PRIMARY IN VITRO CYTOTOXIC T CELL RESPONSE TO NON-MAJOR HISTOCOMPATIBILITY COMPLEX ALLOANTIGENS IN NORMAL MICE*

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Primary in vitro cytotoxic T cell (T_c)[1] responses to non-major histocompatibility complex (non-MHC) alloantigens in normal mouse strains have not, to date, been convincingly demonstrated, although strong in vitro secondary responses are readily obtained after in vivo priming (1–3). However, responder lymphocytes from mice of the autoimmune strain, NZB, have been shown to mount a primary T_c response to H-2-compatible BALB/c stimulator cells (4–8). This activity has been interpreted as being due to a hyperactive helper cell response in the NZB mice (7). One group (8) described primary in vitro non-MHC alloantigen-specific T_c responses in cells from B10.BR mice, but emphasized that this occurred in only a few of their experiments. All this work (4–8), however, was carried out in culture media supplemented with xenogeneic serum, usually fetal calf serum (FCS), which has been shown to stimulate helper cell activity (9), and of greater concern, has been shown to stimulate a polyclonal T_c response in which self as well as allogeneic determinants are recognized by T_c (10). It has also been claimed that FCS, most commonly used as a culture medium serum supplement, contains antigenic determinants that can stimulate and be recognized by T_c (11).

In the present paper, we have found that, whereas FCS-supplemented medium supported the development of anti-non-MHC T_c responses in NZB mice, no such activity could be observed in autologous mouse serum (MS) -supplemented media. This finding suggests that if the responsiveness to non-MHC alloantigens is indeed due to hyperactive helper cell activity in the NZB mice as postulated (7), then these helper cells are FCS dependent.

Furthermore, we also demonstrate for the first time, using a limiting dilution assay (LDA) (12), that it is possible to generate a strong specific primary T_c response to a non-MHC alloantigens using responding cells from a normal mouse strain (BALB/c) in culture medium without FCS supplemented with low amounts of autologous MS, provided that there is an adequate helper stimulus or source of exogenous help. This response is not caused by the nonspecific effect of added interleukin 2 (IL-2) on unstimulated cells, and the cytotoxic activity itself is specific. However, actual

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[1] *Abbreviations used in this paper:* C, complement; Con A, concanavalin A; Con A SN, lectin-free supernatant from Con A-stimulated spleen cells; FCS, fetal calf serum; IL-2, interleukin 2; LDA, limiting dilution assay; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; MR, maximal release; MS, mouse serum; SR, spontaneous release; T_c, cytotoxic T cell; 2° MLR SN, supernatant from secondary MLR culture.

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suppression of cytotoxicity was observed at higher responder cell numbers. This may explain or contribute to the absence of a primary in vitro T_c response to non-H-2 alloantigens in conventional bulk cultures with MS-supplemented media, as well as the inconsistent responses in FCS-supplemented media (8). Furthermore, we have demonstrated that indeed a high level of nonspecific suppressor activity, mediated by Lyt-1^+2^- cells, is generated in conventional bulk primary anti-non-H-2 cultures.[2]

Materials and Methods

Mice. The BALB/c, DBA/2, CBA/J, and NZB mice used for the experiments were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were 6–12 wk old and were sex matched in all experiments.

Bulk Culture Generation and Assay of T_c. T_c were generated in bulk mixed lymphocyte reactions (MLR) cultures. Normal spleen responder cells (2.5×10^6) were incubated with 5×10^6 x-irradiated (2,000 rad) stimulator spleen cells in a final vol of 2.5 ml, consisting of medium only or medium plus an exogenous source of IL-2. The medium was either (*a*) FCS medium: RPMI 1640 supplemented with 10% FCS, 1% glutamine (200 mM), 1% nonessential amino acids, 1% penicillin (10^4 μg/ml), 1% streptomycin (10^4 μg/ml), and 5×10^{-5} M 2-mercaptoethanol; or (*b*) MS medium: complete extra-high amino acids (EHAA) (13) supplemented with 0.6% MS isologous to the responder (no FCS) and the same nutrients and antibiotics used for FCS medium. All media components were obtained from Gibco Laboratories (Grand Island Biological Co., Grand Island, NY), except FCS, which was purchased from Sterile Systems (Logan, UT). After 6 d of culture in a humidified atmosphere of 5% CO_2, the effector cells were harvested, counted, appropriately diluted, and dispensed together with 10^4 ^{51}Cr-labeled target cells (P815 mastocytoma cells or concanavalin A [Con A] blast cells [2]) in the wells of U-bottomed microtiter plates (Flow Laboratories, Rockville, MD) and incubated for 4 or 8 h in a final vol of 0.2 ml. At the end of the incubation, the plates were centrifuged for 5 min at 1,000 *g*, and 0.1 ml of supernatant was removed from each well and individually counted in a gamma counter. Controls containing 0.1 ml of medium or 1 N HCl instead of effector cells gave the values of spontaneous release (SR) and maximal release (MR), respectively. Percent cytotoxicity was calculated as follows: percent cytotoxicity = ([experimental (cpm) − SR (cpm)]/[MR (cpm) − SR (cpm)]) × 100. Data shown are the arithmetic means of four replicate samples ± standard deviation.

LDA. Varying numbers of BALB/c responder cells were cultured with 5×10^5 x-irradiated (2,000 rad) DBA/2 stimulator cells in FCS medium or MS medium in a final vol of 0.2 ml in the wells of V-bottomed mictotiter plates (Flow Laboratories), containing, where stated, 50% secondary MLR supernatants (2°MLR SN) as a source of IL-2 (see section below on sources of IL-2 for details). After 6 d of culture, 0.1 ml of medium was removed from each well and 5×10^3 ^{51}Cr-labeled P815 cells were added in 0.1 ml. 20 replicate samples were set up for each responder cell number. Percent cytotoxicity was calculated for each well as described above, using the mean obtained from wells containing stimulators and no responders as the value for SR. Wells showing levels of cytotoxicity >3 SD above the SR were scored positive. Only experiments in which the data conformed to a Poisson distribution could be used to calculate the frequency of T_c precursors responding to non-H-2 alloantigens (12). We accept as usable those results in which the observed chi-square value exceeds the criterion of $P = 0.10$. The natural logarithm of the fraction of nonresponding wells is plotted against the number of responding cells cultured. The intercept of the straight line produced estimates the number of distinct cell types required for a positive response over the dilutions tested. If the straight line goes through the origin, one cell type is required and the slope of the line gives the frequency of such T_c precursors in the responding population (12).

To calculate cytotoxic effect per precursor, values of 100 N α t (14) were computed for each positive well. This value was found to be directly proportional to the number of T_c in a given population, whereas percent cytotoxicity was not. 100 N α t is calculated by the formula: 100

[2] Macphail, S., and O. Stutman. Suppressor T cells activated in a primary in vitro response to non-major histocompatibility antigens. Manuscript submitted for publication.

$N \alpha t = -100\ 1N\ (1\text{-}P)$, where N = number of sensitized cells, α = a constant chosen to fit the data, t = the time of incubation, and P = percent cytotoxicity ÷ 100.

The total cytotoxic effect per responder cell group was calculated by adding the 100 $N \alpha t$ values for all positive wells. The total number of precursors in the group was computed from the frequency data. From these values, the cytotoxic effect generated per precursor, in each group, was estimated.

Sources of IL-2. Two sources of exogenous IL-2 were used. (*a*) Lectin-free IL-2 from Con A-activated DBA/2 spleen cells (Con A SN) was prepared by washing the cells three times after a 2-h exposure to Con A and culturing them for a further 24 h (15). The cell suspensions were harvested and centrifuged at 1200 *g* for 10 min. The supernatants were aspirated, millipore filtered, and stored at −20°C. (*b*) Supernatants from 2°MLR SN (16) were harvested and stored as for (*a*). IL-2 preparations were carried out in both FCS and MS media and were used appropriately. Thus, all the experiments in MS medium were done with IL-2 prepared in MS medium.

Results

Generation of T_c in Bulk Culture. Initially, we attempted to generate primary T_c responses to non-MHC alloantigens by supplementing bulk one-way MLR cultures with Con A SN as a source of IL-2, a lymphokine shown to provide the required helper stimulus for anti-H-2 T_c responses (17), support the development of anti-tumor T_c (18), and induce the differentiation of immature cells to T_c precursors (19). The mouse MLR combination we used was BALB/c ($H\text{-}2^d$, Mls^b) anti-DBA/2 ($H\text{-}2^d$, Mls^a), which included an Mls incompatibility capable of inducing a strong proliferative response (20) as well as many non-H-2 differences. Culture media were supplemented with either FCS or MS. In some experiments, cytotoxic activity was observed in the IL-2-supplemented cultures (see Table I). IL-2 was prepared in MS and FCS medium as described in Materials and Methods. In the MS cultures, cytotoxic activity was very low and was observed in only two out of eight experiments, even after an 8-h incubation. Addition of IL-2 to BALB/c anti-DBA/2 cultures in FCS medium led to a T_c response against DBA/2 antigens in 4 out of 10 experiments, as shown by the lysis of P815 and DBA/2 blast target cells (Table IA). However, in these FCS cultures, a nonspecific effect was also observed, in that BALB/c blast target cells were also significantly lysed by the T_c. Cells recovered from syngeneic (BALB/c anti-BALB/c) cultures supplemented with IL-2 also showed a nonspecific cytotoxic activity that was as high on BALB/c as it was on DBA/2 blast target cells (Table IA). These effects reflected either a polyclonal or FCS-specific response to FCS determinants (9, 10), as they were never observed in MS-supplemented cultures (Table IB). In 2 out of 10 experiments using FCS medium, low levels of cytotoxic activity were observed without IL-2 supplementation, as shown by others (8), and this was increased by IL-2 addition (data not shown).

The data in Table II show that although we were able to obtain a primary response to non-MHC alloantigens in bulk MLR cultures of autoimmune strain NZB ($H\text{-}2^d$) responder cells and BALB/c ($H\text{-}2^d$) stimulator cells when FCS medium was used, no such response was seen in MS medium. The same NZB responder cells were, however, perfectly capable of mounting a strong anti-$H\text{-}2^k$ response to CBA/J stimulator cells in MS medium. Thus, it seems that the primary response to non-MHC alloantigens observed with NZB responder cells (4–8) is FCS dependent. It is not clear whether the cytotoxicity observed is directed against FCS or FCS-altered cell surface determinants, or whether the FCS merely provides a strong auxiliary helper stimulus.

TABLE I

Cytotoxic Activity of T_c Recovered from Bulk MLR of Spleen Cells Stimulated across Non-MHC (Including Mls) Incompatibilities with and without Added IL-2

A

Cultures Supplemented with FCS

MLR combination*	Exogenous IL-2 concentration	Percent cytotoxicity		
		P815	DBA/2 blasts	BALB/c blasts
BALB/c + DBA/2x	0	2.7 ± 1.4	0.0 ± 2.1	0.0 ± 4.8
BALB/c + DBA/2x	25%	51.0 ± 7.6	37.2 ± 5.9	12.2 ± 3.7
BALB/c + BALB/cx	0	5.3 ± 1.1	0.0 ± 1.6	0.0 ± 7.3
BALB/c + BALB/cx	25%	35.2 ± 3.9	18.4 ± 6.2	15.8 ± 5.1

B

Cultures Supplemented with MS

MLR combination	Exogenous IL-2 concentration	Percent cytotoxicity P815
BALB/c + DBA/2x	0	2.2 ± 3.2
BALB/c + DBA/2x	25%	15.2 ± 2.7
BALB/c + BALB/cx	0	0.0 ± 2.4
BALB/c + BALB/cx	25%	0.0 ± 2.3

* T_c were recovered from bulk cultures of normal BALB/c responder cells and x-irradiated DBA/2 or BALB/c stimulator cells on day 5 of culture and tested for cytotoxic activity in a ^{51}Cr release assay as described in Materials and Methods. A 4-h incubation was used for data shown in (A), and an 8-h incubation was carried out for those shown in (B). The effector/target cell ratio of data shown was 40:1.

TABLE II

Failure of NZB Responder Cells to Mount a T_c Response to Non-H-2 Alloantigens in MS-supplemented Bulk Cultures

Media	MLR combination*	Effector/target ratio	Percent cytotoxicity‡	
			BALB/c	CBA/J
MS	NZB anti-NZB_x	50:1	0.0 ± 0.9	0.0 ± 2.7
		10:1	0.0 ± 2.9	0.0 ± 3.6
MS	NZB anti-$BALB/c_x$	50:1	0.0 ± 1.4	—
		10:1	0.0 ± 3.8	—
MS	NZB anti-CBA/J_x	50:1	—	87.4 ± 8.4
		10:1	—	63.3 ± 4.3
FCS	NZB anti-NZB_x	50:1	0.0 ± 9.2	—
		10:1	0.0 ± 2.3	—
FCS	NZB anti-$BALB/c_x$	50:1	65.5 ± 8.4	—
		10:1	47.6 ± 2.7	—

* T_c were recovered from bulk cultures of normal NZB responder cells and x-irradiated NZB, BALB/c, or CBA/J stimulator cells on day 5 of culture and tested for cytotoxic activity in a ^{51}Cr release assay (4-h incubation) at two effector/target cell ratios as described in Materials and Methods.

‡ Target cells were BALB/c or CBA/J Con A blast cells.

LDA. In a previous paper (21) we showed that by pretreating a responder cell population with anti-Ia antiserum, which contains strong anti-I-J activity, and complement (C) before stimulation with non-MHC-incompatible stimulator cells, it was possible to generate a significant level of cytotoxic activity against target cells of the stimulator cell strain. Although this experiment was carried out in FCS medium and the specificity of it was not thoroughly studied, the interpretation of the result was that suppressor cells were eliminated by the antiserum and C pretreatment; thus a primary response to non-MHC alloantigens was facilitated. We have also shown that a high level of nonspecific suppressive activity, which is T lymphocyte mediated, is observed in cells recovered from a bulk BALB/c anti-DBA/2 MLR culture.[2] It thus seemed possible that by carrying out the T_c-generating MLR in a limiting dilution procedure instead of bulk culture, a responder cell number might be found at which suppressor cell precursors were present at low enough concentrations to allow a T_c response. This approach was attempted using both FCS- and MS-supplemented media. Cultures were set up in microtiter plates using various responder cell numbers and a constant number of x-irradiated stimulator cells (5×10^5/well). 20 replicate wells were set up at each responder cell number. One set of cultures contained an exogenous source of IL-2 (2°MLR SN prepared in media supplemented with the appropriate serum) and another did not. After 6 d of culture in a final vol of 0.2 ml, 0.1 ml of medium was removed from each well and ^{51}Cr-labeled P815 (DBA/2 mastocytoma) cells were added. After a 4-h incubation at 37°C, the plates were processed as described in Materials and Methods. Percent cytotoxicity was calculated for each well, and these were then scored as positive or negative as described in Materials and Methods.

In the FCS-supplemented cultures, a high number of positive wells was observed at responder cell numbers of 3×10^5, 10^5, and 3×10^4, whether or not the cultures were supplemented with IL-2 (Fig. 1 a and b). In the IL-2-supplemented cultures, suppression of the response at the highest responder cell numbers was readily observable, although the total number of positive wells was the same (Figure 1 a). If the cytotoxic effect per T_c precursor at each responder cell concentration is calculated (as described in Materials and Methods), this suppressive effect can be observed in the unsupplemented as well as in the IL-2-supplemented cultures (Table III).

Of greater importance, however, is the high level of activity observed in the MS-supplemented cultures (Fig. 1 c). Virtually no cytotoxicity was seen in cultures without added IL-2 (Figure 1 d), whereas high activity was observed in IL-2-supplemented cultures containing 3×10^5 and 10^5 responder cells (Fig. 1 c), although it was not as high as that observed in FCS-supplemented cultures. In the MS-supplemented culture, the responder cell number was taken up to 5×10^5/well, and here the suppressive effect was even more striking than in the FCS cultures, although it was still obvious at a responder cell number of 3×10^5/well when compared with the 10^5 values (Fig. 1 c). This effect is more apparent when the cytotoxic effect per T_c precursor is calculated (Table III). Thus the lack of response observed in bulk culture stimulated cells is probably due, partially at least, to the generation of suppressor cells which, preliminary experiments indicate, may act by either absorbing out or inactivating IL-2.[2]

Specificity. The data shown in Table IV demonstrate that the response is not due to the nonspecific effect of exogenously added IL-2 on the unstimulated cells. Various

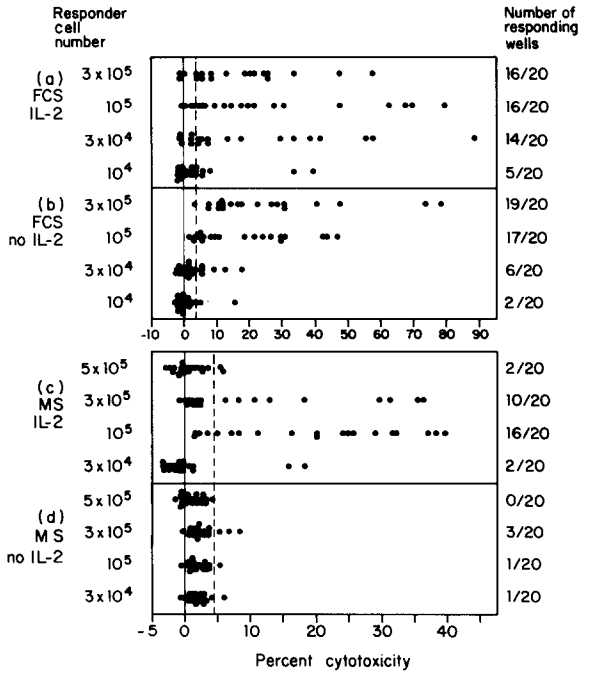

FIG. 1. Cytotoxic activity of T_c generated in a LDA in media supplemented with FCS (a and b) or MS (c and d) with (a and c) and without (b and d) a source of IL-2 (2° MLR SN). Varying numbers of BALB/c responder cells were cultured with 5×10^5 DBA/2 irradiated stimulator cells per well in V-bottomed microtiter plates, and on day 6 ^{51}Cr-labeled P815 target cells were added to the individual wells as described in detail in Materials and Methods. The dotted lines indicate the value above which wells were scored positive (three times SD of the mean value of wells without responders above this mean).

numbers of BALB/c responder cells were cultured with irradiated DBA/2 or BALB/c stimulator cells in the presence of IL-2 in MS medium. Cytotoxicity was tested against ^{51}Cr-labeled P815 target cells of DBA/2 origin. A high number of responding wells was observed at responder cell numbers of 10^5 and 3×10^4 in cultures containing DBA/2 stimulator cells, whereas insignificant activity was seen in those containing BALB/c stimulator cells. The cytotoxic activity per se was shown to be specific by comparing the activity of two identical dilution series in MS medium against DBA/2 and BALB/c Con A blast as well as P815 target cells (Table V). Positive wells were observed at three responder cell concentrations against DBA/2 target cells, whereas an insignificant number (zero or one) of positive wells were observed at the same responder cell numbers when tested against BALB/c target cells.

Discussion

By carrying out the generation of T_c in an MLR under limiting dilution conditions, in which the number of responder cells per well is between 10- and 1,000-fold lower than in conventional bulk cultures, it was possible to observe a strong primary T_c response to non-MHC alloantigens in BALB/c mouse spleen responder cells. Such a response is not observed in conventional primary bulk MLR cultures. Decreased

TABLE III

Suppression is Observed at High Responder Cell Numbers in the LDA *

Responder cell number	IL-2‡	Cytotoxic effect per precursor	
		FCS	MS
		100 N α t/precursor	
5×10^5	Yes	ND§	0.06
3×10^5	Yes	1.7	2.04
10^5	Yes	9.3	11.0
3×10^4	Yes	22.8	3.1
10^4	Yes	14.0	0
3×10^5	No	11.5	—‖
10^5	No	21.7	—
3×10^4	No	10.0	—
10^4	No	0.0	—

* LDA were carried out using both FCS- and MS-supplemented media as described in Materials and Methods. The cytotoxicity per precursor is expressed as 100 N α t/precursor and is computed as described in Materials and Methods. No positive wells were observed at 10^4 responders/well in MS medium, so this point is not shown in Fig. 1.

‡ The sources of IL-2 were 2° MLR SN prepared in media supplemented either with FCS or MS.

§ Not determined.

‖ In the MS medium cultures, without added IL-2, there were not sufficient responding wells to calculate a precursor frequency, and thus it was not possible to compute values of 100 N α t/precursor.

TABLE IV

The Afferent (Generating) Phase of the T_c Response to Non-MHC Alloantigens Is Specific

Responder cell number*	Percent positive wells‡	
	BALB/c + DBA/2x	BALB/c + BALB/cx
5×10^5	20 (4)	0 (0)
3×10^5	25 (5)	5 (1)
10^5	30 (6)	5 (1)
3×10^4	45 (9)	0 (0)
10^4	30 (5)	0 (0)

* Two series of LDA cultures were set up, one containing DBA/2 and the other BALB/c irradiated stimulator cells. The assays were carried out in MS medium and contained a final concentration of 50% 2° MLR SN. After 6 d of culture all wells were tested for cytotoxic activity against ^{51}Cr-labeled P815 target cells.

‡ Data are presented as the percent positive wells. Figures in parentheses indicate the number, out of 20, positive wells. Cytotoxicity was calculated and the number of positive wells computed as described in Materials and Methods.

responses in the LDA were detected at the high responder cell numbers whether FCS or MS medium was used (Fig. 1 a and Table III). This phenomenon is most readily explained by the presence of relatively infrequent suppressor cell precursors that inhibit the response at the high cell concentrations but are diluted out at the low cell numbers to reveal a primary T_c response to the non-MHC alloantigens. These

TABLE V
The Efferent (Effector) Phase of T_c Response to Non-MHC Alloantigens Is Specific

Responder cell number*	Percent positive wells‡		
	P815	DBA/2 blasts	BALB/c blasts
5×10^5	35 (7)	60 (12)	0 (0)
3×10^5	70 (14)	35 (7)	5 (1)
10^5	35 (7)	10 (2)	0 (0)
3×10^4	0 (0)	0 (0)	0 (0)

* Three identical LDA series were set up in MS medium and 50% 2° MLR SN. The three series were tested against ^{51}Cr-labeled P815 and DBA/2 and BALB/c Con A blast target cells.

‡ Data are presented as the percent positive wells. Figures in parentheses indicate the number, out of 20, positive wells. Cytotoxicity was calculated and the number of positive wells computed as described in Materials and Methods.

putative suppressor cells probably also play a role in the lack of a response in primary conventional bulk MLR against non-H-2 alloantigens (Table I). Indeed, we have found that in such bulk cultures a high level of nonspecific T cell-mediated suppression is generated.[2] It has been shown in LDA, carried out to estimate the frequency of IL-2-producing cells in normal spleen cells responding to H-2 and non-H-2 alloantigens, that the amount of IL-2 produced per cell at the lower responder cell numbers was far greater than the amount produced per cell at the higher cell numbers or in conventional bulk MLR cultures (22). These inhibitor effects detected in bulk culture and at high cell numbers in the LDA in terms of IL-2 production or T_c activation are possibly mediated by a similar suppressive mechanism. Suppressor cells could act by directly inhibiting activation of T_c precursors or more likely, as suggested by the study described above (22), by inhibiting activation of IL-2-producing cell precursors, absorbing, or inactivating IL-2. Of course, IL-2 need not be the only lymphokine involved, and the suppressor cells could act by any of the mechanisms suggested above on other required, but still undefined, lymphokines. These possibilities are presently being investigated in our laboratory. That suppressor cells mediated the decreased T_c responses at the high responder cell numbers in the LDA is supported by the recent report that suppression of anti-H-2 T_c subpopulations, present at different frequencies in normal spleen, was observed as the responder cell numbers were increased in a LDA (23).

It may be argued that, in the LDA in MS medium, the response is not a strictly conventional primary one, as an external source of help was required. We were interested to see whether, if the balance between help and suppression was tipped (even artificially) in the favor of help, putative T_c precursors in normal unprimed spleens could be induced to differentiate into T_c. We have shown that they can. The exogenous help is probably required because at the responder cell dilutions at which suppressor precursors are sufficiently diluted to allow a response, the helper cell precursors are not present at a high enough frequency to facilitate a response. In the LDA experiments in FCS medium, exogenous IL-2 was not required because FCS determinants provided a powerful nonspecific helper stimulus (9, 10) sufficient to induce the required amount of help even when the helper cell precursors are present

in relatively low numbers. No such stimulus is provided in medium supplemented with MS, and the exogenous source of help (IL-2) is required. Indeed, the LDA has been extensively used to estimate the frequency of T_c precursors in primary anti-H-2 T_c responses, usually in the presence of an external source of help (Con A SN or 2° MLR SN) (16, 23, 24). For an accurate estimation of the T_c precursor frequency at the low responder cell numbers used, T_c precursor frequency (and not help) should be the only limiting parameter (12, 16, 23, 24).

Although we have previously demonstrated a low level of cytotoxic activity against non-MHC alloantigens in T_c obtained from in vivo immunized mice (25), this is the first demonstration of such a phenomenon in a primary in vitro response of normal mouse responder cells in the absence of xenogeneic serum. This is an important finding because, to date, primary non-H-2 alloantigen specific cytotoxicity has only been convincingly demonstrated using responder cells from the abnormal autoimmune mouse strain NZB (4–8) and medium supplemented with xenogeneic serum. This was usually FCS, the problems of which have been described by other groups (9–11) and are again highlighted by data presented in this report (Table I). It seems that FCS determinants not only activate nonspecific helper cells, but also elicit either a polyclonal (9, 10) or FCS-determined specific T_c response (11). In the latter study (11), guinea pig cytotoxic cells generated in medium containing FCS killed tumor target cells grown in FCS medium, whereas tumor cells grown in medium containing guinea pig serum were not susceptible to lysis. However, in another report (26), it was shown that murine T_c generated in FCS medium would kill self as well as allogeneic targets whether the targets were generated in medium containing FCS or not. Forni and Green (11) indeed suggest that FCS determinants were recognized by T_c precursors, and effectors with specificity for these FCS determinants were generated, whereas the findings of Peck et al. (26) would best be explained by a polyclonal response of T_c. Interestingly, in the second study, the response observed, although not antigen specific, was H-2 restricted (26). Thus, whether the response observed is FCS-determinant specific or polyclonal, it would appear that FCS is not only enhancing the response by providing additional help; we feel this is a matter for concern.

In this regard we have shown that, although an excellent anti-H-2^k T_c response to CBA/J stimulators was observed in MS medium using responder cells from the autoimmune strain NZB (H-2^d), no activity could be detected against BALB/c (H-2^d) H-2-compatible, minor loci-incompatible stimulators (Table II). The same responders, however, did mount a non-H-2-specific response to BALB/c stimulator cells in FCS medium. Thus, if the cytotoxic activity is facilitated by hyperactive helper activity in NZB mice as postulated (7), and not by FCS or by FCS-altered cell surface T_c-activating determinants, such putative hyperactive helper cells are FCS dependent (Table II).

The response of the BALB/c responder cells to non-H-2 alloantigens in the LDA in autologous MS-supplemented medium was shown to be specific in both the afferent (generating) and efferent (effector) phases of the response (Tables IV and V). In other words, merely culturing the responder cells in the presence of exogenous IL-2 without allogeneic stimulators did not result in the generation of T_c (Table IV). Also, the T_c generated with DBA/2 stimulators killed target cells of the stimulator strain (P815 tumor cells and DBA/2 Con A blast cells) but not target cells of the responder cell strain (BALB/c Con A blasts) (Table V). As the two mouse strains used as sources of

responder cells (BALB/c) and stimulator cells (DBA/2) differ at a variety of (seven out of eight studied) non-H-2 loci (27) for which congenic strains in the BALB/c background are not currently available, accurate mapping of the specificities being detected by T_c is not possible. Tentative mapping may be feasible by studying the susceptibility to lysis of target cells from a variety of strains differing at various minor loci. However, even this will be difficult if the response proves to be H-2 restricted, as is the secondary response observed in bulk cultures after in vivo immunization of the responder mice (1, 2). Experiments to test for H-2 restriction are in progress.

The lower response observed in MS medium, in comparison to that in FCS medium (Fig. 1), could be explained by the finding that serum from normal mice contains anti-IL-2 activity (28). We did attempt to generate an anti-non-H-2 T_c response in IL-2-supplemented bulk cultures with medium containing serum from *nu/nu* mice, which lacks this anti-IL-2 activity (28), but were unsuccessful (data not shown). We also tested for enhanced anti-H-2 T_c activity in cells recovered from anti-H-2 MLR cultures carried out in medium supplemented with *nu/nu* mouse serum. No higher activity was observed in comparison with T_c generated in medium containing *nu/+* serum (data not shown). However, these findings do not completely exclude a role for possible anti-IL-2 activities of normal mouse serum in the lower responses observed in MS medium in comparison with those in FCS medium in the LDA.

In MS-supplemented LDA cultures, the putative suppressor cells present at the higher responder cell numbers appeared to decrease both the effective T_c precursor frequency (Fig. 1 c) and the cytotoxic effect per T_c precursor (Table III), whereas in the FCS medium there seemed only to be a decrease in cytotoxicity generated per precursor (Fig. 1 a and b). This suggests that in the MS cultures, the suppressor cells might be inhibiting T_c precursor activation as well as expansion, whereas only expansion is affected in FCS medium. However, this finding might also reflect quantitative differences in the medium conditioning activities rather than qualitative differences between the two sera and required further investigation.

No attempt was made to purify the IL-2 from the supernatants used to supplement the cultures, and it is possible that there are factors other than IL-2 present that facilitate the response. In the experiments shown, Con A SN were used as a source of IL-2 for the bulk cultures, whereas 2° MLR SN were used to supplement the LDA cultures. Experiments were run to test whether the failure to obtain good responses in bulk cultures was caused by the different source of exogenous IL-2. Either 2° MLR SN or Con A SN were added to bulk cultures of BALB/c responder cells and DBA/2 stimulator cells, but T_c activity was not recovered from either culture (data not shown). Thus, the enhanced cytotoxic activity observed using the LDA was not because of the fact that a different source of IL-2 was used. The IL-2-containing supernatants used to supplement MS medium were always prepared with medium containing MS.

Primary in vitro T_c responses to syngeneic tumor in autologous serum-supplemented media have not been described. It was thought that this was caused by the weak antigenic strength of the tumor antigens. It is possible that by using LDA procedures in the presence of exogenous IL-2, suppressor cells similar to those implicated in the non-H-2-specific allogeneic system described in this paper could be diluted out to reveal a highly regulated tumor specific T_c response. It will also be interesting to see if a similar approach may be useful in obtaining in vitro T_c responses to defined non-

H-2 alloantigenic determinants by using congenic mouse strain combinations. As the T_c activity generated in MS medium was highly specific in both the afferent and the efferent phases, it seems highly unlikely that the effector was not a T lymphocyte. However, experiments are in progress to analyze the Lyt phenotype and define the specificity and potential H-2 restrictedness of the killer cells. We propose to compare these parameters to those of T_c generated in secondary responses to minor locus antigens that are readily observed in bulk cultures (1–3).

Summary

We have shown for the first time that it is possible to consistently generate a primary in vitro cytotoxic T cell (T_c) response to non-major histocompatibility complex alloantigens using responder cells from a normal mouse strain. This was achieved by carrying out, in the generating phase, a limiting dilution procedure in which it appears that suppressor cells that inhibit T_c activation or expansion are too dilute to manifest their effect. Moreover, the response was observed in mouse serum- (MS) as well as fetal calf serum- (FCS) supplemented media, an important finding in the light of the anomalous nonspecific effects induced by FCS. The cytotoxic response produced in MS-supplemented media was shown to be highly specific in both the generating and effector phases, whereas the responses in FCS had a strong nonspecific component.

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