0022-1767/82/1281-0061$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 128, No. 1, January 1982
Printed in U.S.A.

INTERLEUKIN 2 IS NOT SUFFICIENT AS HELPER COMPONENT FOR THE ACTIVATION OF CYTOTOXIC T LYMPHOCYTES BUT SYNERGIZES WITH A LATE HELPER EFFECT THAT IS PROVIDED BY IRRADIATED I-REGION-INCOMPATIBLE STIMULATOR CELLS[1]

MATTHIAS REDDEHASE, WERNER SUESSMUTH, CORNELIA MOYERS, WERNER FALK, AND WULF DROEGE

From the Institut für Immunologie und Genetik, Deutsches Krebsforschungszentrum, D-69 Heidelberg, West Germany

Interleukin 2-containing supernatants from concanavalin A-activated spleen cells (CSCS) were found to provide strong helper activity for cytotoxic T lymphocyte (CTL) responses against allogeneic stimulator cells in microculture systems, but provided usually insufficient help for CTL responses against I-region compatible allogeneic or TNP-haptenated syngeneic stimulator cells. The interleukin 2-containing supernatant from HGG-activated AODH 7.1 hybridoma cells also mediated only relatively weak CTL responses against TNP-haptenated syngeneic cells in microcultures. Both types of supernatants, however, supported substantial responses against TNP-haptenated syngeneic stimulator cells if irradiated allogeneically activated syngeneic T cells or irradiated allogeneic spleen cells were added to the cultures. The allogeneic cells and the activated syngeneic T cells provided little helper activity if they were added in the absence of the interleukin 2-containing supernatants, thus demonstrating a synergistic effect between these 2 helper components. An I-region difference was sufficient for the helper effect of the allogeneic cells and control experiments showed that the presence of foreign I-region determinants could not be substituted for the TNP-haptenated stimulator cells.

The AODH 7.1 supernatant was shown to provide a helper effect only if it was added at day 0, and had no effect at day 3 of the culture; but its helper effect was enhanced by irradiated allogeneic cells irrespective of whether these cells were added at day 0 or day 3. This indicated that the allogeneic cells provided a helper effect that was required during the late phase of the CTL response and therefore was qualitatively different from the early helper effect of interleukin 2. The late addition of allogeneic cells could not be substituted by the AODH 7.1 supernatant.

Some of our CSCS batches and concentrated CSCS preparations were found to provide the late helper effect and to support CTL responses equally well in the presence or absence of I-region-incompatible cells. This indicated that the late helper effect was also mediated by a soluble mediator.

Finally, our experiments showed that the helper cells that responded to the I-region differences and synergized with CSCS were provided by the irradiated allogeneic spleen cell populations and also by the responder cells from lymph nodes, but were less frequent in thymic responder cells. Alloreactive helper cells were not detected in the irradiated trinitrophenylated stimulator cell population, indicating that these helper cells were sensitive to the trinitrophenylation procedure but were relatively resistant to irradiation.

It is well established that the activation of cytotoxic T lymphocyte (CTL)[2] precursor cells requires not only antigen, but also helper T cells and non-T accessory cells (1–15). Previous reports have shown 1) that accessory cells plus helper T cells of the phenotype Ly-1^+2^- are both required for the production of costimulator factor (interleukin 2) (11, 16), 2) that the accessory cells can be substituted by lymphocyte-activating factor (LAF, interleukin 1) from a macrophage-like cell line (11, 16), and 3) that the costimulator (interleukin 2) is required as a 2nd signal for the activation of CTL precursor cells (11, 17–20). The CTL precursor cells recognize antigen mainly in association with cell surface structures encoded by the K- or D-region of the murine major histocompatibility complex (MHC) whereas helper T cell precursors (i.e., Ly-1^+2^- cells), recognize antigen mainly (3, 21), but not exclusively (18, 19), in association with I-region-encoded structures.

Our present studies on the activation of CTL against MHC-compatible target cells reveal that interleukin 2 is insufficient to support the activation of CTL against MHC-compatible stimulator cells, but synergizes with a helper effect from allogeneic (I-region) stimulated helper T cells. This latter type of helper effect in allogeneic responses is usually provided by the irradiated allogeneic stimulator cells, and conventional concanavalin A-activated spleen cell supernatant (conventional CSCS)[3] preparations have therefore been successfully employed in the limiting dilution analysis of alloreactive CTL precursor frequencies (22). But this helper effect becomes limiting in CTL precursor frequency studies for MHC-compatible target cells.

MATERIALS AND METHODS

Animals. The A.TH, A.TL, and SJL mice were obtained from OLAC, Bicester, England, or in some cases from The Jackson Laboratory, Bar Harbor, ME. All other strains of mice were obtained from G1. Bomholtgaard, Ry, Denmark. The responder mice were usually 2 to 3 mo old.

Irradiation. Stimulator cells were irradiated with 1500 rad using a ^{60}Co source if not indicated otherwise.

The preparation of CSCS. Two types of preparations were used: conventional CSCS (CSCS I) was prepared by culturing spleen cells from mice or

Received for publication March 4, 1981.
Accepted for publication September 17, 1981.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] This work was supported in part by the Deutsche Forschungsgemeinschaft (SFB 136).

[2] Abbreviations used in this paper: CSCS, concanavalin A-activated spleen cell supernatant; CTL, cytotoxic T lymphocyte(s); CyP, cyclophosphamide; MHC, major histocompatibility gene complex; HEPES, *N*-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; HGG, human γ-globulin.

[3] The term "conventional CSCS" is used for the crude supernatant after addition of methylmannoside without further concentration or purification.

rats at a density of 3×10^8 cells in 30 ml GIBCO medium 199 containing 0.15 mg Con A (GIBCO) and 3×10^{-5} M 2-mercaptoethanol for 24 hr. The supernatant was then isolated by centrifugation at 300 × G for 10 min and stored frozen. Individual batches were tested and titrated before the application in experiments. α-Methylmannoside at a final concentration of 1 mm (0.194 mg/ml) plus HEPES[2] (5×10^{-3} M) was added to the supernatant in order to neutralize the contaminating Con A. CSCS II was prepared according to the procedure of Warren *et al.* (23) with minor modifications. In brief, spleen cells from (C3H × DBA/2)F_1 mice were cultured at a density of 10^7 cells/ml in 30-ml aliquots using RPMI 1640 GIBCO medium 199 containing glutamine, streptomycin-penicillin, HEPES, and 3×10^{-5} M 2-mercaptoethanol plus 0.005 mg/ml of Con A (GIBCO), but no fetal calf serum (FCS). The cells were cultured in 75 cm^2 plastique culture flasks (Falcon 3024) for 2 hr, the supernatants were discarded, and the cells were washed carefully 3 times with BSS and then cultured again in serum-free medium for 16 to 20 hr at 37°C. The supernatant was harvested, centrifuged to remove all cells, and concentrated 10× using an Amicon PM10 membrane. This factor was sterilized by filtration and stored at −20°C.

The preparation of an interleukin 2-containing supernatant from the hybridoma line AODH 7.1. The hybridoma line AODH 7.1 was originally produced by Kappler *et al.* (24) as an antigen-inducible interleukin 2-producing quadruple hybrid, and was kindly presented to us by Dr. C. G. Fathman. This line was maintained in RPMI 1640 containing 5×10^{-5} M 2-mercaptoethanol in tissue culture flasks at cell densities below 8×10^5/ml. For interleukin 2 production, 8×10^5 cells/ml were cultured in the presence of 10% FCS, 1 mg/ml human γ-globulin (HGG; SIGMA), and 5×10^6/ml 1500-rad irradiated B10.D2 spleen cells for 24 hr, and the supernatants were recovered by centrifugation and stored at −20°C.

Assay for interleukin 2 activity. The amount of interleukin 2 in the supernatants was measured by their ability to support the growth of the T cell clone W-2 (C57BL/6 spleen origin) (cloned by Michael Stöck, Hannover, West Germany). The cells were grown in RPMI 1640 containing 30% Con A-stimulated rat spleen (Wistar) cell supernatant, and 5% FCS. The cells were harvested by centrifugation, washed once with RPMI 1640, resuspended in RPMI 1640 with 10% FCS, and finally adjusted to a concentration of 10^5 cells/ml. The culture supernatants were titrated by serial 2-fold dilutions in flat-bottom tissue culture plates (Flow Laboratories) in a volume of 100 μl of RPMI 1640. One hundred microliters of the cell suspension were added per well, and the plates were incubated at 37° in 5% CO_2 for 20 to 24 hr. One microcurie of ^{3}H-TdR in 25 μl of RPMI 1640 was added per well, and the plates were incubated for another 4 to 6 hr. The cells were then harvested with a Skatron cell harvester (Flow Laboratories), and the filters were counted in a liquid scintillation counter. Each point was assayed in duplicate.

Induction and assay of CTL. Our procedures for the induction and assay of CTL in microcultures have previously been described in detail (25, 26) and were based on procedures of Shearer (27) and Teh *et al.* (15) with minor modifications.

The microcultures contained the indicated numbers of responder cells together with 3×10^5 TNP-haptenated irradiated (1500 rad) stimulator cells in the presence or absence of additional 3×10^5 irradiated (1500 rad) allogeneic spleen cells and/or the indicated amount of CSCS in 4 to 6 parallel microcultures of 0.2 ml if not indicated otherwise. The cultures were incubated for 5 days at 37°C under 5 % CO_2 and then tested for cytotoxic activity in a 4-hr ^{51}Cr-release assay (15, 27) using 2×10^4 haptenated or unmodified target cells.

The target cells were prepared by culturing spleen cells together with 0.002 mg/ml Con A (Pharmacia, Uppsala, Sweden) for 2 to 3 days as described previously (26). Stimulator and target cells were haptenated with 10 mM 2,4,6-trinitrobenzene sulfonic acid (Sigma) for 10 min at 37°C as described by Shearer (27).

The data from cytotoxic assays were indicated as specific ^{51}Cr release or as cytotoxic activities (*Nat*), which were computed according to the equation (15)

$$Nat = -\log_e\left(1 - \frac{P}{100}\right)$$

P = % specific ^{51}Cr release.

Macrocultures containing 1×10^7 responder cells and 1×10^7 irradiated allogeneic stimulator cells in 4.5 ml culture medium (26) were used to preactivate helper cells in some of our experiments.

RESULTS

Characterization of the various batches of CSCS and AODH 7.1 supernatant that were used in the subsequent experiments. The present experiments were essentially done with 2 preparations of AODH 7.1 supernatant (batches No. 1 and No. 2.7.) and 3 conventional CSCS preparations. The experiments in the subsequent tables and figures were performed mostly with batch No. 9 MR if not indicated otherwise. This preparation was obtained from DBA/2 spleen cells. The CSCS batch No. 2 was obtained from spleen cells of Sprague Dawley rats. CSCS batch No. 10 finally was obtained from (C3H × DBA/2)F_1 spleen cells. All these interleukin 2 preparations revealed only moderate differences with respect to the interleukin 2 titers (Fig. 1), but marked differences with respect to their helper effect in cytotoxic responses (see Figs. 2 and 6). The interleukin 2 titers were relatively reproducible in different experiments, but the height of the ^{3}H-TdR incorporation varied considerably. The exceptionally high cpm value for batch No. 2 in Figure 1 is therefore not meaningful.

The synergistic effect of irradiated I-region-incompatible cells and CSCS as helper components for CTL responses in microcultures. CSCS were previously shown to provide a convenient source for interleukin 2 and to support the long-term growth of T cell lines (28) as well as the primary activation of CTL in a number of systems (28–30). CSCS has also been successfully employed in studies on alloreactive CTL precursor frequencies (22), where it compensates for the insufficient numbers of helper T cells in the limiting-dilution conditions. In accordance with these reports, we found that small numbers of thymocytes or peripheral lymphocytes generated cytotoxic activity against allogeneic cells in microcultures in the presence, but not in the absence, of conventional CSCS (Fig. 2 and Table I). The 2 conventional CSCS preparations, No. 2 and No. 10, generated strong CTL activity in CBA lymph node cells against allogeneic SJL stimulator cells, but only batch No. 10 mediated a substantial CTL response against the TNP-haptenated syngeneic stimulator cells (Fig. 2). Experiments with a variety of CSCS batches revealed that most of our conventional CSCS preparations from rat or mouse spleen cells behaved like batch No. 2, whereas only a few preparations showed the

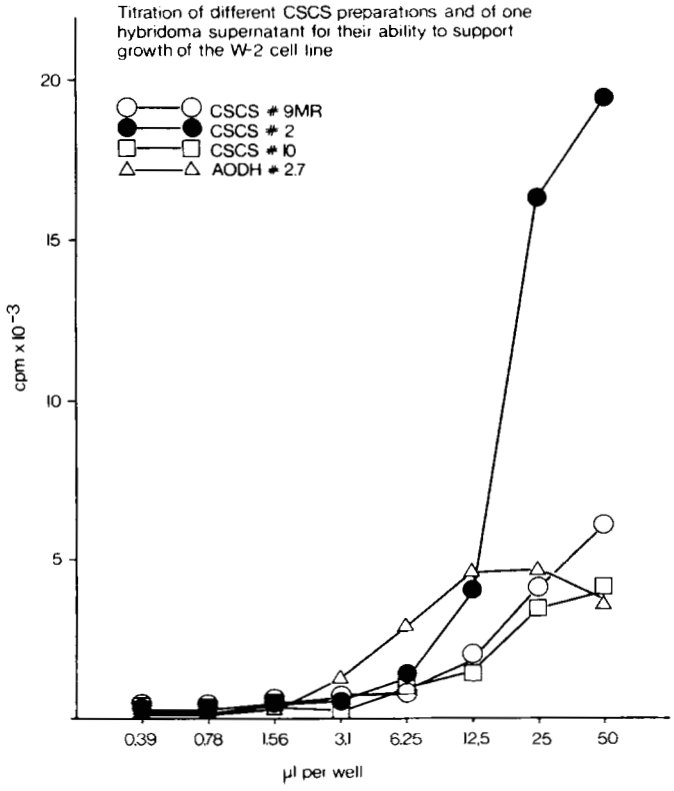

Figure 1. Titration of different CSCS preparations and of a AODH 7.1 hybridoma supernatant (batch No. 2.7.) for their ability to support growth of the T cell clone W-2. For details see *Materials and Methods*.

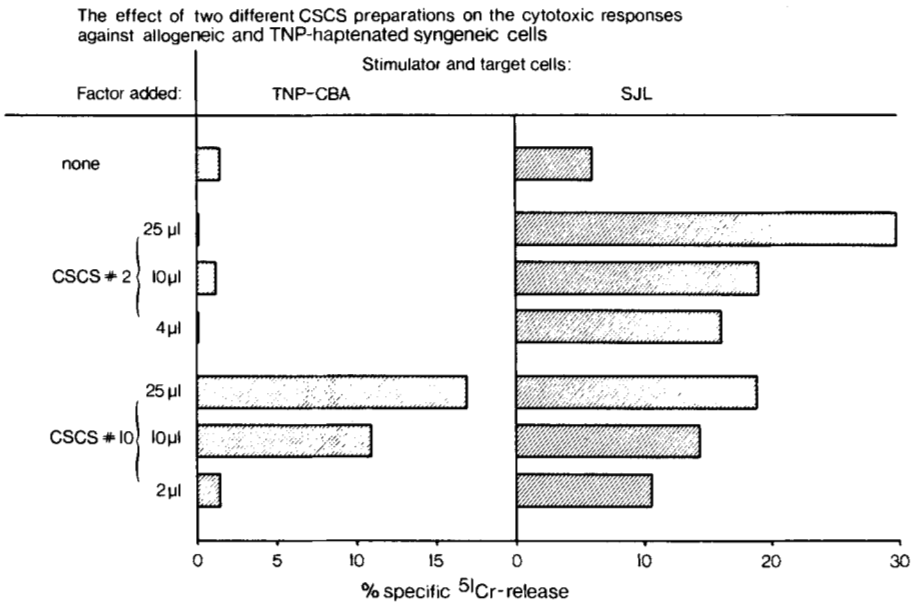

Figure 2. The effect of 2 different CSCS preparations on the cytotoxic responses against allogeneic and TNP-haptenated syngeneic cells. Five × 10^4 CBA lymph node cells were cultured together with 3 × 10^5 irradiated (1500 rad) SJL spleen cells or TNP-haptenated CBA spleen cells in the presence of the indicated factors for 5 days at 37°C and tested for cytotoxic activity on the homologous target cells. Both factors were found to support the allogeneic response, but only the factor No. 10 supported the cytotoxic response against the TNP-haptenated syngeneic cells.

TABLE I

Synergism between CSCS and an I-region disparity in the CTL responses of A.TH and A.TL cells against allogeneic cells[a]

Gr	Stimulator and Target Cells	CSCS Added	Responder Cells from			
			Lymph nodes		Thymus	
			A.TH ($K^sI^sD^d$)	A.TL ($K^sI^kD^d$)	A.TH ($K^sI^sD^d$)	A.TL ($K^sI^kD^d$)
1	CBA/J (H-2^k)	+	75.9 (1424 ± 104)	8.1 (85 ± 16)	74.6 (1370 ± 60)	10.4 (110 ± 21)
2	SJL (H-2^s)	+	8.2 (86 ± 15)	49.5 (684 ± 81)	9.4 (99 ± 16)	68.8 (1167 ± 177)
3	C57BL/6 (H-2^b)	+	70.3 (1213 ± 68)	35.5 (439 ± 61)	64.0 (1021 ± 115)	46.1 (618 ± 48)
4	C57BL/6 (H-2^b)	−	0.7 (7 ± 6)	0.4 (4 ± 5)	3.4 (35 ± 10)	2.5 (26 ± 9)

[a] Five × 10^4 lymph node cells or 2 × 10^5 thymus cells from A.TH or A.TL mice were cultured as responder cells together with the indicated stimulator cells (3 × 10^5) in the presence or absence of CSCS. The cytotoxic activities against haptenated syngeneic target cells are expressed as % specific ^{51}Cr-release (data in brackets indicate the cytotoxic activities Nat × 10^3 ± SE).

exceptional property of batch No. 10. The basis for this exceptional behaviour is presently under study.

Our experiments with responder cells from A.TH and A.TL mice (Table I) and A/J mice (Table II) revealed, moreover, that the conventional CSCS preparation (in this case, batch No. 9 MR) was not able to support the CTL responses against isolated H-2K or H-2D differences, but mediated strong responses against differences in the entire H-2 complex. This demonstrated a synergistic effect between the conventional CSCS and an I-region disparity in the system.

A similar synergy was also observed in CTL responses against TNP-haptenated syngeneic cells (Fig. 3). 4 × 10^4 (C3H × DBA/2)F_1 lymph node responder cells plus 3 × 10^5 TNP-haptenated and 1500-rad irradiated syngeneic stimulator cells generated strong CTL responses against TNP-haptenated target cells if cultured in the presence of 3 × 10^5 irradiated SJL spleen cells and 0.03 ml conventional CSCS (batch No. 9 MR); but even the 2- to 3-fold higher concentration of the same CSCS preparation in the absence of the irradiated allogeneic cells mediated only weak responses and allogeneic cells without CSCS also mediated no substantial responses (Fig. 3). This indicated that CSCS and irradiated allogeneic cells acted synergistically and contributed helper effects of different quality.

The concentrated CSCS II preparation that was prepared according to the procedure of Warren *et al.* (23) (Fig. 3) and the conventional CSCS preparation No. 10 (for example, see Fig. 6) were found to mediate equally strong responses in the presence or absence of allogeneic cells, indicating that the helper effect of the allogeneic stimulus could also be substituted by a soluble factor.

TABLE II

Requirement of an I-region difference for optimal cytotoxic responses of A/J cells against allogeneic cells in the presence of conventional CSCS[a]

Gr	Responder Cells	Stimulator and Target Cells	Responder Cells from	
			Lymph node	Thymus
1	A/J ($K^kI^kD^d$)	C57BL/6 ($K^bI^bD^b$)	31.6 (380 ± 36)	10.6 (112 ± 16)
2	A/J ($K^kI^kD^d$)	A.TH ($K^sI^sD^d$)	22.9 (260 ± 12)	7.6 (79 ± 19)
3	A/J ($K^kI^kD^d$)	A.TL ($K^sI^kD^d$)	6.0 (62 ± 24)	−0.4 (−4 ± 7)

[a] Three × 10^4 lymph node cells or 2 × 10^5 thymus cells from A/J mice were cultured as responder cells together with 0.04 ml CSCS and 3 × 10^5 stimulator cells of the indicated strain.

The requirement for additional allogeneic cells in the microculture response against TNP-haptenated syngeneic cells was found to be satisfied by irradiated I-region-incompatible cells (Table III). The response of A.TL responder cells against haptenated syngeneic stimulator cells in the presence of CSCS (batch No. 9 MR) was equally well enhanced by irradiated A.TH cells, which differed only in the I-region of the H-2 complex, as by C56BL/6 cells, which differed in the entire H-2 complex. Conversely, A.TH responder cells were also helped by irradiated A.TL cells.

The experiments in Figure 4 illustrated, finally, that the synergy between CSCS (batch No. 9 MR) and allogeneic cells already occurred at the relatively small dose of 3 × 10^4 allogeneic cells. A strong synergistic effect was also observed between CSCS No. 9 MR and syngeneic helper T cells (7, 8) that had been activated for 2 days in macrocultures against irradiated SJL cells, fractionated over nylon wool columns, and then irradiated with 1500 rad (indicated as MLR in Fig. 4).

The specificity of the CTL response in the presence of

Effect of conventional and concentrated interleukin preparations with or without allogeneic cells on the CTL responses against TNP-haptenated syngeneic cells

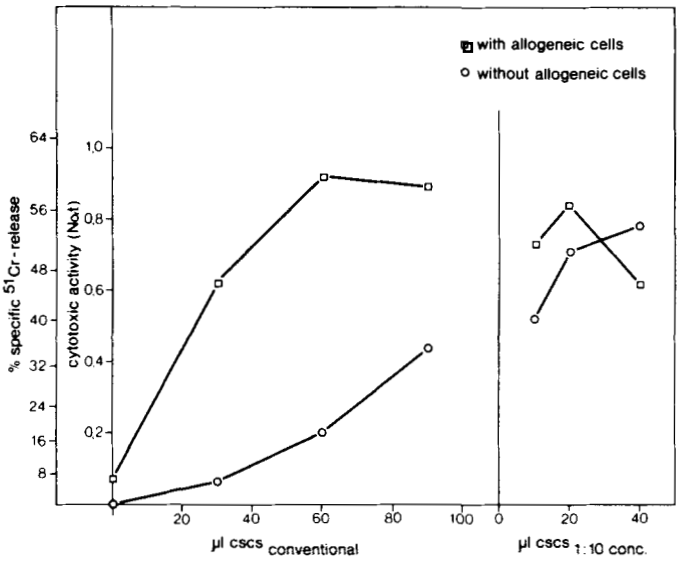

Figure 3. The effect of conventional concanavalin A-activated spleen cell supernatant (Batch No. 9 MR) and of a concentrated preparation (CSCS type II) on the CTL response against TNP-haptenated syngeneic cells in the absence or presence of additional irradiated allogeneic cells. Four × 10^4 lymph node cells from C3D2F1 mice were incubated in microcultures together with 3 × 10^5 1500 rad irradiated TNP-haptenated syngeneic stimulator cells with or without 3 × 10^5 1500 rad irradiated SJL spleen cells in the presence of the indicated amount of CSCS I preparation from C3D2F1 mice (*left panel*) or a CSCS II preparation that was prepared from DBA/2 spleen cells by the procedure of Warren *et al.* (23) (*right panel*).

TABLE III

Demonstration that an I-region disparity is sufficient for the allogeneic cells to synergize with CSCS[a]

Gr	Responder Cells	Allogeneic Cells Added	CSCS Added	Responder Cells from	
				Lymph node	Thymus
1	A.TL ($K^sI^kD^d$)	None	+	11.7 (125 ± 13)	9.5 (100 ± 11)
2	A.TL ($K^sI^kD^d$)	A.TH	−	1.2 (12 ± 17)	−0.7 (−7 ± 9)
3	A.TL ($K^sI^kD^d$)	A.TH	+	38.1 (479 ± 43)	43.0 (562 ± 37)
4	A.TL ($K^sI^kD^d$)	C57BL/6	+	49.6 (686 ± 35)	40.5 (520 ± 29)
5	A.TH ($K^sI^sD^d$)	None	+	4.1 (42 ± 11)	8.9 (94 ± 17)
6	A.TH ($K^sI^sD^d$)	A.TL	+	18.5 (204 ± 32)	40.2 (515 ± 41)
7	A.TH ($K^sI^sD^d$)	C57BL/6	+	22.0 (248 ± 27)	34.4 (421 ± 47)

[a] Eight × 10^4 lymph node cells or 4 × 10^5 thymus cells were cultured as responder cells together with 3 × 10^5 TNP-haptenated syngeneic 1500 rad irradiated stimulator cells in the presence or absence of 0.04 ml CSCS and/or additional 3 × 10^5 1500 rad irradiated allogeneic cells as indicated and were tested on haptenated syngeneic target cells 5 days later (for other details see legend to Table I).

allogeneic I-region determinants. The work of Billings *et al.* (31) indicated that allogeneic I-region determinants stimulate CTL precursor cells with specificity for TNP-haptenated syngeneic I-region determinants. This raised the possibility that the allogeneic determinants amplified the response by recruiting additional CTL precursor cells rather than by stimulating helper cells. The experiments in Table IV showed, however, that the cytotoxic activity of A.TH responder cells against TNP-haptenated syngeneic target cells after stimulation with TNP-haptenated A.TL stimulator cells was primarily directed against the haptenated H-2D^d determinants and not against the haptenated I^s or K^s determinants (compare groups 1, 2, and 4, or groups 12 and 13), although a strong cytotoxic activity against the haptenated or unhaptenated foreign I-region determinants (groups 3 and 14) was also observed. Thus, these experiments again supported the notion that the allogeneic determinants enhanced the response against the haptenated syngeneic targets by stimulating helper T cells rather than the self-MHC-restricted CTL precursor cells under test.

This conclusion was also supported by the fact that the allogeneic I-region determinants did not substitute for the TNP-haptenated stimulator cells. Again unhaptenated stimulator cells with allogeneic I-region determinants stimulated some cytotoxic activity against these I-region determinants, but not against TNP-haptenated syngeneic target cells (Table V).

The alloreactive helper T cells are located in the responder and in the irradiated allogeneic cell population. Combinations of homozygous responder cells and irradiated semi-allogeneic F_1 cells were used to locate the alloreactive helper T cell precursor in the system. It was found that thymic responder cells from DBA/2 mice responded against TNP-haptenated syngeneic stimulator cells only in the presence of conventional CSCS (batch No. 9 MR, 0.04 ml) plus irradiated allogeneic C3H, but not semi-allogeneic (C3H × DBA/2)F_1 spleen cells (Fig. 5). This experiment showed that the thymocytes contained substantial numbers of TNP-specific CTL precursor cells, but that even the large number of 10^5 thymocytes did not contain sufficient alloreactive helper T cell precursors to recognize the H-2^k determinants on the semi-allogeneic cells. It was therefore concluded that the substantial response in the presence of the allogeneic C3H cells was mediated by alloactivated helper T cells within this irradiated allogeneic cell population. DBA/2 lymph node cells, on the other hand, responded equally well in the presence of allogeneic and semi-allogeneic irradiated cells, indicating that even the small number of 12.5 × 10^3 peripheral lymphocytes contained sufficient numbers of alloreactive helper T cell precursors to recognize the foreign determinants on the semi-allogeneic cells (Fig. 5). CSCS in the absence of allogeneic cells may therefore help to identify and to assay helper cell precursors in the responder population. Control experiments also showed in this case that no cell combinations produced any detectable responses in the absence of CSCS (data not shown). This demonstrated again the synergistic effect. The experiment in Figure 5 implied also that the hapten-

Effect of additional irradiated allogeneic or MLR activated cells on the CTL response against TNP-haptenated syngeneic cells

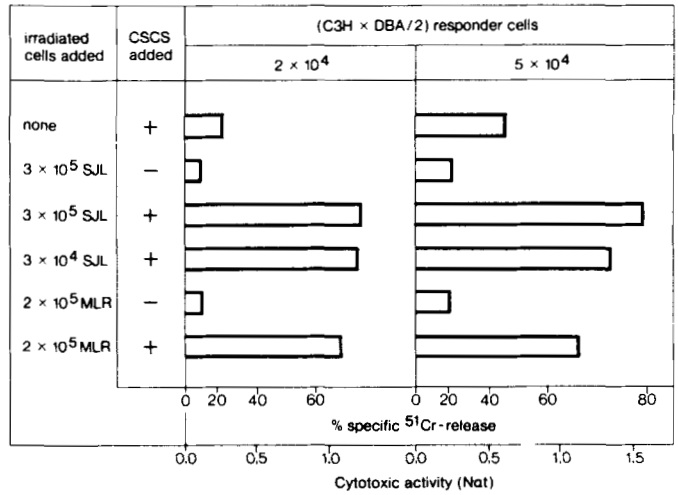

Figure 4. Effect of small numbers of allogeneic cells and of preactivated syngeneic helper T cells on the CTL response against TNP-haptenated syngeneic cells. The indicated numbers of responder cells from (C3H × DBA/2)F_1 lymph nodes were cultured together with 3 × 10^5 TNP-haptenated syngeneic stimulator cells with or without 0.08 ml conventional CSCS (batch No. 9 MR) and the indicated numbers of 1500 rad irradiated SJL spleen cells or preactivated syngeneic helper T cells. These syngeneic helper T cells have been activated by co-culturing with irradiated SJL-stimulator cells for 2 days in macrocultures and have been enriched for T cells by a fractionation over nylon wool columns and subsequently irradiated with 1500 rd (8).

TABLE IV

Specificity of the CTL responses against TNP-coupled haptenated stimulator cells with an I-region difference[a]

	Gr	Responder Cells	Stimulator Cells	Target	Responder Cells from	
					Lymph node	Thymus
Expt. 1	1	A.TH ($K^kI^kD^d$)	TNP-ATL ($K^kI^kD^d$)	TNP-ATH ($K^kI^kD^d$)	51.0 (713 ± 80)	67.4 (1121 ± 124)
	2	A.TH ($K^kI^kD^d$)	TNP-ATL ($K^kI^kD^d$)	TNP-SJL ($K^kI^kD^k$)	11.7 (125 ± 12)	14.5 (156 ± 24)
	3	A.TH ($K^kI^kD^d$)	TNP-ATL ($K^kI^kD^d$)	TNP-CBA ($K^kI^kD^k$)	31.8 (382 ± 51)	64.9 (1048 ± 106)
	4	A.TH ($K^kI^kD^d$)	TNP-ATL ($K^kI^kD^d$)	TNP-DBA/2 ($K^dI^dD^d$)	35.0 (430 ± 26)	58.9 (890 ± 56)
	5	A.TH ($K^kI^kD^d$)	TNP-ATL ($K^kI^kD^d$)	TNP-AJ ($K^kI^kD^d$)	39.7 (506 ± 104)	58.5 (880 ± 83)
	6	A.TH ($K^kI^kD^d$)	TNP-ATL ($K^kI^kD^d$)	TNP-BL/6 ($K^bI^bD^b$)	12.3 (131 ± 24)	21.8 (224 ± 36)
	7	A.TL ($K^kI^kD^d$)	TNP-ATH ($K^kI^kD^d$)	TNP-ATL ($K^kI^kD^d$)	28.0 (329 ± 93)	58.0 (866 ± 123)
	8	A.TL ($K^kI^kD^d$)	TNP-ATH ($K^kI^kD^d$)	TNP-SJL ($K^kI^kD^k$)	15.6 (170 ± 30)	36.8 (458 ± 71)
	9	A.TL ($K^kI^kD^d$)	TNP-ATH ($K^kI^kD^d$)	TNP-CBA ($K^kI^kD^k$)	7.9 (82 ± 16)	14.2 (153 ± 27)
	10	A.TL ($K^kI^kD^d$)	TNP-ATH ($K^kI^kD^d$)	TNP-DBA/2 ($K^dI^dD^d$)	19.0 (211 ± 62)	51.1 (716 ± 122)
	11	A.TL ($K^kI^kD^d$)	TNP-ATH ($K^kI^kD^d$)	TNP-BL/6 ($K^bI^bD^b$)	9.7 (102 ± 25)	15.9 (173 ± 36)
Expt. 2	12	A.TH ($K^kI^kD^d$)	TNP-ATL ($K^kI^kD^d$)	TNP-SJL ($K^kI^kD^k$)		0.4 (4 ± 12)
	13	A.TH ($K^kI^kD^d$)	TNP-ATL ($K^kI^kD^d$)	TNP-DBA/2 ($K^dI^dD^d$)		19.9 (221 ± 46)
	14	A.TH ($K^kI^kD^d$)	TNP-ATL ($K^kI^kD^d$)	A.TL ($K^kI^kD^d$)		29.6 (350 ± 37)

[a] One × 10^5 lymph node cells or 4 × 10^5 thymus cells from the indicated responder strains were cultured in the presence of 0.05 ml CSCS and 3 × 10^5 TNP-haptenated stimulator cells, and were finally tested on the indicated target cells. For other details see legend to Table I.

TABLE V

Specificity of the CTL response of A.TH responder cells against nonhaptenated stimulator cells with an I-region difference[a]

Gr	Target Cells	Cytotoxic Activity
1	CBA ($K^kI^kD^k$)	11.7 (125 ± 10)
2	C57BL/6 ($K^bI^bD^b$)	0.0 (1 ± 10)
3	A.TH	0.0 (0 ± 10)
4	TNP-A.TH	0.1 (1 ± 9)

[a] Three × 10^5 thymic responder cells from A.TH donors were cultured together with 0.05 ml CSCS and 3 × 10^5 nonhaptenated A.TL stimulator cells and tested on the indicated target cells. For other details see legend to Table I.

ated syngeneic stimulator cells, in contrast to the irradiated allogeneic cells, were not able to provide this type of helper effect even in the presence of I-region differences.

Effect of the supernatant from an interleukin 2-producing hybridoma cell line and the demonstration of a helper effect in the late phase of the cytotoxic response. The hybridoma line AODH 7.1 was produced by Kappler *et al.* (24) and was derived as a quadruple hybrid from the fusion of HGG-activated DBA/2 T cell blasts to an inducible interleukin 2-producing hybridoma line. The particular advantage of this line for our studies is that it produces interleukin 2 after activation with HGG, and that the resulting supernatant is therefore not contaminated with Con A. It is also expected that this supernatant contains a smaller variety of lymphokines than the conventional CSCS preparation, which is derived from a heterogenous mixture of cell types.

The AODH 7.1 supernatant batch No. 1 (A) and the conventional CSCS preparation No. 10 (C) that was selected to mediate equally good CTL responses in the presence or absence of allogeneic cells were added at day 0 and/or day 3 as helper components, with or without irradiated SJL cells (S), to cultures of CBA lymph nodes or thymus cells and irradiated TNP-haptenated CBA stimulator cells (Fig. 6). One hundred microliters of the culture supernatants were removed after centrifugation at day 3 and were replaced by 100 μl of fresh culture medium containing the helper component indicated in Figure 4. The experiment revealed that the AODH 7.1 supernatant by itself supported a clear-cut CTL response only in the case of 5 × 10^4 lymph node responder cells, but not with 2 × 10^4 lymph node or 2 × 10^5 thymic responder cells (Fig. 6, lines 3 and 4). The CSCS No. 10 preparation, in contrast, supported considerable responses also in the case of the thymic responder cells or the smaller numbers of lymph node cells (lines 8 and 11), indicating that sufficient numbers of CTL precursor cells were present in the thymus, and that endogenous helper cells in the lymph nodes were required to synergize with the AODH 7.1 supernatant. A more direct demonstration of a synergistic effect was revealed by lines 1 to 3: The CBA lymph node cells generated substantial responses when interleukin 2 (A) was given at day 0 and allogeneic cells (S) at day 3 (see line 2), whereas either component alone generated only weak or no substantial responses (lines 1 and 3). The AODH 7.1 supernatant provided a detectable helper effect only if added at day 0 and not at day 3 (compare lines 3, 4, and 5), and the same applied to its synergistic effect (compare lines 2 and 7). Moreover, the addition of the AODH 7.1 supernatant at day 3 did not substitute for the irradiated SJL cells (compare lines 2 and 4). The irradiated SJL cells, on the other hand, synergized equally well whether added at day 3 or day 0 (compare lines 1 and 6), indicating that their synergistic contribution was required only in the late phase of the response and was therefore qualitatively different from the early effect of the AODH 7.1 supernatant. The selected CSCS preparation No. 10, on the other hand, supported equally strong CTL responses in the absence and presence of the allogeneic cells (lines 8 to 11), but again only if added at day 0 and not at day 3 (lines 12 and 13).

The experiments in Figure 7 showed finally that CSCS batch No. 2 behaved like the AODH 7.1 supernatant (batch No. 2.7.) and provided no detectable helper effect in the late phase of the cytotoxic response, whereas CSCS batch No. 10 and the irradiated allogeneic SJL cells mediated a strong enhancement when added at day 3 to the microcultures (Fig. 7).

DISCUSSION

Our experiments revealed that the conventional CSCS preparations are sufficient to support cytotoxic responses against allogeneic cells but are usually insufficient as helper components to support CTL responses against MHC- or I-region-compatible stimulator cells in microcultures and the same applied to the interleukin 2-containing supernatant from HGG-activated AODH 7.1 hybridoma cells. However, both types of supernatants mediated substantial CTL responses in the presence of irradiated I-region-incompatible cells or allogeneically activated irradiated syngeneic T cells, which by themselves were not able to support substantial CTL responses in microcultures. This observation indicated that interleukin 2 synergized with a 2nd type of helper effect that was activated in the presence of an I-region difference. Only some exceptional CSCS preparations (i.e., batch No. 10) or the concentrated

The effect of irradiated allogeneic and semiallogeneic cells on the CTL responses against TNP-haptenated syngeneic cells

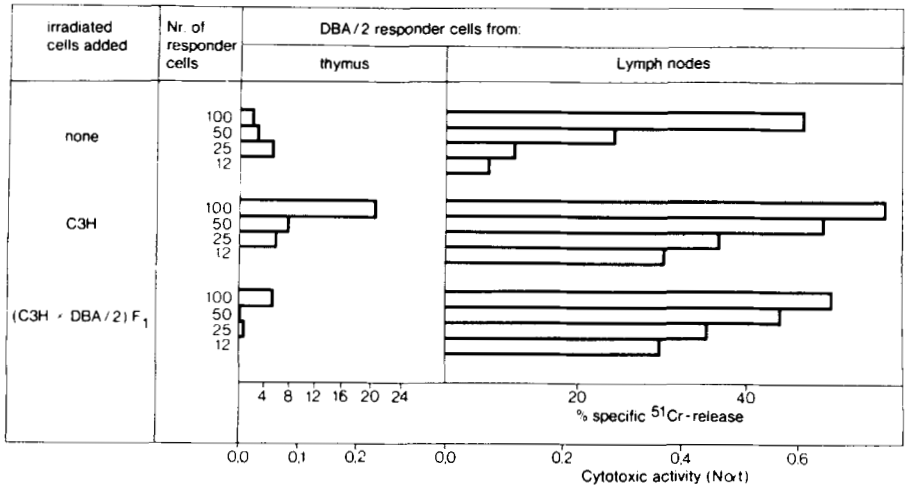

Figure 5. The synergistic effect of allogeneic and semi-allogeneic cells with conventional CSCS (batch No. 9 MR) on the CTL responses of thymic and peripheral lymphocytes against TNP-haptenated syngeneic cells. Graded numbers of responder cells from DBA/2 lymph nodes or thymus were cultured together with 3×10^5 TNP-haptenated syngeneic stimulator cells and 0.04 ml CSCS No. 9 MR with or without 3×10^5 irradiated C3H or (C3H × DBA/2)F_1 spleen cells. The cytotoxic activity was tested after 5 days on TNP-haptenated DBA/2 target cells. Control cultures without CSCS were included in the experiment for all experimental groups but generated in all cases not more than background cytotoxicity.

Synergy between AODH supernatant and allogeneic cells after addition at different times

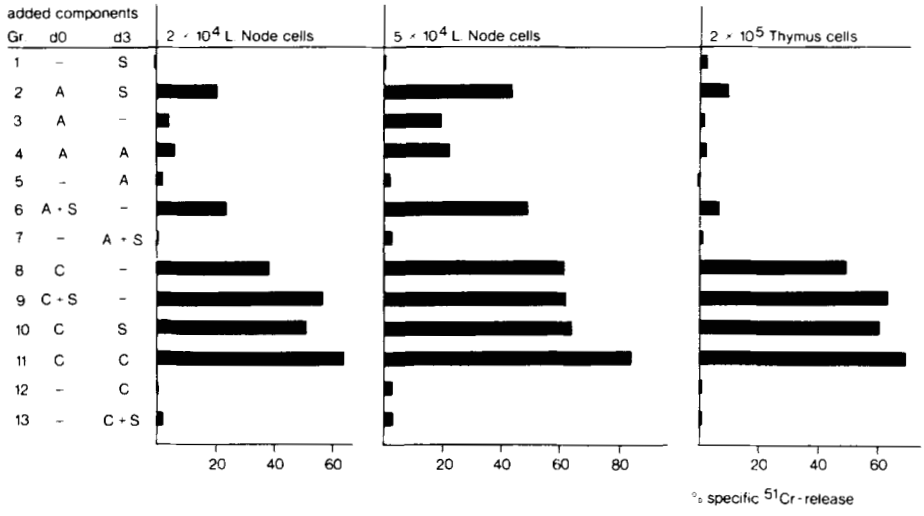

Figure 6. Synergy between AODH 7.1 supernatant batch No. 1 and allogeneic cells after addition at different times. Responder cells from CBA lymph nodes (2 or 5×10^4) or thymus (2×10^5) were cultured together with 3×10^5 TNP-haptenated and 1500 rad irradiated syngeneic spleen cells either alone (—) or together with 0.05 ml AODH 7.1 supernatant (*A*), 0.05 ml of the CSCS preparation No. 10 (*C*), 3×10^5 1500 rad irradiated SJL spleen cells (*S*) or combinations of these as indicated (*d0*). Three days later, the cultures were centrifuged, 0.1 ml culture supernatant was removed and replaced by 0.1 ml fresh culture medium containing the indicated components (*d3*). The cultures were incubated for another 2 days and then tested for CTL activity on TNP-CBA targets.

and partially purified CSCS preparation according to the procedure of Warren *et al.* (23) were able to support equally good CTL responses in the presence or absence of I-region-incompatible cells (see Figs. 3 and 6), indicating that the allogeneically activated helper effect is also mediated by a soluble factor.

Several experimental observations indicated that the MHC disparity in the culture provided an antigenic stimulus mainly for helper T cell precursors rather than for the CTL precursor cells under test. First, it was established that an I-region disparity was required, sufficient for the synergistic effect to occur (Tables I–III), and it was previously shown (4–6) that helper T cell precursors preferentially recognize I-region determinants, whereas CTL precursor cells preferentially recognize K- or D-region determinants. The synergistic effect was observed regardless of whether the foreign I-region determinants were presented on the stimulator cells for the CTL precursor cells (i.e., on the TNP-haptenated cells) or on separate cells (unlinked recognition). This observation was at variance with the model proposed by Paetkau *et al.* (17), but was in line with the earlier experiments of Bach, Alter, *et al.* (4–6) and of others (18), and demonstrated that this helper function is nonspecific in its effect. Our experiments also showed that the allogeneic cells could not be substituted for the TNP-haptenated stimulator cells, indicating again that they did not stimulate the TNP-specific CTL precursor cells under test, but rather some helper cells (Table V). Previous experiments of Billings *et al.* (31) suggested the possibility that the allogeneic I-region determinants might have recruited additional CTL precursor cells with specificity for TNP-haptenated syngeneic I-region determinants. Our experiments on this point showed, however, that the cytotoxic activity on TNP-haptenated syngeneic I-region determinants was low and would not have contributed significantly to the strong responses against the TNP-haptenated syngeneic K- or D-region determinants (see Table IV). The experiments of Billings *et al.* (31) were performed in macrocultures and indeed showed substantial kill on TNP-haptenated syngeneic I-region determinants only at relatively high killer-to-target cell ratios (i.e., 100:1).

Two lines of evidence supported the assumption that interleukin 2 and the additional I-region disparity provided helper signals of different quality and not merely quantity. First, the various conventional CSCS preparations and the AODH 7.1 supernatant showed similar interleukin 2 titers (Fig. 1) but differed strikingly in the ability to support a CTL response in an MHC-compatible system (Fig. 2). Moreover, titration of the

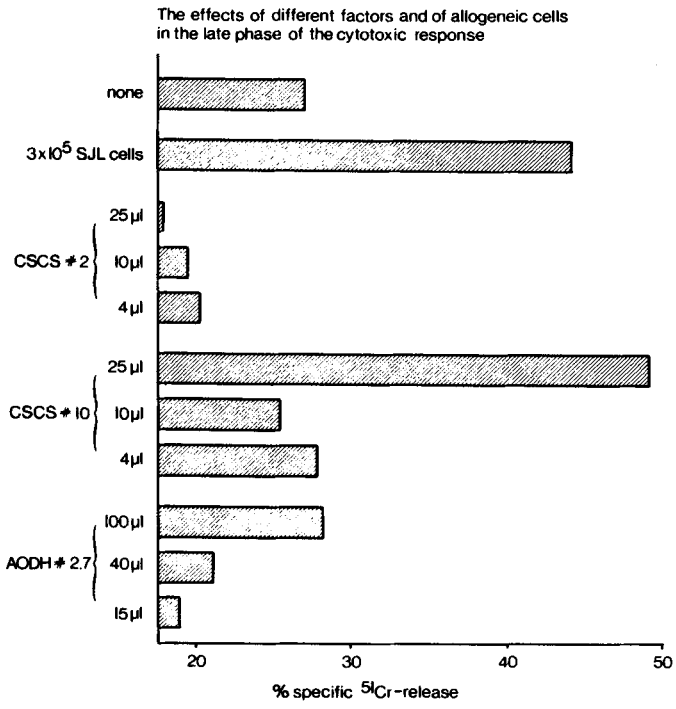

Figure 7. The effect of different factors and of allogeneic cells in the late phase of the cytotoxic response. Two × 10^4 CBA lymph node cells were cultured together with 3 × 10^5 TNP-haptenated CBA spleen cells and 0.02 ml CSCS No. 10 in 0.2 ml microcultures for 3 days. The microculture plates were then centrifuged, 0.1 ml supernatant was removed from each culture and replaced by 0.1 ml fresh culture medium containing the indicated helper factors or allogeneic cells. The cultures were incubated for another 2 days and assayed for cytotoxic activity on TNP-haptenated CBA target cells.

CSCS No. 9 MR in the presence and absence of allogeneic cells (Fig. 3) revealed that the combination of allogeneic cells and a low concentration (i.e., 30 µl) of CSCS supported a substantial CTL response, whereas even the 2- or 3-fold higher amount of CSCS alone or the allogeneic cells alone mediated only weak responses. Second, experiments with the AODH 7.1 supernatant revealed that the supernatant provided a helper effect only if applied at day 0 and no effect if given at day 3, whereas the synergistic effect of allogeneic cells was equally strong regardless of whether they were added at day 3 or day 0 (Fig. 6). Since the AODH 7.1 supernatant was known to contain high interleukin 2 activity (see *Materials and Methods*), it was concluded that interleukin is required relatively early and that the allogeneic stimulus provided a 2nd type of helper effect in the late phase of the CTL response. Expectedly, the CSCS batch No. 10 also provided this late type of helper effect (Fig. 7). The latter finding indicated that the late helper effect is also mediated by a soluble factor.

Our experiments showed, furthermore, that the synergizing helper cells that respond to the I-region difference are located both in the responder cell population (except for thymic responder cells) and in the irradiated stimulator cell population. The addition of irradiated semi-allogeneic F_1 cells to parental responder cells (Fig. 5) showed clearly that alloreactive helper cells were present in responder cells from lymph nodes but were not detectable in the thymus or in the trinitrophenylated cell population. This was consistent with the observation of Pilarski and Baum (7, 8) and Wagner *et al.* (32) that thymic responder cells produced poor CTL responses unless they were supplemented with an additional source of help. The substantial response of thymocytes in the presence of the completely allogeneic, but not semi-allogeneic, cells showed, on the other hand, that the irradiated allogeneic population was able to recognize the MHC-incompatible responder or TNP-haptenated stimulator cell population and to respond to this antigen by delivering the helper effect. This also showed that these helper cells were relatively radiation resistant even before their activation (see also Ref. 7). The observation that helper effects can be obtained from irradiated helper cells with specificity for determinants on the CTL precursor cells under test has previously been reported by Corley *et al.* (33). The fact that the irradiated TNP-haptenated stimulator cells were obviously not able to mediate a helper effect in the presence of irradiated semi-allogeneic cells (Fig. 5) suggested, on the other hand, that the trinitrophenylation procedure destroyed the potential helper activity of these cells.

Conventional CSCS preparations have previously been used successfully for the determination of alloreactive CTL precursor cell frequencies in limiting-dilution experiments (22). These studies were obviously not affected by the insufficient helper effect of these interleukin 2 preparations, because irradiated allogeneic cells were present in the cultures and presumably supplied the synergistic helper effect. Our experiments with I-region-compatible, K-/D-region-incompatible stimulator cells indicated, on the other hand, that allogeneic I-region determinants stimulate this synergistic helper effect more efficiently than K- or D-region determinants. This is in line with earlier observations on the specificity of helper T cells (4–6), but not in line with 2 more recent reports that helper T cells are also activated to some extent by K- or D-region determinants (18, 19).

Plate (34) reported recently that the activation of TNP-specific CTL in macrocultures requires an MHC-restricted helper factor if Ia^+ T cells are removed from the responder cell population. These studies thus suggested that the activation of CTL requires different helper components but it was not clear from the experiments whether the MHC-restricted helper factor was needed in addition to interleukin 2, or whether it was a part of the same helper pathway and able to overcome the requirement for interleukin 2. Our observation that the CSCS batch No. 10 from (C3H × DBA/2)F_1 spleen cells, in contrast to most other CSCS preparations, was able to support the response of the CBA responder cells in the absence of I-region-incompatible stimulator cells suggested the possibility that an MHC-restricted 2nd helper factor was indeed detected in these experiments. But the helper effect of C3H cells on DBA/2 responder cells (Fig. 5) was not consistent with this interpretation. Clearly, this point needs further investigation.

REFERENCES

1. Wagner, H. 1973. Cell-mediated immune responses in vitro: interaction of thymus-derived cells during cytotoxic allograft responses in vitro. Science 181:1170.
2. Wagner, H. M., M. Röllinhoff, and G. J. V. Nossal. 1973. T-cell mediated immune responses induced in vitro: a probe for allograft and tumor immunity. Transplant. Rev. 17:3.
3. Cantor, H., and E. A. Boyse. 1975. Co-operation between subclasses of Ly bearing cells in the generation of killer activity. J. Exp. Med. 141:1390.
4. Bach, F.-H., C. Grillot-Courvalin, O. J. Kuperman, H. W. Sollinger, C. Hayes, P. M. Sondel, B. J. Alter, and M. L. Bach. 1977. Antigenic requirements for triggering of cytotoxic T lymphocytes. Immunol. Rev. 35:76.
5. Bach, F. H., M. Segall, K. S. Zier, P. M. Sondel, B. J. Alter, and M. L. Bach. 1973. Cell-mediated immunity: separation of cells involved in recognitive and destructive phases. Science 180:403.
6. Alter, B. J., D. J. Schendel, M. L. Bach, F. H. Bach, J. Klein, and J. Stimpfling. 1973. Cell-mediated lympholysis: importance of serologically defined H-2 regions. J. Exp. Med. 137:1303.
7. Pilarski, L. M. 1977. A requirement for antigen-specific helper T cells in the generation of cytotoxic T cells from thymocyte precursors. J. Exp. Med. 145:709.

8. Baum, L. L., and L. M. Pilarski. 1978. In vitro generation of antigen-specific helper T cells that collaborate with cytotoxic T-cell precursors. J. Exp. Med. 148:1579.
9. Melief, C. J., M. Y. van der Meulen, B. J. Christiaans, and P. De Greeve. 1979. Cooperation between subclasses of T lymphocytes in the in vitro generation of cytotoxicity against a mutant H-2K difference. An analysis with anti-Lyt antisera. Eur. J. Immunol. 9:7.
10. Zinkernagel, R. M., G. N. Callahan, A. Althage, S. Cooper, J. W. Streilein, and J. Klein. 1978. The lymphoreticular system in triggering virus plus self-specific cytotoxic T cells: evidence for T help. J. Exp. Med. 147:897.
11. Shaw, J., B. Caplan, V. Paetkau, L. M. Pilarski, T. L. Delovitch, and F. C. McKenzie. 1980. Cellular origins of co-stimulator (IL2) and its activity in cytotoxic T lymphocyte responses. J. Immunol. 124:2231.
12. Schilling, R. M., R. A. Phillips, and R. G. Miller. 1976. Requirement for non-T-cells in the generation of cytotoxic T lymphocytes in vitro. I. Use of nude mice as source of non-T cells. J. Exp. Med. 144:241.
13. Miller, R. G., R. M. Schilling, and R. A. Phillips. 1977. Requirement for non-T-cells in the generation of cytotoxic T lymphocytes (CTL) *in vitro*. II. Characterization of the active cells in the spleen of nude mice. J. Immunol. 118:166.
14. Lutz, C. T., and F. W. Fitch. 1979. Accessory cell requirements for the generation of cytolytic T lymphocytes. J. Immunol. 122:2598.
15. Teh, H.-S., E. Harley, R. A. Phillips, and R. G. Miller. 1977. Quantitative studies on the precursors of cytotoxic lymphocytes. I. Characterization of a clonal assay and determination of the size of clones derived from single precursors. J. Immunol. 118:1049.
16. Farrar, J. J., S. B. Mizel, J. Fuller-Farrar, W. L. Farrar, and M. L. Hilfiker. 1980. Macrophage-independent activation of helper T cells. I. Production of interleukin 2. J. Immunol. 125:793.
17. Paetkau, V., J. Shaw, G. Mills, and B. Caplan. 1980. Cellular origins and targets of co-stimulator. Immunol. Rev. 51:157.
18. Wagner, H., C. Hardt, K. Heeg, K. Pfitzenmaier, W. Solbach, R. Bartlett, H. Stockinger, and M. Röllinghoff. 1980. T-T cell interactions during cytotoxic T lymphocyte (CTL) responses: T cell-derived helper factor (interleukin 2) as a probe to analyze CTL responsiveness and thymic maturation of CTL progenitors, Immunol. Rev. 51:215.
19. Lafferty, K. J., L. Andrus, and S. J. Prowse. 1980. Role of lymphokine and antigen in the control of specific T cell responses. Immunol. Rev. 51:279.
20. Lalande, M. E., M. J. McCutcheon, and R. G. Miller. 1980. Quantitative studies on the precursors of cytotoxic lymphocytes. VI. Second signal requirements of specifically activated precursors isolated 12 hr after stimulation. J. Exp. Med. 151:12.
21. Nagy, Z., B. E. Elliott, and M. Nabholz. 1976. Specific binding of K- and I-region products of the H-2 complex to activate thymus-derived (T) cells belonging to different Ly subclasses. J. Exp. Med. 144:1545.
22. Fischer-Lindahl, K., and D. B. Wilson. 1977. Histocompatibility antigen-activated cytotoxic T lymphocytes. II. Estimates of the frequency and specificity of precursors. J. Exp. Med. 145:508.
23. Warren, H.-S., J. A. Woolnough, and K. J. Lafferty. 1978. Cytotoxic T cell responses to a syngeneic tumor. Conditions for primary activation in vitro. Austr. J. Exp. Biol. Med. Sci. 56:247.
24. Kappler, J. W., B. Skidmore, J. White, and P. Marrack. 1981. Antigen-inducible, H-2-restricted, interleukin-2-producing T cell hybridomas. Lack of independent antigen and H-2 recognition. J. Exp. Med. 153:1198.
25. Dröge, W., M. Reddehase, and M. Wolf. 1981. Expression of subregion and haplotype preference for H-2K^k restricted cytotoxic T-cell responses in vivo and at the level of the precursor frequencies. Cell. Immunol. 63:53.
26. Galli, H. P., and W. Dröge. 1980. Development of cytotoxic T lymphocyte precursors in the absence of the thymus. Eur. J. Immunol. 10:87.
27. Shearer, G. M. 1974. Cell-mediated cytotoxicity to trinitrophenyl-modified syngeneic lymphocytes. Eur. J. Immunol. 4:527.
28. Reviewed in: Göran Möller, ed. 1980. "T cell stimulating growth factors," Transpl. Rev. 51.
29. Talmage, D. W., J. A. Woolnough, H. Hemmingsen, L. Lopez, and K. J. Lafferty. 1977. Activation of cytotoxic T cells by nonstimulating tumor cells and spleen cell factor(s). Proc. Natl. Acad. Sci. 74:4610.
30. Rulon, K., and D. W. Talmage. 1979. Enhancement of the primary cytotoxic response to membrane by a lymphokine costimulator. Proc. Natl. Acad. Sci. 76:1994.
31. Billings, P., S. Burakoff, M. E. Dorf, and B. Benacerraf. 1977. Cytotoxic T lymphocytes induced against allogeneic I-region determinants react with Ia molecules on trinitrophenyl-conjugated syngeneic target cells. J. Exp. Med. 146:623.
32. Wagner, H., M. Röllinghoff, K. Pfitzenmaier, C. Hardt, and G. Johnscher. 1980. T-T cell interactions during in vitro cytotoxic T lymphocyte (CTL) responses. II. Helper factor from activated Lyt 1$^+$ T cells is rate limiting i) in T cell responses to nonimmunogenic alloantigen, ii) in thymocyte responses to allogeneic stimulator cells, and iii) recruits allo- or H-2 restricted CTL precursors from the Lyt 123$^+$ T subset. J. Immunol. 124:1058.
33. Corley, R. B., K. A. Switzer, D. E. Hudson, and M. A. Cooley. 1980. Multiple pathways of T-T interaction in the generation of cytotoxic T lymphocytes to alloantigens. J. Exp. Med. 51:1125.
34. Plate, J. 1981. Major histocompatibility complex restriction of soluble helper molecules in T cell responses to altered self. J. Exp. Med. 153:1102.