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THE OPTIMAL ACTIVATION OF CYTOTOXIC T LYMPHOCYTES REQUIRES METABOLICALLY INTACT STIMULATOR CELLS NOT ONLY FOR THE ACTIVATION OF THE INTERLEUKIN 2-PRODUCING HELPER CELLS

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Primary cytotoxic responses in macrocultures against UV-treated stimulator cells or glutaraldehyde-fixed stimulator cells in the presence of third party stimulator cells were studied to investigate whether metabolically active stimulator cells are only required for the activation of interleukin 2-producing helper cells. Cultures containing splenic responder cells in a mixture with allogeneic UV-treated stimulator cells of a mouse strain X plus conventional (i.e., only γ-irradiated) stimulator cells of a third strain Y were found to generate strong cytotoxic activity against cells of strain Y but not strain X. Macrocultures with conventional stimulator cells of strain Y plus UV-treated stimulator cells of (X × Y)F_1 hybrid mice also failed to generate substantial CTL activity against cells of strain X. The response against antigens of strain X was not reconstituted by interleukin 2- (IL 2) containing factors. UV-treated stimulator cells did not suppress the response against conventional stimulator cells of the same H-2 haplotype. These results provide suggestive evidence that CTL precursor cells are optimally activated in primary cytotoxic responses when their receptors interact with antigen on metabolically active stimulator cells. The experiments excluded the possibility that the metabolically active stimulator cells were *only* required for the activation of an unlinked helper effect (i.e., for the stimulation of interleukin 2 production) because the response against the third party stimulator cell proceeded in the same culture at normal magnitude. Our experiments with the (X × Y)F_1 stimulator cells also excluded the possibility that the metabolically active (i.e., UV-sensitive) stimulator cells were only required for the stimulation of a type of helper cell (or for the production of an antigen-specific helper factor) that interacts with the CTL precursor cell through a cellular antigen bridge (antigenically linked helper effect). A antigenically linked helper effect through a molecular antigen bridge was not formally excluded. Cold target competition experiments revealed that the antigenic structures were not detectably destroyed by the UV-irradiation procedure.

Cultures that received UV-treated stimulator cells on day 0 and an optimal dose of corresponding conventional stimulator cells on day 2 generated still weaker cytotoxicity than control cultures with conventional stimulator cells on day 0, indicating that the metabolically active (UV-sensitive) stimulator cells were required in the early phase of the culture to achieve optimal cytotoxic responses.

Responses against TNP-haptenated syngeneic stimulator cells were, in contrast to the allogeneic responses, only marginally affected by the UV treatment. Spleen cells from mice that carried the K end of the H-2^k haplotype responded in the presence of third party stimulator cells about equally well against TNP-haptenated H-2K^k-bearing UV-treated stimulator cells and conventional stimulator cells, suggesting the possibility that TNP-haptenated antigen was transferred to nonirradiated cells in the cultures, which then served as metabolically active stimulator cells. This phenomenon was only seen when both the responder cells and the UV-treated stimulator cells carried the K end of the H-2^k haplotype.

Primary cytotoxic immune responses require that the cytotoxic T lymphocyte (CTL)[1] precursor cells become exposed to several activating signals, including antigen and at least two soluble helper factors (1–7). Figure 1 illustrates four possible mechanisms of how these activating signals may be transmitted to the CTL precursor cells: 1) The antigenically unlinked helper effect (Fig. 1*A*) is transmitted by a soluble helper factor from a helper cell to a given target cell (i.e., a CTL precursor cell) of unrelated antigen specificity. The T cell growth factor interleukin 2 can be delivered in this unlinked fashion, at least in conventional macroculture systems (8). 2) The antigenically linked helper effect (see the example in Fig. 1*B*) is delivered from a helper cell to its target cell via an antigen bridge. This bridge maintains a close proximity between the CTL precursor cell and a helper cell or an antigen-specific helper factor. The carrier effect in the T cell-B cell cooperation (9) is the best known example of this type of helper effect, but there is suggestive evidence that an antigenically linked helper effect may also operate in the *in vivo* priming of CTL responses (10–12). 3) A third type of activating signal may be produced by the stimulator cell itself and delivered to the CTL precursor cell during the stimulation process (Fig. 1*C*). A requirement for this type of interaction is suggested by the experiments in this report. This mode of signal transmission was already known to operate in the activation of helper T cells by interleukin 1-producing macrophages or dendritic cells (13): the stimulating activity of the macrophages and dendritic cells was also found to be abrogated by glutaraldehyde fixation and was (at least partially) reconstituted by the addition of an interleukin 1-containing factor (13). Lafferty and Cunningham (14) and Davidson (15) have hypothesized that this mode of activation (Fig. 1*C*) might apply to all T cells, but so far there is no evidence that it also applies to CTL precursor cells. 4) The antigen receptor of the responding cell is also believed to provide an activating signal to

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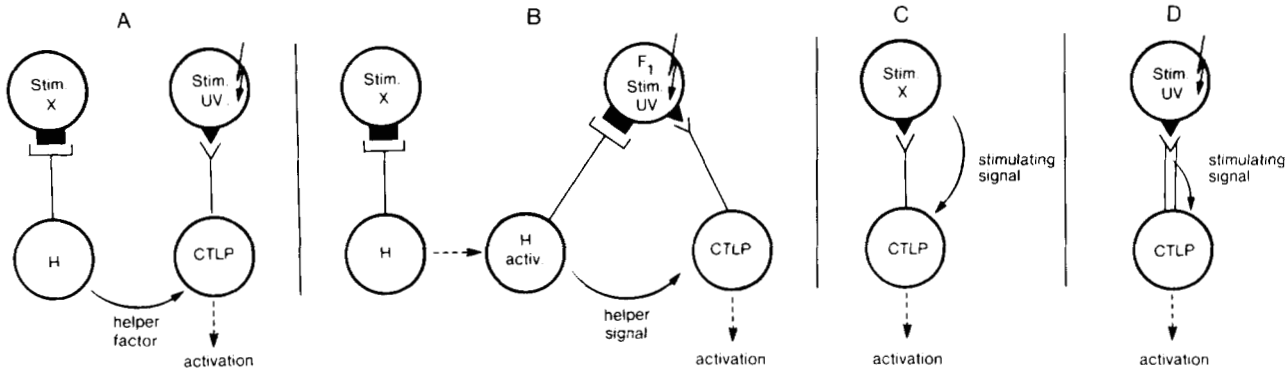
[1] Abbreviations used in this paper: CTL, cytototoxic T lymphocyte; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PMA, phorbol myristic acetate.

Four possible mechanims for signal transmission to CTL precursor cells and possible explanations for the failure of UV. treated stimulator cells to activate cytotoxic responses



Stim. X	stimulator cell functions only if metabolically active
Stim. UV	stimulator cell not required to be metabolically active
CTLP	Cytotoxic T lymphocyte precursor cell
H	Helper cell
H activ	antigenically activated helper cell or antigen specific helper factor

Figure 1. Four possible mechanisms for signal transmission to CTL precursor cells and possible explanations for the failure of UV-treated stimulator cells to activate cytotoxic responses. Model *A* illustrates the unlinked helper effect (i.e., the helper effect of interleukin 2-producing helper cells in conventional macrocultures (2)). This model assumes that the CTL precursor cells are readily stimulated by metabolically inactivated (i.e., UV-treated) stimulator cells and that the metabolically active (i.e., UV-sensitive) stimulator cells are only required for the activation of the helper cells. Model *B* illustrates the antigenically linked helper effect. There is suggestive evidence that an antigenically linked helper effect may operate in cytotoxic responses *in vivo* and involves a helper cell (or an antigen-specific helper factor) that interacts with the CTL precursor cell through a common stimulator cell (antigen bridge) (8–10). Model *B* assumes also that the CTL precursor cell is not required to interact with metabolically active (i.e., UV-sensitive) stimulator cells, whereas metabolically active stimulator cells are required for the stimulation of this hypothetical type of helper cell. Model *C* illustrates the transmission of an activating signal from a metabolically active stimulator cell to the CTL precursor cell. The antigen receptor serves in this case only the purpose of concentrating the signal producing cells in the proximity of the CTL precursor cell. Model *D* illustrates the possibility that the antigen receptor produces a stimulating signal for the CTL precursor cells after contact with antigen. The antigen-displaying cell is in this case not required to be metabolically active. The present experiments provide suggestive evidence for the requirement for an activating signal of model *C*.

that cell if it merely interacts with antigen regardless of whether this is metabolically active or not (Fig. 1*D*). This was concluded from the observation that antigen in liposomes as well as UV-treated or glutaraldehyde-fixed stimulator cells can stimulate cytotoxic responses in the presence of helper factor(s) or third party stimulator cells (13, 16–22). This implied that the antigen receptor does not function exclusively as a concentrating device for activating signals from other cells.

In this series of experiments we have examined whether CTL precursor cells must interact with metabolically active stimulator cells in primary cytotoxic responses in the absence of external factors (Fig. 1, *model C*). For this purpose, we studied cytotoxic responses in cultures with two types of stimulator cells. At least one of these stimulator cell populations was always a conventional γ-irradiated spleen cell population, to ensure a sufficient activation of interleukin 2-producing helper cells in the culture. The experiments revealed that the cytotoxic response against UV-treated or glutaraldehyde-fixed stimulator cells in the presence of conventional γ-irradiated third party stimulator cells was demonstrable but clearly inferior to the response against corresponding conventional gamma-irradiated stimulator cells and was also inferior to the response against the third party stimulator cells. This suggested that an activating signal from a metabolically active (i.e., UV-sensitive) stimulator cell is probably also required in primary cytotoxic responses for the CTL precursor cells, and not only for the helper T cells, as suggested previously (16, 18, 20). Hurme *et al.* (23) reported recently that cytotoxic responses against TNP-haptenated syngeneic target cells can be readily obtained with UV-treated stimulator cells. We also found that mice that carried the K end of the H-2^k haplotype produced cytotoxic responses against TNP-haptenated syngeneic stimulator cells, which were in contrast to the allogeneic responses only marginally affected by UV-treatment of the stimulator cells.

Macrophage-like cells and dendritic cells have recently been shown to function as optimal stimulator cells in complex reactions such as proliferative T cell responses and cytotoxic responses (13, 24–30), but there were strong indications that cytotoxic responses require dentritic cells or macrophages primarily as stimulator cells for the activation of helper T cells (13, 29–31).

MATERIALS AND METHODS

Animals. Mice of the strains C3H, DBA/2, BALB/c, C57BL/6, A/J, C3H nu/nu, BALB/c nu/nu, and C57BL/6 nu/nu were purchased from Bomholtgard, Ry, Denmark. The congeneic strains and the CBA mice were maintained at the German Cancer Research Center and were originally obtained from The Jackson Laboratory, Bar Harbor, ME.

Cytotoxic responses in macrocultures. Twenty million responder cells were incubated in a total volume of 4.5 ml culture medium (RPMI 1640, GIBCO medium containing 10 mM L-glutamine, streptomycin/penicillin [100 U/ml], 0.5% HEPES[1] [all the above from GIBCO, Grand Island, NY]; 10% fetal calf serum [Paesel, Frankfurt, West Germany], and 3×10^{-5} M 2-mercaptoethanol [see Reference 32]) together with various combinations of irradiated (1500 rad) allogeneic stimulator cells for 5 days at 37°C in 5% CO_2 if not indicated otherwise. Responder and stimulator cells were spleen cells if not indicated otherwise. Some cultures received stimulator cells that were fixed with glutaraldehyde or treated with UV light in addition to the standard irradiation of 1500 rad; most cultures received more than one type of stimulator cell, and some of the cultures received additional helper factors or stimulator cells on different days of culture. The cultures were tested after 5 days for cytotoxic activity as described (3, 32).

Enrichment for T cells. Lymphoid cells were incubated in nylon wool columns and eluted as described (33).

Irradiation of stimulator cells. All stimulator cells were irradiated with 1500 rad from a ^{60}Co source. Some of the stimulator cells were additionally irradiated with 254 nm UV light (30 W, UV source from VETTER, Wiesloch, Germany; irradiation time 15 min, distance 5 cm). This latter treatment was shown in control experiments to completely abrogate the capacity of spleen cells to proliferate and to produce interleukin 2 and interferon in response to concanavalin A; but the cells were still viable by the trypan blue exclusion test (data not shown).

Fixation of stimulator cells with glutaraldehyde. The fixation procedure was essentially performed as described by Bing *et al.* (34). In brief, 1 ml of a 25% glutaraldehyde solution was dissolved in 24 ml BSS at 4°C. Spleen cells from 20 mice (about 2×10^9 cells) were added to the freshly prepared solution and were incubated for 30 min at 4°C. The cells were resuspended every 10 min. After this incubation period, the cells were washed three times with serum-free BSS, twice with RPMI 1640 plus 10% fetal calf serum, and were finally adjusted with culture medium to 5×10^7/ml and stored at 4°C. In control experiments, this treatment was shown to completely abrogate the capacity of spleen cells to proliferate and to produce interleukin 2 and interferon in response to concanavalin A (data not shown).

The preparation of interleukin 2-containing supernatants from EL-4 thymoma cells. Supernatants from EL-4 thymoma cells were obtained from an interleukin 2-producing EL-4 subline (kindly provided by Dr. J. Farrar) as described (35). Briefly, 10^6 EL-4 cells/ml were incubated with 10 ng/ml of phorbol myristic acetate (PMA) for 48 hr. The supernatant was collected and stored frozen at −20°C.

RESULTS

Cytotoxic responses against mixtures of conventional and UV-irradiated or glutaraldehyde-fixed allogeneic stimulator cells of different H-2 haplotypes. As few as 5×10^4 conventional γ-irradiated BALB/c stimulator cells stimulated substantial cytotoxic responses against BALB/c target cells if cultured in conventional macrocultures with 2×10^7 C3H spleen cells as responder cells and 5×10^6 γ-irradiated C57BL/6 spleen cells as third party stimulator cells (Fig. 2). The response against the small dose of stimulator cells was higher than the response against 5×10^5 or even 5×10^6 glutaraldehyde-fixed or UV-irradiated BALB/c stimulator cells (Fig. 2). The response against third party stimulator cells (C57BL/6), on the other hand, was not detectably affected by UV treatment or glutaraldehyde-fixation of the BALB/c stimulator cells. High doses of conventional γ-irradiated stimulator cells inhibited nonspecifically the response against BALB/c and C57BL/6 cells (Fig. 2).

A reduced stimulator activity of UV-treated cells was also demonstrated in several other strain combinations (Table I). Cultures containing 2×10^7 C57BL/6 responder cells and 5×10^6 conventional γ-irradiated C3H stimulator cells plus 5×10^6 γ-irradiated and UV-treated DBA/2 stimulator cells generated strong CTL activity only against antigens of the conventional, and not the UV-treated, stimulator cells. Similar results were obtained in other combinations (Table I). The UV-treatment of the stimulator cell did not produce delayed responses but reduced the peak response on day 5 (Fig. 3). Control experiments established that our procedure of UV irradiation did not reduce the viability in the trypan blue exclusion test but completely abrogated the capacity of spleen cells to proliferate and to produce interleukin 2 and interferon in response to concanavalin A. Moreover, when conventional and UV-irradiated stimulator cells were placed in culture without responder cells and counted at various time intervals of up to 4 days, there was practically no difference in either the number of persisting cells or the proportion of trypan blue-positive cells (data not shown). These results suggested the possibility that the CTL precursor cells develop optimal cytotoxic activity only if they interact with metabolically active stimulator cells.

The experiments in Table I indicated also that the T cell-enriched nylon wool-nonadherent fraction of spleen cells (NW-T) was also able to stimulate CTL precursor cells in a UV-sensitive fashion. This observation suggested the possibility that macrophage-like cells and dendritic cells may be required in cytotoxic responses as stimulator cells for helper T cells but not as immediate stimulator cells for the CTL precursor cells (13). This point, however, certainly needs more extensive studies with more rigorous cell fractionation procedures.

Failure to reconstitute the cytotoxic responses against the UV-treated stimulator cells with interleukin 2-containing helper factors. Various interleukin 2-containing helper factors including several batches of concanavalin A-activated spleen cell supernatant and supernatants from PMA-activated EL-4 thymoma cells (35) were tested for their ability to reconstitute the cytotoxic responses against UV-treated stimulator cells. One such experiment with various responder and stimulator cell populations is illustrated in Table II. The addition of the EL-4 supernatant enhanced the cytotoxic response at least slightly in most of the cases. However, the difference between UV-treated and conventional stimulator cells was not reduced but rather enhanced by the addition of this helper factor. Similar results were obtained with concanavalin A-activated spleen cell supernatants (data not shown).

Demonstration that the UV-treated stimulator cells carry intact alloantigens. A reconstitution of the responses against UV-treated stimulator cells by soluble factors would have provided the ideal control to ensure that the alloantigens on the UV-irradiated stimulator cells were still physically intact. Because such efforts have failed, we tested the antigenic properties of

Cytotoxic responses against different concentrations of glutaraldehyde fixed, UV-irradiated or conventional gamma irradiated stimulator cells in the presence of third party stimulator cells

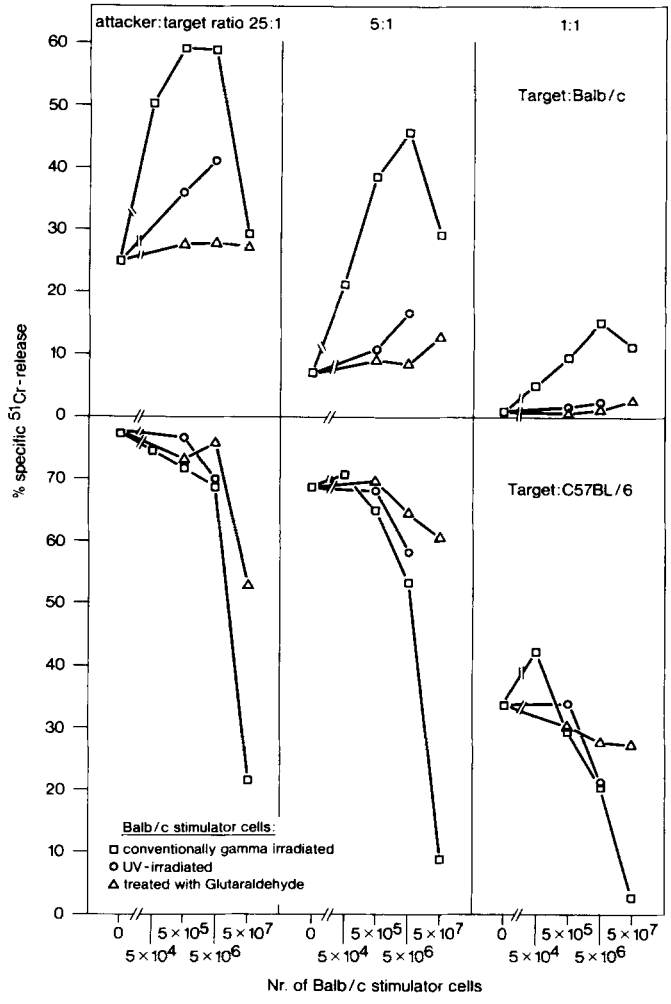


Figure 2. Cytotoxic responses against different concentrations of glutaraldehyde-fixed, UV-irradiated, or conventional γ-irradiated stimulator cells in the presence of third party stimulator cells. 2×10^7 C3H spleen cells were cultured as responder cells together with 5×10^6 conventional γ-irradiated C57BL/6 stimulator cells and the indicated numbers of conventional γ-irradiated, UV-irradiated, or glutaraldehyde treated BALB/c stimulator cells. The data indicate the percent of specific ^{51}Cr release by the indicated target cells at attacker to target cell ratios 25:1, 5:1, and 1:1.

TABLE I

Cytotoxic responses against UV-treated stimulator cells in the presence of conventional γ-irradiated third party stimulator cells[a]

Groups	Responder Cells	Stimulator Cells	Attacker to Target Cell Ratios						
			25:1	5:1	1:1	25:1	5:1	1:1	
					Target: DBA/2			Target: C3H	
1	C57BL/6	DBA/2	+ C3H	50.6 ± 2.4	18.1 ± 1.4	2.2 ± 0.9	46.2	21.9	5.3
2	C57BL/6	DBA/2-UV	+ C3H	12.5 ± 1.8	0.2 ± 1.1	0.2 ± 1.0	43.9	16.4	3.6
3	C57BL/6	DBA/2-UV + DBA/2-NW-T	+ C3H	31.8 ± 1.9	5.7 ± 1.4	0.4 ± 0.8	42.5	12.6	2.1
4	C57BL/6	DBA/2-NW-T	+ C3H	43.0 ± 1.7	9.5 ± 1.1	−1.0 ± 0.7	48.8	25.0	3.4
5	C57BL/6	DBA/2-NW-T-UV	+ C3H	15.7 ± 1.8	0.6 ± 0.7	−2.9 ± 1.0	44.7	17.7	4.7
					Target: C57BL/6			Target: C3H	
6	DBA/2	C57BL/6	+ C3H	50.1 ± 2.7	16.1 ± 1.0	1.3 ± 0.8	50.3	20.8	4.0
7	DBA/2	BL/6-UV	+ C3H	18.2 ± 1.0	2.8 ± 1.2	0.2 ± 0.6	49.8	22.8	4.3
8	DBA/2	BL/6-UV + BL/6-NW-T	+ C3H	41.4 ± 2.1	11.8 ± 0.7	2.4 ± 2.9	52.4	25.1	4.6
9	DBA/2	BL/6-NW-T	+ C3H	59.9 ± 3.0	21.9 ± 1.2	1.7 ± 0.8	53.4	30.5	10.6
10	DBA/2	BL/6-NW-T-UV	+ C3H	21.5 ± 1.0	5.6 ± 1.1	0.1 ± 1.0	52.0	25.6	6.3
					Target: DBA/2			Target: C57BL/6	
11	C3H	DBA/2	+ BL/6	54.3 ± 1.3	19.5 ± 1.1	3.3 ± 0.7	47.6	17.1	3.1
12	C3H	DBA/2-UV	+ BL/6	33.7 ± 2.0	8.7 ± 1.2	1.7 ± 1.5	70.9	32.8	31.7
13	C3H	DBA/2-UV + DBA/2-NW-T	+ BL/6	57.8 ± 1.8	21.0 ± 1.3	4.9 ± 0.8	60.6	27.2	4.8
14	C3H	DBA/2-NW-T	+ BL/6	52.5 ± 2.5	19.0 ± 1.2	1.4 ± 1.3	62.1	25.5	4.2
15	C3H	DBA/2-NW-T-UV	+ BL/6	38.3 ± 1.9	7.2 ± 0.9	0.5 ± 0.9	68.1	26.2	7.0

[a] 2×10^7 splenic responder cells from the indicated strains were cultured together with 5×10^6 of each of the indicated stimulator cells. Some groups of stimulator cells were treated with UV light in addition to γ irradiation, and some of the cultures received the T cell-enriched nylon wool-nonadherent fraction (NW-T) of spleen cells as stimulator cells. The data indicate the percent of specific ^{51}Cr-release on the indicated target cells at attacker to target cell ratios 25:1, 5:1, and 1:1. Experiments with BL/6 and DBA/2 responder cells in combination with UV-treated C3H stimulator cells and experiments with C3H responder cells and UV-treated BL/6 stimulator cells in the presence of allogeneic third party stimulator cells have also been performed and gave similar results.

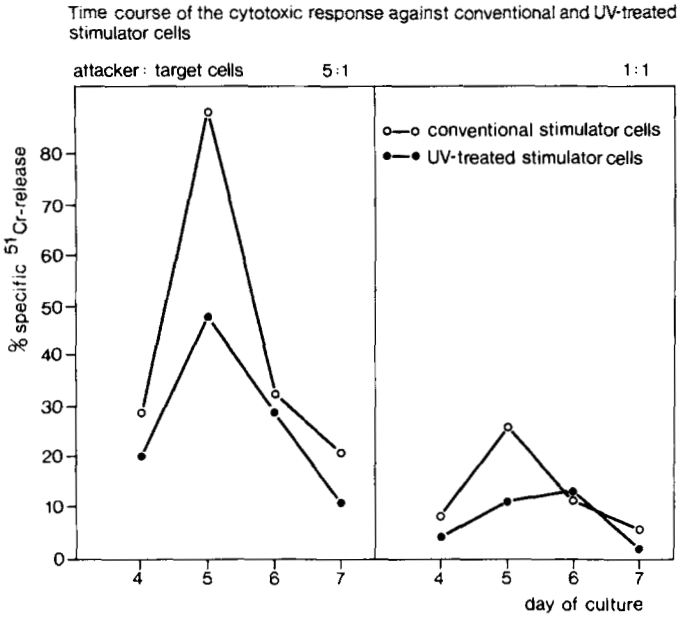


Figure 3. Time course of the cytotoxic response against conventional and UV-treated stimulator cells. 2×10^7 C57BL/6 spleen cells were cultured as responder cells together with 5×10^6 conventional or UV-treated C3H stimulator cells plus 5×10^6 conventional DBA/2 stimulator cells in macrocultures for the indicated time period. The data indicate the percent of specific ^{51}Cr release of C3H target cells at the attacker to target cell ratios 5:1 and 1:1.

UV-treated cells in cold target competition experiments. Using various conventional and UV-treated concanavalin A blasts as cold target inhibitors, we found no difference in the inhibitory activity of normal and UV-treated cells (Fig. 4).

Cytotoxic responses against heterozygous UV-treated stimulator cells in the presence of semi-allogeneic γ-irradiated stimulator cells. The experiments with two unrelated sets of stimulator cells (Table I and Fig. 2) excluded the possibility that the UV-sensitive function of the stimulator cells was *only* required for the activation of an unlinked helper effect (i.e., for the production of interleukin 2) as schematically illustrated in Figure 1*A*. Two possible explanations remained: either the UV-sensitive function of the stimulator cell was required directly for the activation of the CTL precursor cell, for example via a soluble mediator (Fig. 1*C*), or it was required for the activation of an antigenically linked helper effect (Fig. 1*B*). In several recent reports (10–12) it has been suggested that such a linked helper effect operates at least in primary cytotoxic responses *in vivo.* It is believed to involve a common stimulator cell that carries antigenic determinants recognized by the CTL precursor cell and (a potentially different set of) determinants recognized by a helper cell or by an antigen-specific helper factor as schematically illustrated in model B in Figure 1. Experiments with conventional stimulator cells of a given strain X in the presence of UV-treated heterozygous (X × Y)F_1 stimulator cells indicated that such a helper mechanism was not exclusively, if at all, responsible for the inferior activity of UV-treated stimulator cells in our culture systems. This type of experiment was performed again in several combinations, two of which are reported in Table III. These experiments revealed consistently a strong response against target cells of strain X and weak responses against target cells of strain Y. This indicated that the UV-sensitive stimulator cell was not (exclusively) required for the activation of an antigenically linked helper effect via a cellular antigen bridge. Because we have tested only hybrid cells and not hybrid molecules, we cannot formally exclude an antigenically linked helper effect by a noncellular antigen bridge. The simplest and most probable interpretation of these results, however, is that the UV-sensitive property of the stimulator cells was directly required for the activation of the CTL precursor cells and not for the activation of helper cells.

Failure to demonstrate the suppressive effect of UV-treated stimulator cells. The experiments in Table III showed that the UV-treated F_1 cells did not suppress cytotoxic responses with specificity for antigenic determinants on these cells. Similar results were obtained with combinations of homozygous UV-irradiated and conventional stimulator cells (Table IV). These experiments indicated that the reduced stimulatory activity of UV-treated cells does not result from active suppression. Only mixtures of UV-irradiated stimulator cells with γ-irradiated nylon wool-nonadherent splenic stimulator cells often showed a slight degree of suppression (Table I, compare groups 3 and 4 or groups 8 and 9). This phenomenon is under investigation, but hardly provides an explanation for the substantial loss of stimulatory activity in the UV-treated cell population.

The UV-sensitive function of the stimulator cell is most effective

TABLE II

The effect of EL-4 supernatant on cytotoxic response against UV-irradiated stimulator cells[a]

Resp. (2×10^7)	Stim. 1 (6×10^5)	Stim. 2 (5×10^5)	EL-4 Supernatant Added to the Cultures															
			0.0 ml			0.2 ml			0.6 ml			0.0 ml			0.2 ml			0.6 ml	
			25:1	5:1	1:1	25:1	5:1	1:1	25:1	5:1	1:1	25:1	5:1	1:1	25:1	5:1	1:1	25:1	5:1	1:1
			Target: BL/6									Target: BALB/c								
1 C3H	BL/6	BALB/c	41	32	09	46	44	17	44	40	13	46	25	09	43	33	07	40	26	05
2 C3H	BL/6-UV	BALB/c	25	12	01	29	14	01	29	12	02	47	32	14	44	36	10	41	27	06
			Target: BALB/c									Target: C3H								
3 BL/6	BALB/c	C3H	42	37	15	51	48	20	49	42	14	60	49	23	63	57	26	57	57	22
4 BL/6	BALB/c-UV	C3H	37	18	03	43	29	08	39	29	10	58	46	18	65	62	30	51	49	24
			Target: BL/6									Target: C3H								
5 DBA/2	BL/6	C3H	36	30	06	41	39	17	29	21	03	39	25	05	41	31	09	31	18	05
6 DBA/2	BL/6-UV	C3H	14	08	−02	18	06	−02	07	03	−03	44	33	11	43	35	10	38	28	06
			Target: C3H									Target: BL/6								
7 DBA/2	C3H	BL/6	46	35	14	51	36	14	45	29	11	35	29	09	36	24	03	23	13	−02
8 DBA/2	C3H-UV	BL/6	35	19	09	31	18	08	26	13	07	24	21	05	28	23	04	32	17	03

[a] 2×10^7 responder cells were incubated with the indicated stimulator cell combinations together with the indicated amounts of an interleukin 2-containing supernatant from PMA-activated EL-4 thymoma cells. The data indicate the percent of specific ^{51}Cr release at the three attacker to target cell ratios 25:1, 5:1, 1:1. For other details, see footnote to Table I.

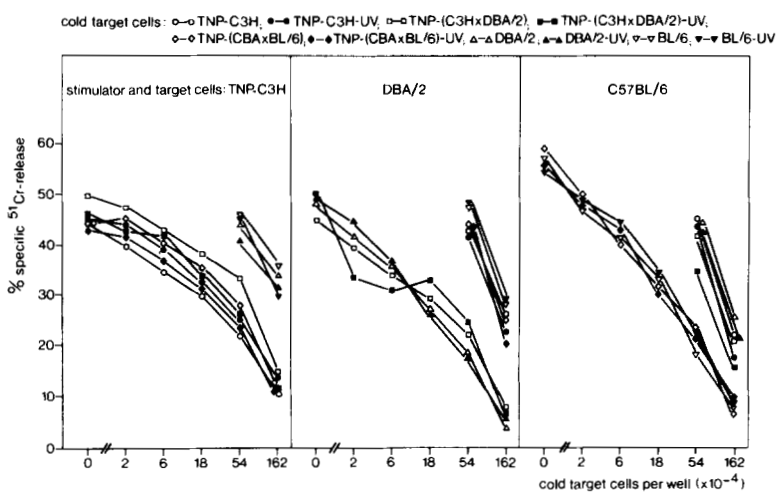


Figure 4. Cold target inhibition by UV-irradiated cells. 1×10^7 C3H responder cells were cultured with 10^7 of the indicated stimulator cells for 5 days in 4.5-ml macrocultures. Then 1×10^5 cultured cells were mixed with 2×10^4 ^{51}Cr-labeled allogeneic or TNP-haptenated syngeneic target cells and graded doses of the indicated cold target cells and were assayed in the conventional 4-hr ^{51}Cr-release assay. The cold target cells were not labeled with ^{51}Cr but were otherwise prepared according to the same procedure as the conventional target cells. Some of the cold target cells were irradiated with UV light.

TABLE III

Cytotoxic responses against heterozygous UV-treated stimulator cells in the presence of semi-allogeneic γ-irradiated stimulator cells[a]

Groups	Stimulator cells	Target					
		CBA			BALB/c		
		25:1	5:1	1:1	25:1	5:1	1:1
1	C3H + (C3H × DBA/2)F_1	64.2 ± 1.3	36.1 ± 1.7	11.4 ± 0.5	40.2 ± 0.7	21.3 ± 1.4	5.5 ± 0.5
2	C3H + (C3H × DBA/2)F_1-UV	66.8 ± 1.3	39.5 ± 0.7	13.9 ± 0.5	28.5 ± 0.7	12.3 ± 0.7	3.7 ± 0.6
3	C3H + DBA/2-UV	67.3 ± 1.8	43.7 ± 1.9	13.3 ± 0.5	28.3 ± 0.9	12.0 ± 0.7	3.4 ± 0.6
4	DBA/2 + (C3H × DBA/2)F_1	54.6 ± 2.0	25.6 ± 0.6	5.6 ± 0.6	51.2 ± 2.1	32.2 ± 1.5	11.6 ± 0.7
5	DBA/2 + (C3H × DBA/2)F_1-UV	33.5 ± 1.1	10.7 ± 0.8	2.5 ± 0.4	50.4 ± 0.7	30.3 ± 0.9	9.8 ± 0.8
6	DBA/2 + C3H-UV	37.5 ± 0.8	15.4 ± 1.2	2.9 ± 0.7	49.8 ± 0.9	26.6 ± 1.1	7.3 ± 0.8

[a] 2×10^7 C57BL/6 responder cells were cultured together with 5×10^6 stimulator cells of each of the indicated strains. For other details, see footnote to Table I.

TABLE IV

Failure to demonstrate a suppressive effect of UV-treated stimulator cells[a]

Groups	Stimulator Cells			Target					
				C57BL/6			BALB/c		
	3×10^5	3×10^5	5×10^5	25:1	5:1	1:1	25:1	5:1	1:1
1	BL/6		+ BALB/c	46.5	34.5	11.6	47.1	32.8	8.8
2	BL/6-UV		+ BALB/c	33.2	17.0	5.4	51.8	38.3	12.5
3	BL/6 +	BL/6-UV	+ BALB/c	50.6	27.6	8.5	42.3	25.7	5.6
4	BALB/c		+ BL/6	47.7	43.7	15.0	40.8	23.5	9.5
5	BALB/c-UV		+ BL/6	47.7	41.0	13.9	25.0	12.7	5.5
6	BALB/c +	BALB/c-UV	+ BL/6	45.5	35.8	10.9	43.4	23.3	5.7

[a] 2×10^7 C3H spleen cells were cultured with the indicated mixtures of stimulator cells. For other details, see footnote to Table I.

in the early phase of the cytotoxic response. UV-treated stimulator cells were inferior to conventional stimulator cells even when the cultures were supplemented with an optimal dose of conventional stimulator cells 2 days later (Table V). This indicated that the metabolically active stimulator cells are required in the early culture period to achieve optimal activation of the cytotoxic response. The addition of conventional stimulator cells on day 1 produced by itself a substantial response; it was therefore not

TABLE V

The UV-sensitive function of the stimulator cell is required in the early phase of the cytotoxic response[a]

Group	Stimulator Cells (day 0)	5×10^6 (day 0)		1×10^7 (day 2)	Target: C57BL/6 25:1	C57BL/6 5:1	C57BL/6 1:1	BALB/c 25:1	BALB/c 5:1	BALB/c 1:1
1		BALB/c	+	BL/6	10.2	5.1	1.6	57.0	49.6	22.1
2	2×10^6 BL/6	BALB/c	+	BL/6	43.7	26.9	7.2	54.5	45.3	17.8
3	5×10^5 BL/6	BALB/c	+	BL/6	24.5	8.8	0.1	56.9	49.2	23.6
4	2×10^6 BL/6-UV	BALB/c	+	BL/6	27.7	8.9	−0.9	58.0	40.4	19.2
5	5×10^5 BL/6-UV	BALB/c	+	BL/6	17.4	2.2	2.0	55.5	40.1	19.0
6		BL/6	+	BALB/c	49.4	32.5	9.6	22.8	7.2	4.6
7	5×10^5 BALB/c	BL/6	+	BALB/c	55.9	30.8	5.8	52.6	27.4	9.0
8	5×10^5 BALB/c-UV	BL/6	+	BALB/c	56.1	29.4	10.1	34.7	8.8	4.3
9	5×10^5 BALB/c NW-T[b]	BL/6	+	BALB/c	44.8	28.8	5.3	47.6	25.6	5.5

[a] 2×10^7 C3H spleen cells were cultured together with the indicated stimulator cells for 5 days and then assayed on the indicated target cells. Variable numbers of stimulator cells were added at the start of the culture (day 0), and 1×10^7 stimulator cells were added 2 days later as indicated. For other details, see footnote to Table I.

[b] NW-T = T cell-enriched nylon wool-nonadherent fraction.

possible to clarify whether the UV-sensitive function was already required during the first 24 hr of the culture. Additional experiments showed, on the other hand, that the addition of metabolically active stimulator cells as late as day 3 may still enhance detectably the cytotoxic response, if the cultures have been started with a suboptimal population of stimulator cells (Table VI). The late addition of stimulator cells with recombinant H-2 haplotypes mediated optimal cytotoxic responses only to determinants that were displayed on these cells (Table VI, groups 16 through 19). This supported our assumption that these stimulator cells interacted directly with the CTL precursor cells under test.

Cytotoxic responses against UV-treated TNP-haptenated stimulator cells. The cytotoxic responses of CBA spleen cells against TNP-haptenated syngeneic stimulator cells were markedly reduced when the haptenated stimulator cells were treated with UV light; but this reduction was only seen in the absence and not in the presence of third party stimulator cells (Table VII). This indicated that a UV-sensitive function was required in this case for the activation of an unlinked helper effect but not for the interaction with the CTL precursor cells under test. Similar results were obtained with responder cells from C3H mice (Table VII) and some other strains with the K end of the H-2^k haplotype, including B10.BR and B10.A, but not with responder cells from other strains, such as C57BL/6 and BALB/c (data not shown). The relatively strong effect of UV treatment on the allogeneic response and the comparably weak effect on the TNP-specific self-restricted response was also observed when the third party stimulator cells were replaced by the interleukin 2-containing EL-4 supernatant (Table VII, Expts. II through IV). These experiments contained the internal control that the addition of the EL-4 supernatant did not abrogate the effect of UV irradiation on the allogeneic stimulator cells. These experiments revealed, moreover, that the relatively strong reduction of the allogeneic response and the mild reduction of the self-restricted response was obtained even when the corresponding antigens (i.e., alloantigen and TNP-hapten) were displayed together on the same F_1 hybrid stimulator cell (Table VII, groups 9 through 12). The relatively small reduction of the self-restricted response was again observed irrespective of whether the stimulator cells were normal spleen cells or T cell-enriched nylon wool-nonadherent spleen cells (Table VII). Control experiments established that this abnormal behavior of the TNP-specific response was not due to

TABLE VI

The effect of conventional and UV-irradiated stimulator cells in the late phase of the cytotoxic response[a]

Expt.	Group	Stimulator Cells: Day 0	Stimulator Cells: Day 3	Attacker:Target Cell Ratios					
				25:1	5:1	1:1	25:1	5:1	1:1
					Target: BL/6			Target: DBA/2	
I	1	5×10^6 BL/6-UV + 5×10^6 DBA/2	2×10^6 BL/6	36	15	06	50	33	09
	2	5×10^6 BL/6-UV + 5×10^6 DBA/2	2×10^6 BL/6-UV	18	04	06	45	30	06
	3	5×10^6 DBA/2	2×10^6 BL/6	16	05	03	44	28	07
	4	5×10^6 DBA/2	2×10^6 BL/6-UV	12	04	05	44	28	05
					Target: BL/6				
II	5	5×10^6 BL/6-UV		49	17	04			
	6	5×10^6 BL/6-UV	1×10^4 BL/6	41	28	07			
	7	5×10^6 BL/6-UV	1×10^5 BL/6	52	47	15			
	8	5×10^6 BL/6-UV	1×10^6 BL/6	52	40	16			
	9	5×10^6 BL/6-UV	1×10^4 BL/6-UV	40	19	05			
	10	5×10^6 BL/6-UV	1×10^5 BL/6-UV	38	11	02			
	11	5×10^6 BL/6-UV	1×10^6 BL/6-UV	39	07	00			
					Target: BALB/c				
III	12	5×10^5 BALB/c		42	16	03			
	13	5×10^5 BALB/c	2×10^6 BALB/c	51	34	21			
	14	5×10^5 BALB/c	2×10^6 BALB/c-UV	41	19	06			
	15	5×10^5 BALB/c	2×10^6 BALB/c-GA	28	13	03			
					Target: B10.A			Target: C3H-H-2°	
	16	5×10^5 BALB/c	2×10^6 B10.A	52	25	05	40	11	04
	17	5×10^5 BALB/c	2×10^6 C3H-H-2°	40	14	02	52	19	06
	18	5×10^5 BALB/c	2×10^6 B10.D2	44	19	04	51	25	09
	19	5×10^5 BALB/c	2×10^6 C57BL/10	26	09	00	33	11	03

[a] 2×10^7 C3H responder cells were cultured with the indicated stimulator cells that were added either on day 0 or on day 3. Cultures in experiments II and III also received 0.2 ml EL-4 supernatant. The cultures were tested at day 5 for cytotoxic activity on the indicated target cells at attacker to target cell ratios 25:1, 5:1, 1:1. For other details, see footnote to Table I.

TABLE VII

Cytotoxic responses against UV-treated TNP-haptenated cells[a]

Expt.	Group Responder	Stimulator Cells	Target													
			TNP-CBA			TNP-BALB/c			TNP-BL6			BALB/c			BL/6		
			25:1	5:1	1:1	25:1	5:1	1:1	25:1	5:1	1:1	25:1	5:1	1:1	25:1	5:1	1:1
I	1 CBA	TNP-CBA	63	41	14	08	02	−02	08	02	03		n.t.			n.t.	
	2 CBA	TNP-CBA-UV	44	22	09	00	−02	00	03	02	02		n.t.			n.t.	
	3 CBA	TNP-CBA + BL/6	75	55	28	28	14	03	72	63	20		n.t.			n.t.	
	4 CBA	TNP-CBA-UV + BL/6	78	55	20	28	14	05	75	60	21		n.t.			n.t.	
	5 CBA	TNP-BALB/c-UV + BL/6	23	12	04	62	29	08	79	66	23	47	19	04	68	50	13
	6 CBA	BALB/c-UV + BL/6	30	24	18	59	33	08	75	64	26	49	25	03	69	50	14
	7 CBA	TNP-BL/6-UV + BALB/c	33	23	13	73	60	19	62	34	08	59	49	18	45	18	03
	8 CBA	BL/6-UV + BALB/c	32	20	08	78	54	21	62	39	10	61	51	17	57	29	09
II	9 C3H	TNP-(CBA × BL/6)	30	22	09		n.t.			n.t.			n.t.		61	37	09
	10 C3H	TNP-(CBA × BL/6)-UV	21	07	02		n.t.			n.t.			n.t.		01	−04	00
III	11 C3H	TNP-(C3H × DBA/2)	63	46	18		n.t.			n.t.		55	35	10		n.t.	
	12 C3H	TNP-(C3H × DBA/2)-UV	57	31	08		n.t.			n.t.		24	08	02		n.t.	
IV	13 C3H	TNP-C3H	58	36	14		n.t.			n.t.			n.t.			n.t.	
	14 C3H	TNP-C3H (UV)	51	31	09		n.t.			n.t.			n.t.			n.t.	
	15 C3H	TNP-C3H (NW-T)[b]	52	37	14		n.t.			n.t.			n.t.			n.t.	
	16 C3H	TNP-C3H (NW-T) (UV)	48	29	09		n.t.			n.t.			n.t.			n.t.	

[a] 2×10^7 responder cells were cultured together with 5×10^6 of the indicated stimulator cells. The cultures of experiments II through IV also received 0.2 ml EL-4 supernatant. The cytotoxic activity at day 5 was tested on the indicated target cells. For other details, see footnote to Table II. The standard errors were calculated for the underlined data and were always smaller than ± 2.2%. n.t. = not tested. TNP-C3H targets in experiments II and IV; DBA/2 targets in experiment III.

[b] NW-T = T cell-enriched nylon wool-nonadherent fraction.

the trinitrophenylation of the target cells or the stimulator cells. Cytotoxic responses against UV-treated and trinitrophenylated allogeneic stimulator cells were reduced to the same extent as the responses against the UV-treated nonhaptenated cells (Table VII, groups 5 through 8). This was also the case when H-2^k-bearing cells were used as allogeneic stimulator cells (data not shown). There were at least two possible explanations for this observation: either the TNP-specific H-2^k-restricted response might have recruited a different set of CTL precursor cells with different stimulation requirements, or the TNP-haptenated antigen was transferred to nonirradiated stimulator cells in the responder population and displayed by these cells to the CTL precursor cells. It was consistently observed, however, that UV-treated and TNP-haptenated allogeneic stimulator cells failed to activate in CBA responder cells a cytotoxic reaction against TNP-haptenated CBA cells. If antigen transfer did operate in these experiments, it did so only when both the haptenated stimulator cells and the responder cells carried the H-2^k haplotype (Table VII).

DISCUSSION

A series of earlier studies established that the activation of CTL precursor cells requires an antigen-nonspecific helper factor (interleukin 2) that is only produced if the helper cells are stimulated by metabolically active (i.e., UV-sensitive) stimulator cells (13, 16, 18, 20, 36). Our present macroculture experiments with two sets of stimulator cells demonstrated that the optimal activation of CTL requires metabolically intact stimulator cells not only for the activation of an unlinked helper effect (i.e., for the activation of interleukin 2 production), but also for the direct stimulation of the CTL precursor cells or for the activation of a helper cell that interacts with a CTL precursor through a noncellular antigen-bridge (antigenically linked helper effect).

We found that a single dose of glutaraldehyde-fixed or UV-treated allogeneic stimulator cells activated only a relatively weak cytotoxic response in comparison with conventional γ-irradiated stimulator cells, even if the cultures contained conventional γ-irradiated stimulator cells of an unrelated allogeneic strain. Control experiments established that our procedures of UV irradiation did not reduce the viability in the trypan blue exclusion test but completely abrogated the ability of spleen cells to proliferate and to produce interleukin 2 and interferon in response to concanavalin A. The UV-irradiated cells did not suppress the cytotoxic response against conventional splenic stimulator cells of the same H-2 haplotype, indicating that the weak stimulatory activity of these cells is not caused by active suppression. A slight suppression was only observed when UV-treated cells were mixed with nylon wool-nonadherent spleen cells. This phenomenon is presently under further investigation. Cold target inhibition experiments established furthermore that UV irradiation does not detectably destroy the antigenic determinants on cold target cells. The observation that the H-2^k-restricted TNP-specific responses were not markedly affected by UV irradiation of the stimulator cells also supported the conclusion that the UV-irradiation procedure did not destroy the physical integrity of the antigenic determinants.

The fact that primary cytotoxic responses can be abrogated by UV treatment of the stimulator cells has been documented (16–20, 23, 36). These published experiments strongly suggested that the metabolically active stimulator cells were mainly required to stimulate helper T cells. The possibility that the CTL precursor cells did not have to interact with metabolically active stimulator cells was especially supported by the previous observations that UV-irradiated (16, 18, 20) or glutaraldehyde-fixed (13) stimulator cells, or even purified histocompatibility antigens in liposomes (29, 30), activated demonstrable cytotoxic responses in the presence of third party stimulator cells (16, 18), soluble factors (13, 20, 29, 30), or activated helper cells (36). Our experiments revealed, however, that the responses against UV-irradiated or glutaraldehyde-fixed stimulator cells in the presence of conventional γ-irradiated third party stimulator cells were demonstrable but still markedly lower than responses against conventional γ-irradiated stimulator cells in corresponding control cultures or within the same cultures. This difference was obviously not explained by a general deficiency of helper factor in the culture on the basis of an unlinked helper effect, because the response against the conventional stimulator cells of the unrelated H-2 haplotype proceeded at a normal magnitude in the same culture. These observations thus excluded the possibility that the UV-sensitive function of the stimulator cells was *only* required for the activation of an unlinked helper effect (as schematically illustrated in Fig. 1*A*). Additional experiments (Table III) also excluded the possibility that the metabolically active (UV-sensitive) stimulator cell was *only* required for the activation of a

type of helper cell (or the production of an antigen-specific helper factor) that interacts with the CTL precursor cell through a cellular antigen bridge (antigenically linked helper effect as schematically illustrated in model B in Fig. 1). It has been suggested in several recent reports (10–12) that an antigenically linked helper effect operates in primary cytotoxic responses *in vivo* and involves a complex of helper cell (or antigen-specific helper factor), the CTL precursor cell, and a common stimulator cell that serves as an antigen bridge (see Fig. 1*B*). The interpretation that the UV-sensitive function of the stimulator cells was only required for the activation of such a helper cell was compatible with our experiments with UV-treated homozygous stimulator cells (Table I and Fig. 2), but was excluded by experiments with UV-treated heterozygous stimulator cells in combination with semi-allogeneic conventional stimulator cells (Table III). Cultures with γ-irradiated stimulator cells of a given mouse strain X plus UV-irradiated $(X \times Y)F_1$ stimulator cells revealed consistently, and in various strain combinations, a normal response against target cells of type X and a reduced response against target cells of type Y. The response against Y was indeed as low as the response of control cultures that contained completely unrelated sets of UV-irradiated and γ-irradiated stimulator cells (Table III). Because we tested only hybrid cells and not hybrid molecules, we cannot exclude that the UV-sensitive stimulator cells were required to activate a linked helper effect that is based on a noncellular antigen bridge. The simplest and most probable interpretation, however, is that the metabolically active (UV-sensitive) stimulator cell was required to interact directly with the CTL precursor cell under test as schematically illustrated in model C in Figure 1. The fact that UV irradiation of the stimulator cells reduced the cytotoxic responses only partly, and not completely, might be explained by the assumption that the activating signal can be delivered partly, although much less efficiently, in an unlinked fashion by the third party stimulator cells in the culture. It seems also possible that the normal spleen cell population contains different CTL precursor pools with different activation requirements. These interpretations may also explain why various types of antigen preparations on inactivated cells or nonliving particles (13, 16, 18, 20, 29, 30, 36–39) can stimulate some degree of cytotoxicity at least *in vitro*. Because cytotoxic responses *in vivo* are generally much weaker than *in vitro*, however, it is likely that only the optimal activation mechanism leads to substantial cytotoxic activity *in vivo*.

Splenic responder cells from CBA mice and some other H-2^k-bearing mice generated in the presence of conventional third party stimulator cells equally strong cytotoxic responses against the optimal dose of 5×10^6 UV-treated or conventional TNP-haptenated syngeneic stimulator cells. The comparably small effect of UV treatment on this kind of stimulator cells was in agreement with experiments of Hurme *et al.* (23). There were at least two possible explanations for this observation: either this type of response might have recruited a different set of CTL precursor cells with different stimulation requirements, or the TNP-haptenated antigen was transferred to nonirradiated antigen-presenting cells in the responder population and then displayed to the CTL precursor cells. The display of *acquired* antigen and a possibly active processing and presentation of antigen to CTL precursor cells was previously reported for the TNP-system by Pettinelli *et al.* (31), and was also inferred from the phenomenon of "cross-priming" in minor H antigen systems (40–43) and from related observations in the HY system (44).

The reason for the requirement of the metabolically active stimulator cells remains unknown. Our experiments combined with the previous reports that UV-irradiated (16, 20) or glutaraldehyde-fixed (13) stimulator cells activate substantial cytotoxic responses in the presence of soluble factor(s) suggested the possibility that the metabolically active stimulator cell delivers a soluble short-range mediator to the CTL precursor cell. However, all attempts to reconstitute the response against the UV-treated stimulator cells with helper factors such as concanavalin A-activated spleen cell supernatant or PMA-activated EL-4 supernatant were not successful. We assume that the previous reports were only dealing with the reconstitution of the response of a subset of CTL precursor cells. Another possibility is that UV-irradiated stimulator cells may be rapidly degraded in the cultures, and thereby cause a lack of antigen in the later phase of the culture. However, our experiments in Table V showed that the defect cannot be reconstituted by the addition of relatively large amounts of intact stimulator cells at day 2 of culture. If rapid degradation of antigen is indeed the explanation for our observations, this degradation would have to occur within hours. Considering the viability of the UV-treated cells in the trypan blue exclusion test, this may seem unlikely but is not formally excluded. Moreover, conventional and UV-treated stimulator cells persisted in practically equal numbers and similar proportions of trypan blue-positive cells when placed in culture without responder cells and counted at various time intervals of up to 4 days (data not shown).

The T cell-enriched nylon wool-nonadherent fraction of spleen cells was found to reconstitute the response against UV-treated stimulator cells and to stimulate by itself as effectively as the normal unfractionated spleen cell population. Additional experiments confirmed that the stimulatory activity of nylon wool-nonadherent spleen cells was also UV sensitive. The most obvious interpretation of these observations was that T cells can serve as stimulator cells for CTL precursor cells in conventional macrocultures. Several recent reports identified dendritic cells as the most potent stimulator cells for cytotoxic responses *in vitro* (13, 24–26), but did not distinguish whether these cells were the most effective stimulator cells for helper cells or CTL precursor cells directly. In one report it was demonstrated that dendritic cells produce interleukin 1 and are thus likely to activate interleukin 2-producing helper T cells (13). These recent reports therefore provide no direct contradiction to our results and conclusions. But the nylon wool-nonadherent fraction is certainly not a 100% pure T cell population. More rigorous cell fractionation experiments are certainly needed to identify the metabolically active stimulator cells that were shown to be required in our present experiments.

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