb did not stimulate cytotoxic responses at these cell concentrations. The strong stimulatory potential of the Cspl preparation was reduced by treatment with anti-Thy-1 antibody plus complement, whereas the stimulatory activity of the AC preparation was resistant to this treatment. All cell types tested expressed class I major histocompatibility antigens. Nonactivated NWT cells, in contrast to the CNWT preparation, showed no detectable staining with anti-I-E or anti-I-A antibodies and also a slightly weaker staining with class I antisera. Experiments with the tumor cell lines revealed, however, that there was no strict correlation between stimulatory potential and density of class I alloantigens or the expression of I-E determinants. Experiments on primary cytotoxic responses *in vivo* gave similar results. Experiments in cultures with a single set of stimulator cells and I region-compatible responder cells indicated that AC and Cspl or CNWT also have a markedly stronger capacity than NWT to induce IL 2-dependent DNA synthesis.

It has been reported that proliferative or cytotoxic responses in mixed lymphocyte reactions require non-T, non-B Ia^+ accessory cells as stimulator cells. Dendritic cells (1–6) and macrophage-like cells (7, 8) have been identified as the most effective stimulator cells for these reactions. More detailed studies indicated, however, that the Ia^+ accessory cells are required as stimulator cells for the interleukin 2 (IL 2)-producing helper T cells (5, 9); it was suggested that in the presence of sufficient helper factor (IL 2) cytotoxic T lymphocyte (CTL)[1] precursor cells do not require a special type of stimulator cells (9), and that they may be activated even by metabolically inactivated cells (10–13) or by noncellular antigen (14–19). Unfortunately, these studies contained no quantitative comparison of graded numbers of stimulator cells. The work of Austyn *et al.* (20) indicated, on the other hand, that dendritic cells are the principal stimulator cells for IL 2 production as well as for the induction of IL 2 responsiveness.

To resolve this apparent discrepancy, we studied the capacity of a number of different cell types to activate CTL in the *presence of IL 2*. Our experiments revealed that plastic-adherent cells (AC) (enriched for dendritic cells) are indeed on a per cell basis 10- to 30-fold more effective than nylon wool-nonadherent spleen cells (NWT) and are more effective than unfractionated spleen cells (Spl) or several lines of tumor cells, including P815 mastocytoma cells, Meth A fibrosarcoma cells, and some lymphoma lines. Concanavalin A-activated T cells and some T cell lymphoma lines, on the other hand, were found to stimulate as effectively as the AC preparation. The experiments indicated that the presence of antigen on the surface of stimulator cells (in this case allogeneic H-2 determinants) is not sufficient for efficient stimulation of the CTL precursor cells. Some types of stimulator cells obviously provide an *additional activating signal*, possibly a soluble mediator that mediates relatively efficient stimulation of the CTL precursor cells or the stimulation of a yet unknown antigenically linked helper effect that is required for the cytotoxic response in addition to antigen and IL 2.

It is quite conceivable that the stimulator cells produce (or stimulate the production of) one of the helper factors that are required in addition to IL 2 for the activation of CTL (21–28). However, the reports on these additional factors (21–28) have not dealt with antigen-specific aspects of these helper effects, i.e., they did not indicate whether the factors are delivered by a cognate interaction that involves the antigen receptor of the CTL precursor cell. The experiments in this report demonstrate the antigen-specific aspect of the additional activating signal.

Previous studies provided essentially two explanations

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[1] Abbreviations used: AC, plastic-adherent spleen cells; CTL, cytotoxic T lymphocyte(s); CNWT, NWT cultured in the presence of Con A; Cspl, spleen cells cultured in the presence of Con A; NWT, T cell-enriched nylon wool-nonadherent spleen cells; PMA, phorbol myristic acetate; Spl, unfractionated spleen cells; FACS, fluorescence-activated cell sorter.

for the usual failure of tumor cells to stimulate cytotoxic responses *in vivo*. First, the cytotoxic responses may be inhibited by suppressor cells (29–33), and secondly, tumor cells may be inadequate stimulator cells for the IL 2-producing helper T cells (12). The experiments in this report indicate that tumor cells may be weak stimulator cells for cytotoxic responses even in the absence of detectable suppression and in the presence of IL 2-containing cell supernatants and/or an unrelated set of allogeneic stimulator cells.

MATERIALS AND METHODS

Animals. Mice of the strain C3H, DBA/2, BALB/c, and C57BL/6 were purchased from Bomholtgard, Ry, Denmark. The C3H.OH, A.TL, and A.SW mice were maintained at the German Cancer Research Center and were originally derived from the Jackson Laboratory, Bar Harbor, ME.

Tumor lines. The P815 mastocytoma line, of DBA/2 origin (34, 35) was kindly provided by Dr. G. Hämmerling, Heidelberg. Eb 737 is a cloned subline of the methylcholanthrene-induced DBA/2 T cell lymphoma Ly 5178 YEb (36); ESb 721 is a cloned subline of the ESb T cell lymphoma, which developed spontaneously as a metastasizing variant from the weakly metastasizing Eb tumor (36, 37). The tumor line ESbM was isolated by M. Fogel and V. Schirrmacher as a plastic-adherent spontaneous variant of the ESb tumor line, and showed only weak metastasizing potential (38). The ESbD line was prepared by mutagenization as a ouabain and thioguanin-resistant variant of the ESb line by Drs. J. W. Dennis, R. S. Kerbel, and V. Schirrmacher. All these tumor lines together with the methylcholanthrene-induced Meth A fibrosarcoma line (BALB/c) (39) and the Rauscher leukemia virus-induced T cell lymphoma line RBL/5 (C57BL/6) (40) were kindly provided by Dr. V. Schirrmacher, Heidelberg. ESbW was another ouabain and thioguanin-resistant variant of the ESb line and was also produced by mutagenization by A. Wehrmaker. The corresponding H-2 genotype of these tumors was confirmed with a panel of alloreactive CTL preparations (data not shown) and with monoclonal anti-H-2 antibodies (Tables VIII and IX).

Activation of cytotoxic responses in vitro. Twenty million responder cells (usually C3H spleen cells) were incubated in a total volume of 4.5 ml culture medium (RPMI 1640, GIBCO medium supplemented with 10 mM L-glutamine (GIBCO, Grand Island, NY) streptomycin/penicillin (GIBCO; 100 U/ml), 0.5% HEPES (GIBCO), 10% fetal calf serum (GIBCO), and 3×10^{-5} M 2-mercaptoethanol together with 0.4 ml of an IL 2 (about 100 U/ml)-containing EL-4 supernatant and various combinations of irradiated (1500 rad) or mitomycin C-treated allogeneic stimulator cells for 5 days at 37°C in 5% CO_2 if not indicated otherwise. The cultures usually received a variable set of stimulator cells (irradiated tumor cells or spleen cells at graded doses) plus a constant set of 5×10^5 irradiated allogeneic spleen cells from an unrelated H-2 haplotype. Responder and stimulator cells were Spl if not indicated otherwise. Unless indicated, the cultures were tested after 5 days for cytotoxic activity against concanavalin A-activated spleen cell blasts in a 4-hr ^{51}Cr-release assay as described (21, 41).

Activation of cytotoxic responses in vivo. Mice (usually 8- to 12-wk-old male C3H mice) were treated with 3 mg cyclophosphamide (Endoxan, Asta, Brackwede, Germany) in 0.15 ml saline i.p. 2 days before immunization if not indicated otherwise. The immunization was performed by distributing the indicated allogeneic cell preparations equally into the four footpads in a total volume of 0.2 ml. Five days after immunization, the mice were sacrificed, and the pooled inguinal and axillary lymph nodes were directly tested in a 4-hr ^{51}Cr-release assay for cytotoxic activity on concanavalin A-activated lymphoblasts as target cells if not indicated otherwise. The attacker to target cell ratios were 100:1, 30:1, 11:1, 3.6.1, and 1.2:1.

Irradiation of stimulator cells. The stimulator cells were irradiated with a dose of 1500 rad (unless indicated otherwise) either with a Gammatron (Siemens, Erlangen, Germany) designed for human therapy and equipped with a ^{60}Co source or with a Gammacell 1000 D (Atomic Energy of Canada Limited, Commercial Products) equipped with a 2300 Ci ^{137}Cs source (1700 rad/min).

Treatment of stimulator cells with mitomycin C. Splenic stimulator cells (max. 10^7 cells/ml) were incubated with 0.04 mg/ml of mitomycin C (Sigma Chemical Co., St. Louis, MO) for 45 min and were then washed three times. Tumor cells were treated with 0.08 mg/ml mitomycin C.

The preparation of IL 2-containing supernatants from EL-4 thymoma cells. Supernatants were obtained from an IL-2-producing EL-4 subline (kindly provided by Dr. J. Farrar) after induction with phorbol myristic acetate (PMA) as described (42). Briefly, 10^6 EL-4 cells/ml were incubated together with 10 ng/ml PMA (Sigma) for 48 hr. The supernatant was collected and stored frozen at −20°C.

Preparation of T cell-enriched nylon wool-nonadherent spleen cells. Lymphoid cells were incubated in nylon wool columns and eluted as described (43).

The preparation of "plastic-adherent cells" (AC). The term "plastic-adherent spleen cells" was used in this report for a fraction of spleen cells prepared by a procedure that has been reported to enrich especially for dendritic cells (6). Spleen cells from three mice (about 3×10^8 cells) were incubated in 10 ml culture medium in plastic petri dishes with a 10-cm diameter (Greiner, Nürtingen, W. Germany, type TC94/16) at 37°C. After 2 hr, the supernatant was discarded, the nonadherent cells were washed off carefully by extensive rinsing with warm culture medium or BSS, and the AC were again incubated with 10 ml of fresh culture medium for another 18 hr. The cells that detached spontaneously during this time period (usually 1 to 2×10^6 cells) were then collected in the supernatant and used as the "plastic-adherent spleen cell population" in these studies. According to reports from another laboratory (6), this population is a mixture of dendritic cells (80 to 95%) and macrophages.

Preincubation of stimulator cells. In some experiments, the stimulator cells were cultured at a density of 1.5 to 2×10^7 cells per 4.5 ml culture medium for 30 min or 24 hr with variable amounts (0 to 0.08 mg) of concanavalin A (Con A) (Pharmacia) or 45 ng PMA per culture.

Fluorescence staining and cytofluorographic analysis. A series of monoclonal antibodies (see Tables VIII and IX) were kindly provided by Dr. G. Hämmerling, DKFZ, Heidelberg. First, 5×10^5 cells in 0.05 ml were mixed with 0.05 ml of appropriately diluted antibody and then incubated for 30 min at 4°C. The cells were then mixed with 0.1 ml Dulbecco's phosphate saline (PBS; Seromed), centrifuged, and washed twice with 0.2 ml PBS. The resulting cell sediment was then resuspended in 0.05 ml fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin (Sigma F 7506; diluted 1/30) and incubated again for 30 min at 4°C. The cells were finally washed twice with PBS as described before.

The stained cells were analyzed on a cell sorter (NHP Spectrum 3, Ortho Instruments, Westwood, MA). Scatter gates were set to exclude non-viable cells. The positive cells were defined as having a fluorescence intensity above an arbitrary threshold, which was chosen to yield a maximum of positive cells with the relevant antibodies and at the same time a minimum (usually less than 12%) of positive cells with an irrelevant control antibody.

RESULTS

Different subpopulations of spleen cells differ markedly in their capacity to stimulate cytotoxic responses even in the presence of IL 2 and a second unrelated set of stimulator cells. Cytotoxic responses were studied in 4.5-ml cultures with 2×10^7 responder cells and 0.4 ml of an IL 2-containing EL-4 supernatant plus graded numbers of different allogeneic spleen cell subpopulations as stimulator cells. In addition, the cultures routinely contained a second and constant set of unrelated stimulator cells to provide an additional source for antigenically unlinked helper activity. The response against this second set of stimulator cells provided a convenient control for the availability of unlinked helper activity (i.e., IL 2) in the culture and has therefore also been included in the figures and tables. With this system, we found strong differences in the stimulatory potential of different spleen cell fractions. The T cell-enriched nylon wool-nonadherent fraction of spleen cells (NWT) stimulated substantial cytotoxic responses only in numbers of 2×10^5 cells or more, and practically no responses occurred when applied in numbers of 5×10^4 or less (Figs. 1–4). In contrast, a preparation of AC with a high proportion of dendritic cells (see *Materials and Methods*) stimulated substantial cytotoxic responses already at a concentration of 10^4 cells per culture (Figs. 1 and 2). The titration of these two types of stimulator cells revealed an at least 10-fold difference in the stimulatory potential (Fig. 1). The Spl preparations were usually slightly more effective

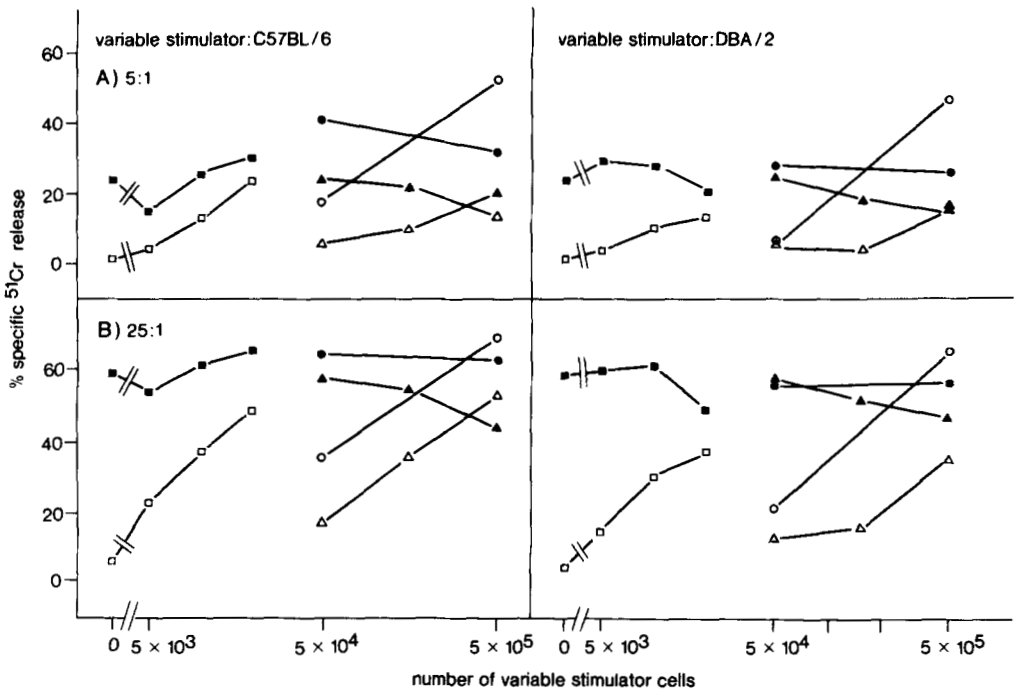

Figure 1. Titration of different stimulator cell preparations in the presence of IL 2 and a second set of allogeneic stimulator cells. C3H responder cells (H-2^k) (2×10^7) were incubated in 4.5-ml cultures with 0.4 ml EL-4 supernatant plus 5×10^5 A.SW stimulator cells (H-2^a) and a variable number of C57BL/6 stimulator cells (H-2^b) (left panel) or DBA/2 stimulator cells (H-2^d) (*right panel*). The stimulator cells had been irradiated with a dose of 1500 rad from the Gammatron. The A.SW cells were always Spl. The variable stimulator cells were either Spl (○——○), NWT (△——△), or AC (□——□). Open symbols, the cytotoxic responses against the variable stimulator cells. They were determined with BL/6 targets (*left panel*) or BALB/c targets (H-2^d) (*right panel*). Solid symbols, corresponding cytotoxic activities on A.SW targets. Data show ^{51}Cr release at attacker to target cell ratios of 5:1 (*top panel*) and 25:1 (*bottom panel*). Figure illustrates the dose-dependent cytotoxic response against the variable sets of stimulator cells and the practically constant response (*solid symbols*) against the constant set of stimulators.

than the NWT fractions (Fig. 1). The experiments revealed also that the type and concentration of the variable stimulator cell had practically no influence on the response against the second (constant) set of stimulator cells (Fig. 1).

The weak stimulatory potential of the NWT fraction is not the result of a suppressive activity. The possibility that the weak stimulatory potential of the NWT might have resulted from active suppression was tested in experiments in which NWT were mixed with the strongly stimulatory preparation of AC (Fig. 2, Table I). These experiments revealed a slight degree of suppression at times, which was nevertheless not sufficient to explain the complete lack of stimulatory activity of the NWT numbers of 5×10^4 or less (Fig. 1).

The NWT fraction can express strong stimulatory activity after incubation with Con A. When NWT were incubated at cell densities of 1.5 to 2×10^7 cells in 4.5 ml together with 0.01 mg Con A per culture for 24 hr, they expressed strong stimulatory activity (Fig. 3). This activity was about as good as the stimulatory potential of the AC population (see Fig. 1). The results from Figures 1 and 3 were obtained in a single experiment and are therefore directly comparable. The experiment in Figure 3 shows also that the incubation of the NWT in the absence of Con A or in the presence of 10 ng/ml of PMA was not sufficient to enhance the stimulatory potential.

Additional experiments showed that a 30-min incubation of NWT with Con A (CNWT) was not sufficient to induce the stimulatory potential (Fig. 4). This result suggested that the expression of stimulatory potential resulted from true activation of the stimulator cells. Incubation of Spl with Con A also enhanced the stimulatory activity of this population (data not shown).

Thymic responder cells in comparison with splenic responder cells showed even greater differences in their cytotoxic responses against Con A-activated (CNWT) *vs* nonactivated NWT stimulator cells (data not shown). This result suggested that the splenic responder cells contain, in contrast to the thymic responder cells, a small subset of CTL precursor cells (possibly the Lyt-1$^-$2$^+$ population), which does not require the additional activating signal from the Con A-activated stimulator cell.

Failure to demonstrate a synergistic effect between different stimulator cell preparations. The experiments described above had shown that a relatively strong stimulatory activity was provided by two apparently different stimulator cell preparations, namely CNWT and the AC fraction of spleen cells (Figs. 1 and 3). This finding raised the question whether the optimal activation of CTL requires the combined action of two functionally different types of stimulator cells, namely activated T cell blasts and dendritic cells or macrophages. The two stimulator cell preparations might have contained predominantly one of these two cell types plus contamination of the other type of cell. One would have expected in this case a strong synergistic effect of CNWT stimulator cells and AC stimulator cell preparations. Such a synergistic effect was not observed (Table II).

Anti-Thy-1 serum plus complement (C) reduced the stimulatory activity of Con A-activated spleen cells (Cspl) but not that of the AC preparation. Another interpretation of the strong stimulatory activity of the two different stimulator cell preparations was that the AC preparation contained a substantial number of T cell blasts. Experiments showed, however, that the stimula-

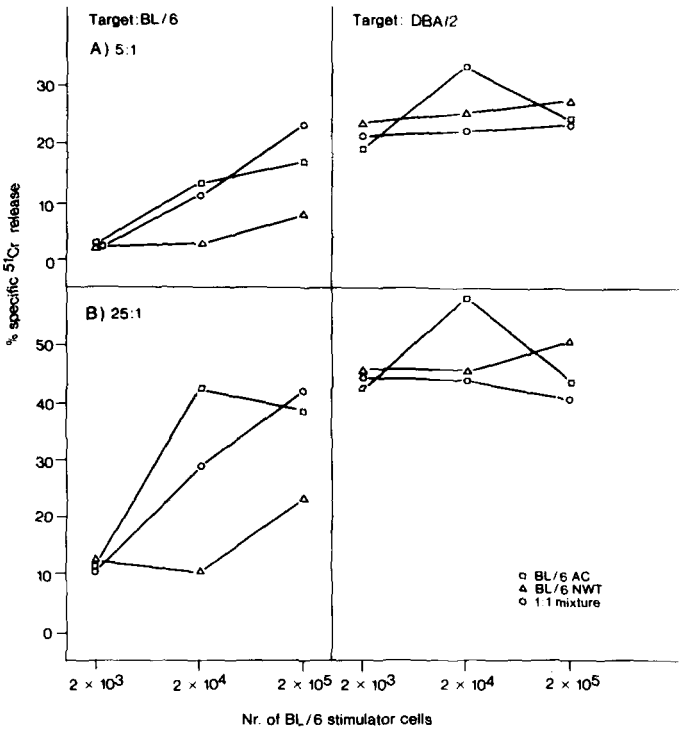

Figure 2. The cytotoxic response against a combination of AC and NWT. C3H responder cells (2×10^7) were incubated with 0.4 ml EL-4 supernatant plus 5×10^5 irradiated DBA/2 spleen cells and variable numbers of irradiated BL/6 AC (□——□) or BL/6 NWT (△——△). A third group of cultures received 1:1 mixtures of these two cell types (each cell type at the indicated cell number per culture) (○——○). Data show the cytotoxic activity against the indicated target cells at the attacker to target cell ratios 5:1 (*top panel*) and 25:1 (*bottom panel*). For other details, see legend to Figure 1.

TABLE I

Cytotoxic responses against combinations of AC and NWT[a]

Constant Stimulator Cells	Variable Stimulator Cells	Targets					
		BL/6		BALB/c		A.SW	
		25:1	5:1	25:1	5:1	25:1	5:1
BL/6	None	50	26	15	09	n.t.[b]	
BL/6	1×10^4 DBA/2-AC	43	22	40	21	n.t.	
BL/6	5×10^3 DBA/2-AC	45	23	24	09	n.t.	
BL/6	2×10^4 DBA/2-NWT	50	27	11	06	n.t.	
BL/6	2×10^4 DBA/2-NWT + 1×10^4 DBA/2-AC	47	29	35	17	n.t.	
BL/6	2×10^4 DBA/2-NWT + 5×10^3 DBA/2-AC	48	26	34	13	n.t.	
A.SW	None	10	04	12	05	44	18
A.SW	2×10^4 BL/6-AC	48	25	21	12	43	15
A.SW	2×10^4 DBA/2-AC	25	09	51	31	41	15
A.SW	1×10^5 BL/6-NWT	23	06	10	05	39	11
A.SW	2×10^4 BL/6-NWT	09	05	08	06	39	13
A.SW	1×10^5 BL/6-NWT + 2×10^4 DBA/2-AC	27	12	31	18	42	15
A.SW	2×10^4 BL/6-NWT + 2×10^4 DBA/2-AC	15	09	39	18	38	10
A.SW	1×10^5 DBA/2-NWT	03	00	18	12	44	11
A.SW	2×10^4 DBA/2-NWT	09	04	09	06	49	14
A.SW	1×10^5 DBA/2-NWT + 2×10^4 BL/6-AC	45	22	17	08	39	11
A.SW	2×10^4 DBA/2-NWT + 2×10^4 BL/6-AC	41	24	14	06	43	17

[a] C3H spleen cells (2×10^7) were incubated as responder cells together with 0.4 ml of an IL 2-containing EL-4 supernatant and 5×10^5 irradiated spleen cells of the constant stimulator type plus the indicated numbers of variable stimulator cells in 4.5-ml macrocultures. All stimulator cells were irradiated with a dose of 1500 rad from the Gammacell 1000 D. Variable stimulator cells were either NWT or AC. Data show percent specific ^{51}Cr release by the indicated target cells at attacker to target cell ratios of 25:1 and 5:1.

[b] Not tested.

tory activity of the AC preparation was not reduced by treatment with anti-Thy-1 serum plus C, whereas the stimulatory activity of Cspl was markedly reduced by this treatment (Table III).

Induction of IL 2 responsiveness and cytotoxic activity in cultures with a single set of I region-compatible stimulator cells. The experiments with two unrelated sets of stimulator cells excluded the possibility that the AC or Con A-activated stimulator cells were required for the activation of an antigenically unlinked helper effect, i.e., for IL 2 production. But these experiments did not allow us to distinguish whether the AC and CNWT or Cspl were required for a maturation signal or for the induction of IL 2 responsiveness, which is believed to be the first event during the induction of the cytotoxic response (44, 45). We therefore tested the capacity of different spleen cell preparations to induce IL 2-dependent DNA synthesis and CTL activity in 0.2-ml microcultures with I region-compatible responder cell populations. Because of the disparity in the H-2 D region between responder and stimulator cells and the absence of third party stimulator cells, we expected that this antigenic stimulus would address mainly the CTL precursor cells rather than the I region-reactive helper cells. The result (Table IV) showed that 3×10^3 AC and Cspl or CNWT induced markedly stronger IL 2-dependent DNA synthesis, and in addition, often stronger cytotoxic activity than 3×10^4 NWT stimulator cells. Moreover, the cytotoxic and proliferative response against NWT was not further enhanced by adding three times more IL 2 (data not shown); small doses of AC and Cspl or CNWT stimulator cells induced cytotoxic and proliferative responses in the presence but not in the absence of external IL 2 (Table IV). This result indicated that the relatively strong stimulatory potential of these cells at relatively small numbers was again not explained by stimulation of internal IL 2 production but rather by a more effective induction of IL 2 responsiveness.

The stimulatory potential of different spleen cell preparations in vivo. Studies of primary cytotoxic responses against allogeneic cells *in vivo* revealed essentially the same differences between the stimulatory activities of the various types of spleen cell preparations (Table V). The mice were immunized with combinations of two unrelated allogeneic spleen cell preparations, which were injected into the four footpads. The simultaneous response against the constant set of stimulator cells was used again as an indicator for antigenically linked helper effects. The mice were also treated with cyclophosphamide 2 days before immunization in most of our experiments to abrogate the effects of a cyclophosphamide-sensitive suppressor cell system (46–48). The strongest stimulatory activity was again expressed by the AC preparation and the CNWT preparation, whereas Spl or NWT stimulated little or no cytotoxic activity (Table V). Mice that have not been treated with cyclophosphamide generated usually only weak cytotoxic activity, but the CNWT preparation stimulated clearly stronger cytotoxic responses than NWT cells or unfractionated spleen cells (Table V, Expt. II). (C57BL/6 mice were used in this case because this strain was generally found to produce stronger responses than C3H mice without cyclophosphamide.)

The CNWT preparation mediated in these *in vivo* ex-

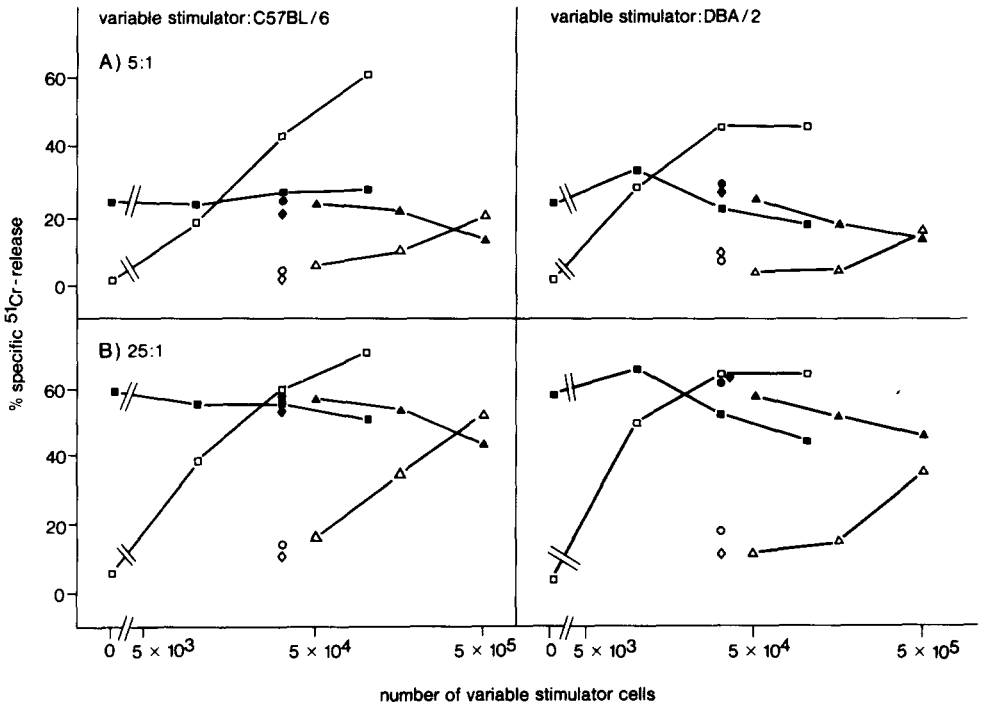

Figure 3. Titration of NWT stimulator cells before or after stimulation with Con A. C3H responder cells (2×10^7) were incubated with 0.4 ml EL-4 supernatant plus 5×10^5 irradiated A.SW spleen cells and a variable number of C57BL/6 stimulator cells (*left panel*) or DBA/2 stimulator cells (*right panel*). The variable stimulator cells were NWT, which were either freshly prepared (Δ——Δ) or cultured overnight at a density of 2×10^7 cells in 4.5 ml medium containing 0.01 mg Con A (□——□) or 45 ng PMA (◇) or neither Con A nor PMA (○). For other details, see legend to Figure 1.

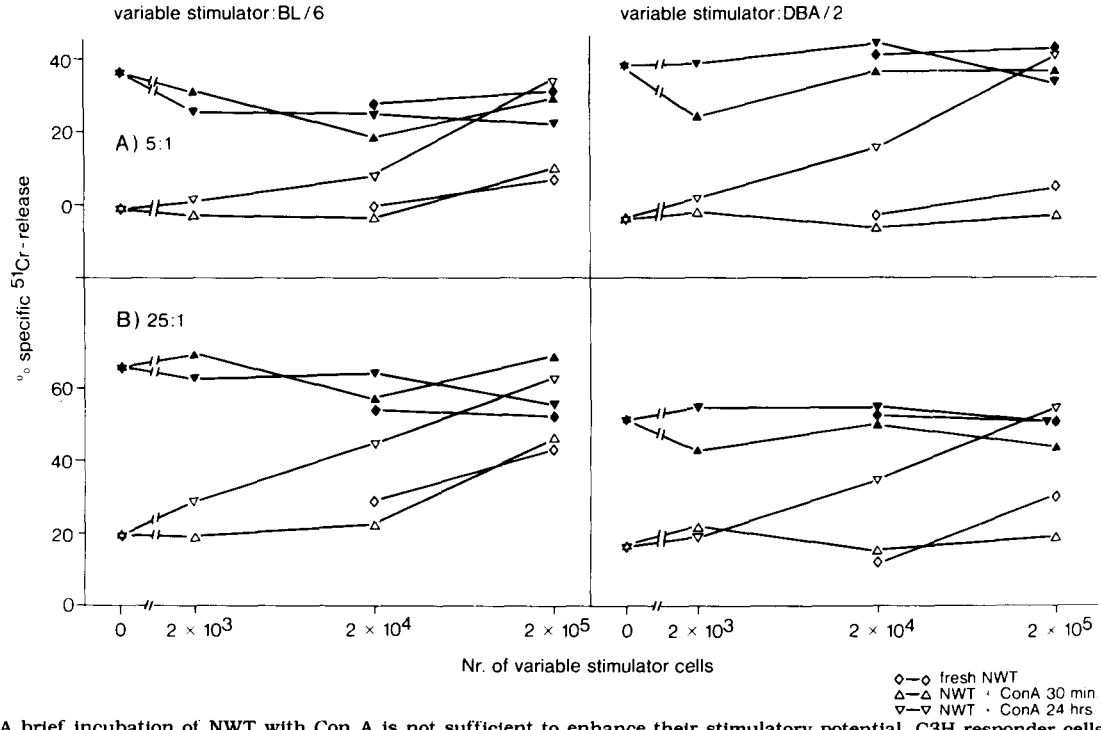

Figure 4. A brief incubation of NWT with Con A is not sufficient to enhance their stimulatory potential. C3H responder cells (2×10^7) were incubated with 0.4 ml EL-4 supernatant plus 5×10^5 irradiated DBA/2 spleen cells (*left panel*) or C57BL/6 spleen cells (*right panel*) and a variable number of BL/6 stimulator cells (*left panel*) or DBA/2 stimulator cells (*right panel*), respectively. The variable stimulator cells were NWT that were either freshly prepared (◇——◇) or were cultured at a density of 1.5×10^7 cells in 4.5 ml with 0.01 mg Con A for 30 min (Δ——Δ) or 24 hr (▽——▽). For other details, see legend to Figure 1.

periments an augmentation of the simultaneous response against the second set of stimulator cells. The CNWT preparations were nevertheless not able to reconstitute the weak stimulatory potential of an unrelated allogeneic NWT preparation (Table V, groups 14 to 18). The activating signal (possibly a helper factor) from the CNWT population was again preferentially delivered to those CTL precursor cells that expressed antigen receptors for antigenic determinants on these CNWT.

Tumor cells as stimulator cells for cytotoxic responses in vitro and in vivo. Tumor cell lines provide, in contrast to spleen cell fractions, relatively homogeneous stimulator cell populations. The comparison of the stimulatory activity of several tumor lines confirmed the basic conclusion from our previous experiments that different cell types differ markedly in their capacity to activate CTL precursor cells even in the presence of IL 2-containing EL-4 supernatant and/or a second set of stimulator

TABLE II

Cytotoxic responses against combinations of small numbers of AC and CspI or CNWT[a]

DBA/2 Stimulator Cells				Targets			
				BALB/c		A.SW	
Group	AC	CspI	CNWT	25:1	5:1	25:1	5:1
1	0	0	0	11.5	5.2	29.5	11.8
2	1×10^4	0	0	32.7	16.4	30.4	14.4
3	5×10^3	0	0	22.6	9.5	34.7	18.3
4	0	1×10^4	0	23.2	13.6	28.8	16.8
5	0	5×10^3	0	16.5	8.6	31.5	13.4
6	0	1×10^3	0	17.2	7.3	35.8	14.0
7	1×10^4	1×10^4	0	36.9	15.3	34.8	15.6
8	1×10^4	5×10^3	0	33.2	12.0	38.0	17.7
9	1×10^4	1×10^3	0	32.6	14.8	32.8	15.7
10	5×10^3	5×10^3	0	28.2	8.8	32.7	13.9
11	5×10^3	1×10^3	0	23.7	9.8	38.0	19.2
12	0	0	1×10^4	18.9	8.1	28.7	16.6
13	0	0	5×10^3	21.8	11.2	38.7	19.3
14	1×10^4	0	1×10^4	37.2	16.2	32.3	14.1
15	1×10^4	0	5×10^3	33.3	12.2	33.3	13.6
16	5×10^3	0	5×10^3	30.9	10.7	39.1	19.5

[a] C3H spleen cells (2×10^7) were incubated in 4.5-ml cultures with 5×10^5 mitomycin C-treated A.SW cells and 0.4 ml EL-4 supernatant plus the indicated numbers of mitomycin C-treated DBA/2 stimulator cells. The DBA/2 stimulator cells were either AC, CspI, or CNWT. Data show the percent specific ^{51}Cr release by the indicated target cells at attacker to target cell ratios of 25:1 and 5:1.

TABLE III

Effect of anti-Thy-1 antibody plus C on the stimulatory potential of AC and CspI[a]

Variable Stimulator Population	Targets			
	C57BL/6		A.SW	
	25:1	5:1	25:1	5:1
Expt. I				
None	5.4	0.7	19.6	7.9
2×10^4 BL/6-CspI/a-Thy-1 plus C	12.6	3.9	19.5	6.2
2×10^4 BL/6-CspI/a-Thy-1 only, no C	29.0	14.1	23.5	6.0
2×10^4 BL/6-CspI/no a-Thy-1, C only	26.5	7.8	18.8	4.6
2×10^4 BL/6-CspI/no a-Thy-1, no C	27.3	11.4	24.7	7.4
	C57BL/6		BALB/c	
Expt. II				
None	14.3	5.2	61.3	30.0
3×10^4 BL/6-AC/a-Thy-1 plus C	56.8	34.6	64.7	36.6
1×10^4 BL/6-AC/a-Thy-1 plus C	38.6	21.2	64.7	37.3
3×10^4 BL/6-AC/a-Thy-1 only, no C	53.2	27.9	64.7	37.3
1×10^4 BL/6-AC/a-Thy-1 only, no C	36.0	15.3	65.8	37.0
3×10^4 BL/6-AC/no a-Thy-1, C only	39.5	14.9	62.9	36.3
1×10^4 BL/6-AC/no a-Thy-1, C only	20.9	8.2	59.6	34.5

[a] C3H responder cells (2×10^7) were incubated with 0.4 ml EL-4 supernatant and 5×10^5 irradiated A.SW (Expt. I) or DBA/2 (Expt. II) spleen cells plus the indicated numbers of Con A-activated BL/6 spleen cells or BL/6 "adherent cells". The BL/6 stimulator cells had been treated in two cycles with anti-Thy-1 antibody (30 min, 4°C) and C (45 min, 37°C). In the control groups, the cells were incubated in culture medium only. The anti-Thy-1 antibody was a monoclonal antibody kindly provided by Dr. G. Hämmerling. Low toxicity M rabbit complement was obtained from Cedarlane, Hornby, Ontario, The efficiency of this treatment was routinely tested on thymocytes and nude spleen cells.

cells.

Irradiated tumor cells of the line ESb mediated a general antigen-nonspecific suppression of the cytotoxic responses against both H-2 haplotypes tested if added in doses of 10^4 cells to the cultures with C3H responder cells and IL 2-containing EL-4 supernatant plus a second set of allogeneic stimulator cells (Table VI). The variant ESbM, in contrast, did not suppress but stimulated at the

TABLE IV

Capacity of different spleen cell preparations to induce IL 2-dependent DNA synthesis and CTL activity in I region-compatible responder cell populations[a]

Stimulator Cells		% ^{51}Cr Release		^3H Incorporation (cpm $\times 10^{-3}$)	
		−EL-4 sup.	+EL-4 sup.	−EL-4 sup.	+EL-4 sup.
Expt. I: responder:C3H.OH ($H\text{-}2K^dI^dD^k$); stimulator: DBA/2 and BALB/c ($H\text{-}2^d$); target:BALB/c.					
None		4.1	3.9	1.4	15.2
1×10^5	DBA/2-NWT	4.9	21.2	2.5	46.5
3×10^4	DBA/2-NWT	4.4	29.7	6.8	32.2
1×10^4	DBA/2-NWT	1.4	16.2	1.5	25.0
1×10^4	DBA/2-AC	8.8	27.4	54.7	140.9
3×10^3	DBA/2-AC	19.3	29.4	35.6	69.9
1×10^3	DBA/2-AC	4.2	13.8	13.0	35.4
1×10^4	BALB/c-CspI	16.3	34.4	27.5	63.9
3×10^3	BALB/c-CspI	17.4	33.1	23.2	46.8
1×10^3	BALB/c-CspI	6.7	26.4	3.1	33.9
3×10^2	BALB/c-CspI	7.2	12.8	1.7	21.7
Expt. II: responder:C3H ($H\text{-}2^k$); stimulator and target: A.TL ($H\text{-}2K^kI^kD^d$)					
None		−2.5	−3.6	3.5	13.9
1×10^5	A.TL-NWT	−1.0	18.7	0.7	47.9
3×10^4	A.TL-NWT	−1.2	18.4	0.9	34.2
1×10^4	A.TL-NWT	−2.2	11.8	1.7	26.5
3×10^3	A.TL-AC	−0.2	14.2	10.9	68.5
1×10^3	A.TL-AC	−1.8	11.9	1.6	24.6
3×10^2	A.TL-AC	−2.4	0.9	1.7	15.6
3×10^3	A.TL-CNWT	−2.1	31.9	16.2	114.4
1×10^3	A.TL-CNWT	−1.7	21.6	6.4	63.0
3×10^2	A.TL-CNWT	−2.0	5.0	2.5	15.0

[a] NWT (5×10^4) were incubated as responder cells with the indicated types of irradiated (1500 rad) stimulator cells in 0.2-ml microcultures with or without 0.01 ml EL-4 supernatant. One set of cultures received 1 μCi [^3H]thymidine 3 days later and was harvested with a Skatron cell harvester after another 8 hr. A parallel set of cultures was incubated for 5 days and tested for cytotoxic activity against the indicated target cells. Similar results were obtained with 0.03 ml EL-4 supernatant per culture.

concentration of 10^4 cells per culture a substantial cytotoxic response against the homologous $H\text{-}2^d$ target; tumor cells of the lines P815, Meth A, and Eb did not suppress or stimulate at these cell concentrations (Table VI). Similar results were obtained with EL-4 and RBL/5 tumor cells: at 10^4 cells per culture they did not stimulate a cytotoxic response against the homologous $H\text{-}2^b$ targets and did not affect the simultaneous response against A.SW stimulator cells in the same culture (data not shown). The ESb mutant lines ESbW and ESbD, on the other hand, had a stimulatory activity similar to the ESbM line (Table VI, Expt. II). Cells of the tumor lines P815 and Meth A were also tested in mixtures with H-2 identical DBA/2 adherent cells as stimulator cells to exclude the possibility of an antigen-specific suppression that would not have been detected in the experiment of Table VI. Again, we observed some antigen-nonspecific suppression at high tumor cell concentration (10^5 cells per culture), but no suppression at lower tumor cell concentrations (data not shown).

Similar differences between the stimulatory activities of the tumor cell lines were observed in primary cytotoxic responses *in vivo* (Table VII). The tumor cells were injected into the footpads of cyclophosphamide-treated mice together with a constant number of allogeneic spleen cells of another H-2 haplotype. Again, only the irradiated (1500 rad) ESbM cells stimulated strong cytotoxic responses, whereas several other tumor lines activated only minute responses against their own H-2 antigens (Table VII). Most tumor cells including the irradiated (1500 rad) ESb cells at higher doses mediated suppression of the response against the second set of stimulator cells if tested in C3H mice. Immunization with combina-

TABLE V

Stimulation of cytotoxic responses in vivo by different types of spleen cell preparations[a]

Group	Injected Cell Preparations	% Specific ^{51}Cr Release with Targets				
		BL/6 (H-2^b)			BALB/c (H-2^d)	
Expt. I:	Cyclophosphamide-treated C3H mice	80:1	27:1	9:1	80:1	27:1
1	3×10^7 BL/6-spl + 3×10^7 BALB/c-spl	14	9	5	9	2
2	6×10^6 3×10^7	2	n.t.[b]	n.t.	n.t.	n.t.
3	1.2×10^6 3×10^7	4	−1	−1	15	6
4	3×10^7 BL/6-NWT + 3×10^7 BALB/c-spl	5	0	0	9	3
5	6×10^6 3×10^7	8	1	2	16	4
6	3×10^7 BL/6-CNWT + 3×10^7 BALB/c-spl	37	26	13	16	7
7	6×10^6 3×10^7	21	9	5	16	6
8	1.2×10^6 3×10^7	10	6	3	24	11
9	2.4×10^5 3×10^7	2	0	2	16	7
10	6×10^6 BL/6-AC + 3×10^7 BALB/c-spl	21	10	5	10	3
11	1.2×10^6 3×10^7	5	0	2	9	3
12	2.4×10^5 3×10^7	3	1	2	18	12
13	— 3×10^7 BALB/c-spl	1	−1	0	14	6
14	6×10^6 BL/6-spl + 6×10^6 BALB/c-CNWT	9	−3	−2	9	2
25	6×10^6 BL/6-NWT 6×10^6	0	−2	−3	10	2
16	6×10^6 BL/6-CNWT 6×10^6	24	7	1	13	4
17	6×10^6 BL/6-AC 6×10^6	26	9	2	13	6
18	— 6×10^6	3	−1	0	5	0
		C3H (H-2^k)			BALB/c (H-2^d)	
Expt. II:	BL/6 mice without cyclophosphamide	100:1	33:1	11:1	100:1	33:1
19	3×10^7 C3H-spl + 3×10^7 Balb/c-spl	4	2	2	3	1
20	6×10^6 3×10^7	2	1	2	0	1
21	1.2×10^6 3×10^7	2	1	1	7	3
22	3×10^7 C3H-NWT 3×10^7	3	1	1	4	3
23	6×10^6 3×10^7	0	0	0	3	1
24	1.2×10^6 3×10^7	1	0	0	3	1
25	3×10^7 C3H-CNWT 3×10^7	11	5	2	4	2
26	6×10^6 3×10^7	4	2	1	5	2
27	1.2 t 10^6 3×10^7	2	1	1	4	1
28	6×10^6 C3H-AC 3×10^7	6	3	1	4	2
29	1.2×10^6 3×10^7	1	0	1	1	0
30	30 — 3×10^7	0	0	0	4	0

[a] Ten-week-old male C3H (H-2^k) mice (Expt. I) and 8-wk-old male C57BL/6 mice (Expt. II) were immunized with the indicated cell preparations; the cytotoxic activity of the pooled axillary and inguinal lymph node cells (two mice per group) was tested 5 days later with a 4-hr ^{51}Cr-release assay with Con A-activated lymphoblasts of the indicated strains used as target cells. Data show the % specific ^{51}Cr release. The C3H mice (Expt. I) were treated with 3 mg of cyclophosphamide i.p. 2 days before immunization. The indicated cell preparations and cell numbers were administered in 0.2 ml BSS and distributed in equal aliquots into the four footpads. The cells had been irradiated with 1500 rad.

[b] Not tested.

TABLE VI

Cytotoxic responses against tumor cells in vitro in the presence of an IL 2-containing EL-4 supernatant[a]

Tumor Cells (Reported Origin)	Targets			
	H-2^d		H-2^b	
	25:1	5:1	25:1	5:1
Expt. I				
None	0	0	24	8
P815 mastocytoma (DBA/2)	6	2	30	19
Meth A fibrosarcoma (BALB/c)	4	0	34	16
Eb T lymphoma (DBA/2)	3	−1	38	17
ESb T lymphoma (DBA/2)	1	2	0	−5
ESbM T lymphoma (DBA/2)	39	26	20	8
Expt. II				
None	5	3	45	27
Eb T lymphoma (DBA/2)	9	4	48	39
ESbM T lymphoma (DBA/2)	19	8	48	25
ESbW T lymphoma (DBA/2)	14	4	47	28
ESbD T lymphoma (DBA/2)	17	4	36	20

[a] C3H spleen cells (2×10^7) were incubated as responder cells in 4.5-ml cultures with 0.4 ml EL-4 supernatants and 5×10^5 irradiated (1500 rad) C57BL/6 spleen cells (H-2^b) plus 1×10^4 irradiated (1500 rad) tumor cells (Expt. I) or 3×10^4 mitomycin C-treated tumor cells (Expt. II) of the indicated lines (all H-2^d). Data show the % specific ^{51}Cr release.

tions of tumor cells and Con A-activated spleen cells of an unrelated haplotype gave similar results (data not shown).

The expression of major histocompatibility class I and class II antigen on the various types of stimulator cells. Two independent strategies were used to ensure that the various types of stimulator cells expressed the expected H-2 antigens, namely i) the analysis of antigen density with monoclonal antibodies in combination with a fluorescence-activated cell sorter (FACS); and ii) the sensitivity against a panel of CTL with known specificities.

The FACS analysis of several pairs of NWT and CNWT preparations from different strains of mice (Fig. 5, Table VIII) revealed that the NWT did contain substantial amounts of class I antigens (L^d and D^d or K^b and D^b), although the antigen density was slightly lower than on the CNWT preparations. Moreover, the CNWT preparations in contrast to the NWT preparation contained detectable amounts of class II antigens (I-E^d or I-A^b). The AC preparations expressed similar densities of class I antigens and relatively high densities of class II antigens (data not shown).

The FACS analysis of several lines of tumor cells revealed that all tumor cells expressed class I alloantigens (Fig. 6, Table IX). The T lymphoma lines ESbM, ESbD, and RBL/5 also expressed detectable amounts of class II alloantigens. The strongly stimulatory line ESbW expressed no detectable amounts of class II antigens and normal levels of class I antigens, similar to the antigen levels of the weakly stimulatory lines P815, Meth A, and Eb. Additional experiments confirmed that the tumor lines with weak stimulatory activity (i.e., Meth A, P815, RBL/5, Eb, and ESb) were effectively lysed by allospecific CTL of the correct specificity H-2^d and H-2^b, respectively, if labeled with ^{51}Cr and used as target (data not shown).

TABLE VII

Stimulation of cytotoxic responses in vivo by tumor cells in mixture with allogeneic spleen cells[a]

Group	Injected Tumor Cells	Injected Allogeneic Cells	Targets					
			100:1	33:1	11:1	100:1	33:1	11:1
1	*RBL/5 (H-2^b)*	*BALB/c (H-2^d)*	*BL/6*			*BALB/c*		
	3×10^7	3×10^7	−3	−4	−4	14	10	3
	3×10^6	3×10^7	−6	−6	−5	14	6	0
	3×10^5	3×10^7	−6	−6	−5	22	12	3
2	*P815 (H-2^d)*	*C57BL/6 (H-2^b)*	*BALB/c*			*BL/6*		
	5×10^7	3×10^7	7	6	1	1	1	−1
	5×10^6	3×10^7	4	2	−4	11	1	−2
	5×10^5	3×10^7	1	5	−1	30	19	6
3	*Meth A (H-2^d)*	*C57BL/6*	*BALB/c*			*BL/6*		
	3×10^7	3×10^7	9	0	1	19	14	9
	3×10^6	3×10^7	8	5	−2	24	20	11
	3×10^5	3×10^7	2	4	−5	20	13	9
4	*Eb (H-2^d)*	*C57BL/6*	*BALB/c*			*BL/6*		
	5×10^7	3×10^7	8	5	2	−1	1	−4
	5×10^6	3×10^7	3	3	1	24	15	3
	5×10^5	3×10^7	3	2	2	30	21	8
5	*ESb (H-2^d)*	*C57BL/6*	*BALB/c*			*BL/6*		
	1×10^7	3×10^7	6	6	0	11	13	2
	1×10^6	3×10^7	3	2	−2	17	20	8
	1×10^5	3×10^7	2	1	0	34	26	12
6	*ESbM (H-2^d)*	*C57BL/6*	*BALB/c*			*BL/6*		
	3×10^7	3×10^7	33	24	9	9	13	4
	3×10^6	3×10^7	14	13	7	22	20	14
	3×10^5	3×10^7	0	1	1	25	19	10
7	*P815 (H-2^d)*	*CBA/J (H-2^k)*	*BALB/c*			*L 929 (H-2^k)*		
	3×10^7	3×10^7	5	2	0	22	18	5
	3×10^6	3×10^7	−1	0	0	25	17	8
	3×10^5	3×10^7	−6	−4	−3	19	11	5
8	*ESbM (H-2^d)*	*CBA/J*	*BALB/c*			*L (29 (H-2^k)*		
	3×10^7	3×10^7	36	24	15	32	21	7
	3×10^6	3×10^7	12	12	5	30	17	11
	3×10^5	3×10^7	11	7	0	31	21	10

[a] Eight- to 12-wk-old male C3H (H-2^k) mice (groups 1–6) or C57BL/6 (H-2^b) mice (groups 7 and 8) received injections of 3 mg of cyclophosphamide i.p. and 2 days later a mixture of the indicated numbers of 1500 rad-irradiated tumor cells and allogeneic spleen cells equally distributed into the four footpads. The cytotoxic activity of the pooled axillary and inguinal lymph node cells was tested 5 days later with a 4-hr ^{51}Cr-release assay by using either Con A-activated lymphoblasts of the indicated strains or L 929 tumor cells (H-2^k) as target cells. Data show the % specific ^{51}Cr release ± SE. Some tumors (i.e., P815, Meth A, Eb, RBL/5, and the El-4 standard line were also injected into C3H mice that had not been treated with cyclophosphamide and were found to stimulate no detectable cytotoxicity. The ESbM tumor cells, on the other hand, were also found to stimulate substantial cytotoxicity in C3H mice without cyclophosphamide (data not shown).

DISCUSSION

Our experiments confirmed and extended our previous observation (49) that the activation of CTL precursor cells requires more than interaction with the physical structure of the antigen plus an antigenically unlinked delivery of IL 2 and other helper factors. The experiments demonstrated that different spleen cell fractions and tumor cell lines differ markedly (on a per cell basis) in their capacity to stimulate cytotoxic responses *in vitro* in the presence of an IL 2-containing cell supernatant and a second set of stimulator cells. (This implied that the various types of stimulator cells differed in their capacity to activate the CTL precursor cell or an unknown type of helper cell that interacts with the CTL precursor through an *antigenically linked* helper effect.) Strong stimulatory activity was expressed by Con A-activated T cell preparations (CNWT), by AC preparations, and by some variant lines of the ESb T lymphoma. These cell types stimulated substantial cytotoxic activity in doses of 10^4 cells per culture or less. Cells of the regular ESb T lymphoma line were found to mediate nonspecific suppression. Several other tumor cell lines and unfractionated spleen cells or the NWT fraction (mostly resting small T lymphocytes) stimulated substantial cytotoxic responses

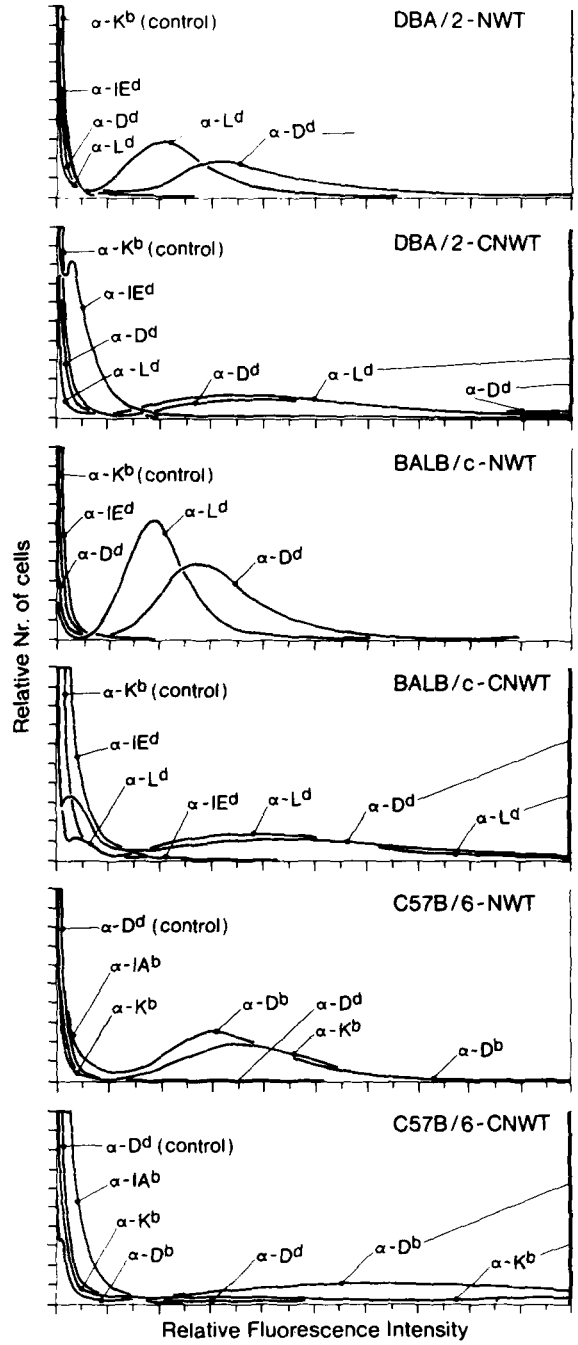

Figure 5. FACS analysis of the expression of class I and class II major histocompatibility antigens on NWT and CNWT cell preparations. The descriptions of the monoclonal antibodies and the proportion of fluorescent cells are shown in Table VIII.

only at higher cell doses. These cell preparations did not actively suppress the cytotoxic response at doses of 10^4 cells per culture.

Complementary *in vivo* experiments confirmed in essence the *in vitro* observations and underlined their significance. The immunization of mice with mixtures of two unrelated types of allogeneic cells revealed practically the same pattern of stimulatory activity: only the CNWT, the AC, and some variants of the ESb T lymphoma stimulated substantial cytotoxic responses if injected in numbers of 6×10^6 cells per mouse or less.

Two independent methods, i.e., fluorescence staining with a monoclonal antibody in combination with cyto-

TABLE VIII

Proportion (%) of fluorescent cells in preparations of NWT and CNWT as defined by a constant arbitrary threshold[a]

Strain	Antibody	NWT	CNWT
DBA/2	Anti-I-E^d (13-4)	8.9	45.2
	Anti-D^d (T19-191)	61.9	65.4
	Anti-L^d (28-14-8)	59.4	63.0
	Anti-K^b (K10-56)	7.7	9.8
BALB/c	Anti-I-E^d (13-4)	5.4	33.6
	Anti-D^d (T19-191)	90.9	89.3
	Anti-L^d (28-14-8)	90.9	72.5
	Anti-K^b (K10-56)	3.4	12.9
C57BL/6	Anti-I-A^b (17-227)	7.9	36.8
	Anti-K^b (K10-56)	65.6	79.3
	Anti-D^b (B22-249)	77.6	89.7
	Anti-D^d (43-2-12)	5.0	17.4

[a] Quantitative data for the analysis in Figure 5.

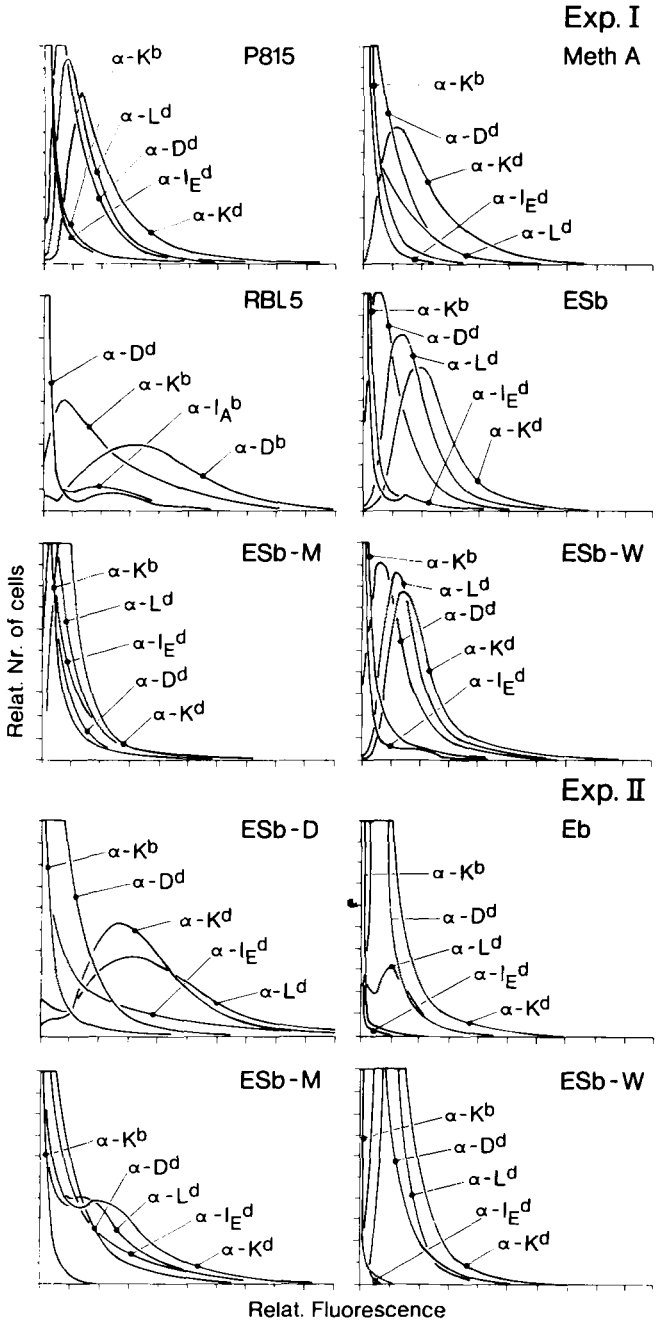

Figure 6. FACS analysis of the expression of class I and class II major histocompatibility antigens on various lines of tumor cells. The description of the monoclonal antibodies and the proportion of fluorescent cells are shown in Table IX.

TABLE IX

Proportion (%) of fluorescent cells in different tumor cell preparations as defined by a constant arbitrary threshold[a]

Tumor Line	Antibody Used						
	Anti-I-E^d (13-4)	Anti-K^d (31-3-4)	Anti-D^d (34-2-12)	Anti-L^d (28-14-8)	Anti-I-A^b (25-5-16)	Anti-K^b (K10-56)	Anti-D^b (B22-249)
Expt. I							
P815	7.8	85.5	46.8	61.7		8.1	
Meth A	3.0	77.3	37.1	33.6		8.2	
RBL/5			11.6		24.3	89.1	96.4
EL-4[b]			8.3		6.3	84.7	89.1
ESb	4.3	95.4	43.2	82.9		6.1	
ESbM	32.9	46.7	17.3	37.9		10.5	
ESbW	6.8	91.3	50.1	80.6		9.8	
Expt. II							
ESbD	54.0	97.4	61.3	94.8		10.9	
Eb	1.0	70.6	38.1	66.3		0.9	
ESbM	60.3	73.0	48.6	65.8		2.4	
ESbW	1.3	94.6	49.3	74.4		3.3	

[a] Quantitative data for the analysis in Figure 6.
[b] The EL-4 data have been included to show a negative control for the anti-I-A^b antibody. These were cells from the IL 2 high producer subline of EL-4 kindly provided by Dr. J. Farrar.

fluorographic analysis and incubation with alloreactive cytotoxic T cells of known specificity were used to ensure that all cell types expressed the expected H-2^d or H-2^b class I alloantigens. The NWT and CNWT preparations differed slightly with respect to the density of class I antigens and also in the expression of class II alloantigens; but the relatively strong stimulatory activity of the ESbW T lymphoma line in comparison with other tumor lines indicated that an exceptionally high density of class I antigen or the expression of class II antigen was not necessary for the expression of strong stimulatory activity. Our experiments suggested that the effective stimulator cells for the CTL precursors provide an *additional activating signal*, possibly an antigen-specific helper factor or a nonspecific short-range factor, in addition to the antigenic stimulus by the relevant class I alloantigen. Complementary experiments with a single set of stimulator cells and I region-compatible responder cells (Table IV) indicated that this additional activating signal is required for the induction of IL 2 responsiveness, which is believed to be the first step in the activation of cytotoxic responses (44, 45).

Of particular importance is our observation that the potent stimulator cells had relatively little or no effect on the magnitude of the bystander response, and conversely that an ongoing bystander response was not sufficient to support the response against the weak stimulator cells. The additional activating signal was obviously delivered only to those CTL precursor cells that carried antigen receptors that could bind to antigens on the signal-delivering cell. This point is expected to have practical implications for attempts to generate cytotoxic responses against tumor cells. It is conceivable that the stimulator cells produce (or stimulate the production of) one of the helper factors that are required in addition to IL 2 for the activation of CTL (21–28). Reports on these additional factors (21–28), however, have not dealt with antigen-specific aspects of these helper effects; i.e., they did not indicate whether the factors are delivered by a cognate interaction that involves the antigen receptor of the CTL precursor cell. The experiments in this report were designed to demonstrate the antigen-specific aspect of the additional activating signal. It has been reported that the stimulation of I-A-restricted T cells requires normally metabolically active stimulator cells, but may be achieved

also with UV-treated stimulator cells if the system is flooded with an IL 1-containing lymphokine preparation (50). This result indicated that the T cells receive the activating factor IL 1 directly from the stimulator cells, i.e., by a *cognate* interaction with the IL 1-producing macrophages. In analogy to this situation, we propose that at least one helper factor for the activation of the K and D region-restricted CTL precursor cells is also usually provided by the stimulator cells, i.e., the factor may be delivered in sufficient concentrations only if the antigen receptors of the CTL precursor cells can bind to antigenic determinants on the factor-producing cells. The hypothesis predicts i) that stimulatory activity is only (or optimally) expressed by certain cell types, and ii) that it is dependent on the metabolic activity of the stimulator cells. Both predictions were indeed confirmed by the experiments in this and a preceding (49) report.

Unexpected was our observation that a relatively high stimulatory potential was provided by two very different types of cell preparations, namely by CNWT and by AC. The former cell preparation contained mainly activated T cell blasts and the latter consisted, according to reports from other investigators (6), essentially of dendritic cells plus a small percentage of macrophages. The stimulatory activity of the AC preparation was not affected by treatment with anti-Thy-1 serum plus C, whereas the activity of Con A-activated spleen cells was strongly reduced by this treatment. A synergistic effect between CNWT and AC was not observed. Taken together, these results suggest that the same activating signal (possibly a factor) may be produced equally well by T cell blasts *and* by AC, or stimulation by the two cell preparations may involve different mechanisms.

Our observation that splenic responder cells in contrast to thymic responder cells (data not shown) generated weak but detectable cytotoxic activity also against the "weak stimulator" cells suggested the possibility that a small subset of splenic CTL precursor cells, i.e., possibly the Lyt-1^-2^+ subset, does not require the additional activating signal for stimulation. The clearly demonstrable stimulation of splenic responder cells by metabolically inactive stimulator cells has previously led to the widely accepted concept of CTL activation (45, 51) that the interaction of antigenic determinants with the antigen receptor of the CTL precursor cell provides by itself a first signal that triggers this cell to express receptors for the growth factor IL 2. The CTL precursor cell was thought to require for its activation neither a special type of stimulator cell (9) nor a metabolically active stimulator cell (5, 10, 11, 44, 52), and it was found to be activated even by noncellular antigen (14–19), provided an IL 2-containing cell supernatant (5, 9, 12, 14–19, 52) or a second set of stimulator cells of an unrelated H-2 haplotype (10, 11) was added to the cultures. We believe that these previous observations on the antigen requirements for cytotoxic responses were misleading and may apply only to a small subset of CTL precursor cells. Considering the relatively large numbers of stimulator cells, stimulation was indeed inefficient in the previous studies. Our experiments, in contrast, were performed with relatively small numbers of stimulator cells. Only the titration of the stimulator cells revealed that different cell types differ markedly in their capacity to stimulate the CTL precursor or an antigenically linked helper effect. Graded doses of stimulator cells have been used to demonstrate that cultures without T cell growth factor require more stimulator cells than cultures with this factor (53); however graded doses have not been used to compare the stimulatory capacity of *different stimulator cell preparations in the presence of T cell growth factor* (53).

It has been suggested (12) that the failure of tumor cells to stimulate an antigenically unlinked helper effect (i.e., IL 2 production) may be the main reason for their weak stimulatory potential. This limitation would have been overcome by activating helper cells of unrelated specificities. Our experiments showed, however, that the activation of CTL precursor cells requires an *antigenically linked* activating signal in addition to the physical structure of the antigen on the stimulator cell. If the weak stimulatory potential is a property of most tumor cells, it is not surprising that CTL play a minor role *in vivo* in tumor allograft rejection and syngeneic tumor rejection as suggested recently by several authors (54, 55).

Macrophage-like stimulator cells are known to process and present antigen, including tumor cell antigen (56), in immunogenic form to T cells. But antigen processing by T cells or T cell blasts has not been observed. The effective activation of CTL precursor cells by antigen-bearing T cell blasts thus may normally be confined to viral antigens, because viruses may be the only pathogens that integrate their components into the surface of T cell blasts. This fact may at least in part explain why CTL were found to be activated *in vivo* by virus (57) and by allografts (58) but usually not by tumors.

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