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The Effects of Cyclophosphamide on in vitro Cytotoxic Responses to a Syngeneic Tumour

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Summary. *We have studied the effects of treating DBA/2 mice with high doses of cyclophosphamide upon their subsequent ability to generate cytotoxic cells in vitro against syngeneic tumour antigens or alloantigens. High doses of cyclophosphamide (100–200 mg/kg body weight) eliminated the response to both antigens. The addition of normal DBA/2 thymocytes into these cultures restored the response to allogeneic cells but not to tumour cells. The anti-tumour response could be restored by the addition of interleukin 2 to the cultures. Treatment with high doses of cyclophosphamide decreased the number of anti-tumour cytotoxic cell precursors in the spleen, but did not affect the capacity of bulk cultures of spleen cells to produce interleukin 2 when stimulated with the mitogen concanavalin A.*

Introduction

Cyclophosphamide (CY) is a non-specific alkylating agent which acts upon actively dividing cells and has been widely used as an immunosuppressive drug [3]. CY seems to show preferential cytotoxic activity for B cells as against T cells [20, 23], but various effects on the T cell subpopulations have been demonstrated [1, 2, 5, 8, 12, 16–18]. Suppressor T cells have been shown to be very sensitive to CY, which may explain the enhancing effects of low doses of CY on cellular immune responses [1, 5, 8, 18]. Higher doses of CY, however, generally suppress these responses [10, 12].

Previous studies by other workers [10, 11] have shown that the cytotoxic response to alloantigens of cells taken from animals treated with high doses of CY is markedly diminished, but that this condition can be reversed by the addition of normal thymocytes to the cultures. According to their results the thymus did not contribute CTLp, and they suggest that CY predominantly affects a helper cell subset normally present in thymus and does not reduce the number of CTLp [11]. Other studies have clearly shown that the thymus contains CTLp to alloantigens [14]; it was not our object to clarify the role of thymocytes in this system, but rather to investigate the effects of CY on the cytotoxic response to a syngeneic tumour cell. The addition of normal thymocytes to cultures of spleen cells from mice treated with high doses of CY did not restore this response. The response could be brought back almost to the normal level by the addition to the cultures of a lymphokine preparation containing IL2. Although this indicates that the precursors to CTLs were not eliminated by treatment of the animals with CY, a direct assay for the frequency of these cells showed that it was significantly decreased.

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Abbreviations used in this paper: CY, Cyclophosphamide; CTL, cytotoxic T cells; CTLp, precursor cytotoxic T cells; IL2, interleukin 2; Con A, concanavalin A; FCS, fetal calf serum

Materials and Methods

Mice and Tumours. Female DBA/2J (1–5 months) and C57Bl/6J (2–4 months) were obtained from the Jackson Laboratory (Bar Harbor, Maine).

PL815.X2 mastocytoma was obtained from Dr Bruce Smith (Institute for Cancer Research, Philadelphia, Pa). EL4 leukemia was obtained from Dr H.-S. Teh (Department of Microbiology, U.B.C.). P815 was maintained as an ascites in DBA/2J (H-2^d) mice and EL4 was similarly passaged in C57Bl/6 (H-2^b) mice.

Cells. Single-cell suspensions were prepared from spleens and thymuses by pressing small pieces of tissue through a stainless steel mesh (60 mesh). Cells were spun through FCS and resuspended in RPMI 1640 culture medium (Grand Island Biological Company, Grand Island, NY) containing 10% FCS (GIBCO), 100 U penicillin/ml, 100 μg Fungizone/ml and 5×10^{-5} *M* 2-mercaptoethanol (complete medium). Viable cells were counted by the Trypan Blue exclusion method.

Cyclophosphamide Treatments. DBA/2 mice received 50–200 mg cyclophosphamide per kilogram body weight (Cytoxan, Bristol Laboratories, Quebec, Canada) in 0.1–0.4 ml saline IP. After 18–48 h their spleen cells were used as a source of responder cells in the generation of cytotoxic cells to P815 or B6 H-2 antigens in vitro.

In vitro Generation of Cytotoxic Cells. Specific cytotoxicity against P815 was generated in vitro as described previously [6].

In short, 3×10^5 spleen cells from normal or CY-treated DBA/2 mice were incubated with 1×10^4 mitomycin C-treated tumor cells in 0.2 ml complete medium, in 96-well, V-bottomed plates (Linbro 76-023-05, Flow Labs, Conn.). After 5 days at 37° C, 48 wells were harvested and pooled, and the viable cells were counted. Cells were resuspended in complete medium at $5–10 \times 10^6$ ml and tested for cytotoxicity by the ^{51}Cr-release method [21].

Cytotoxicity against C57Bl/6 alloantigens was generated in a one-way mixed lymphocyte reaction. Spleen cells (3×10^5)

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from normal or CY-treated DBA/2 mice were incubated with 3×10^5 irradiated spleen cells in 0.2 ml complete medium. After 4 days, 48 wells were pooled and assayed for cytotoxicity against ^{51}Cr-labeled EL4.

Cytotoxicity Test. Target tumour cells at 2×10^7/ml were labelled with ^{51}Cr-sodium chromate at 500 μCi/ml (NEZ-030S, NEN, Canada) for 60–90 min at 37° C. After extensive washing, the cells were adjusted to $5-10 \times 10^4$/ml. Various 2-fold dilutions of in vitro-activated lymphocytes were dispensed in triplicate into U-bottomed plates (LINBRO 76-013-05, Flow Labs, Conn.). A fixed number of target cells ($5-10 \times 10^3$) was added to these, after which the cells were sedimented by centrifugation (120 *g*, 5 min) and plates incubated at 37° C for 4 h (EL4) or 18 h (P815). Of the cell-free supernatant, 0.1 ml was then removed and counted in a gamma counter (Picker Spectroscalar 4R, Picker, Canada). Percent specific cytotoxicity was calculated from the formula:

$$\frac{\%\ \text{cytotoxicity} - \text{test release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100.$$

Maximum ^{51}Cr release was obtained by lysis of targets with 5% Triton X-100. We have previously shown that the effector cell in this system is sensitive to treatment with anti-Thy 1 serum plus complement [21].

Limiting Dilution Assay of CTLp Frequency. A similar technique to that of Teh et al. [22] was used. Microcultures containing serial dilutions of responder spleen cells, a constant number of stimulator tumour cells, and a source of IL2 were set up in V-bottomed plates in a volume of 0.2 ml and incubated for 5 days at 37° C. At this time, the cultures were assayed for cytotoxic activity against ^{51}Cr-labelled P815, by adding 2×10^3 target cells to each well. The plates were centrifuged for 5 min at 120 *g* and incubated for 18 h at 37° C. Percent lysis was calculated as in the normal assay above. Wells were scored as positive if the cpm ^{51}Cr release exceeded the spontaneous by 2.2 times the SD of the spontaneous release. CTL precursor frequencies were calculated using the maximum likelihood method of Porter and Berry [15]. The zero order term of the Poisson distribution describes the percentage of negative wells versus the number of responding cells/well and estimates the frequency of cells which are limiting for a cytolytic response. Plotting the negative ln of the fraction of negative cultures (Fo) against responder cell numbers results in a straight line, and the χ^2 value represents the correlation coefficient for that line. The frequency is calculated from this line as the reciprocal of the number of responding cells/well when the fraction of negative cultures is 0.37, as discussed in detail by Lefkovits and Waldmann [9]. The range for the frequency estimate represents its 95% confidence limits.

Irradiation. Cells in complete medium at 4° C were exposed to a Cobalt^{60} gammairradiation source at a rate of 32R/sec and (Gammacell 220, AECL, Ottawa, Canada). Cells received a total dose of 2000R and were washed once before being added into culture.

Reconstitution Assays. Normal DBA/2 thymocytes (3×10^5 per well) were added to the cultures of CY-treated or untreated DBA/2 spleen cells with stimulator cells, to assess their effect on the generation of cytotoxic cells. Alternatively, rat IL2 was added at a final concentration of 2.5–5 U/ml.

Preparation of IL2. IL2 was prepared from rat spleens [4] by culturing 10^6 cells/ml in complete medium with 4 μg Con A/ml (Miles, Yeda). After 48 h incubation at 37° C, the cultures were centrifuged and the supernatant used as a source of IL2. Con A was removed by adsorption onto Sephadex G25. IL2 in the supernatant was then precipitated with 80% ammonium sulphate and separated by gel filtration on Sephadex G150. Fractions from this separation were tested in the thymocyte proliferation assay [13], by culturing 10^5 CBA/J thymocytes in complete medium containing dilutions of the fraction either with or without 2 μg Con A/ml for 72 h at 37° C. ^{3}H-Thymidine (0.5 μCi; NET-027A, NEN, Boston, Mass.) was added for the last 6–12 h as a measure of proliferation. The active fractions were pooled and used as IL2 in reconstitution experiments and CTLp frequency assays. One unit of IL2 was defined as the amount giving 37% of the maximal thymocyte proliferation. We found the optimal levels of IL2 to be 2.5–5 U/ml in culture.

IL2 Production by Mouse Spleen Cells. Il2 was produced from CY-treated or untreated DBA/2 mice by culturing 5×10^6 spleen cells/ml with 4 μg Con A/ml in complete medium for 18 h at 37° C. The cell-free supernatants were then assayed in the thymocyte proliferation assay as above.

Results

Cyclophosphamide Inhibits the Generation of Cytotoxic T Cells

Normal DBA/2 mice were injected with various doses of cyclophosphamide 24 h before their spleens were used as a source of responder cells in cultures generating cytotoxic T cells to either P815, a syngeneic tumour in DBA/2 mice, or C57Bl/6 alloantigens. Table 1 shows the responses of DBA/2 animals injected with different doses of cyclophosphamide. At 50 mg/kg responses were often enhanced, perhaps due to the removal of suppressor cells normally present in the responding

Table 1. The effect of CY dose on the ability of spleen cells from treated animals to generate in vitro CTL responses

Dose of cyclophosphamide (mg/kg body weight)[a]	% Cytotoxicity[b] (E:T ratio)	
	60 : 1	30 : 1
1. Anti-alloantigen response		
0	60.3 ± 1.6	43.0 ± 3.5
50	75.6 ± 3.2	59.4 ± 1.4
100	27.6 ± 1.0	16.3 ± 0.9
150	0	0
200	0	0
2. Anti-P815 response		
0	20.4 ± 4.5	14.3 ± 2.4
50	31.2 ± 6.0	29.6 ± 2.8
100	2.8 ± 0.3	0
150	0	0
200	0	0

[a] Average weight of mice was 15–25 g, so this represents 1–4 mg CY in saline IP 24 h before cultures were set up

[b] 3×10^5 responder spleens of treated or untreated DBA/2 animals and 1×10^4 mitomycin C-treated P815 or 3×10^5 irradiated C57Bl/6 spleen cells cultured for 4–5 days and then assayed for cytotoxicity against ^{51}Cr-labelled P815 or EL4 targets. Results are expressed as mean % cytotoxicity for 3–6 wells ± SD at effector : target ratios of 60 : 1 and 30 : 1

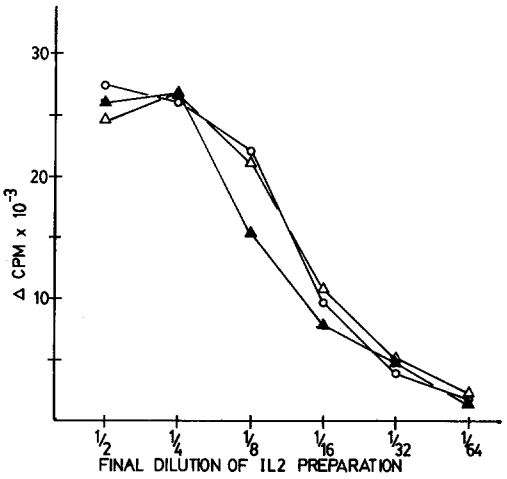

Fig. 1. The effect of treatment with CY on the capacity of spleen cells to produce IL2. Spleen cells (5×10^6) were cultured with Con A at 4 μg/ml for 18 h, and the cell-free supernatant was used as a source of IL2. IL2 activity was measured by the Con A thymocyte stimulation assay. Results are expressed as Δcpm $\times 10^{-3}$ with and without Con A (mean of 3 wells) at different dilutions of the IL2 preparation. IL2 from animals treated with 0 mg/kg (O———O); 50 mg/kg (△———△); and 100 mg/kg (▲———▲). Student's *t*-test: difference between untreated and treated animals is not significant) $P > 0.1$)

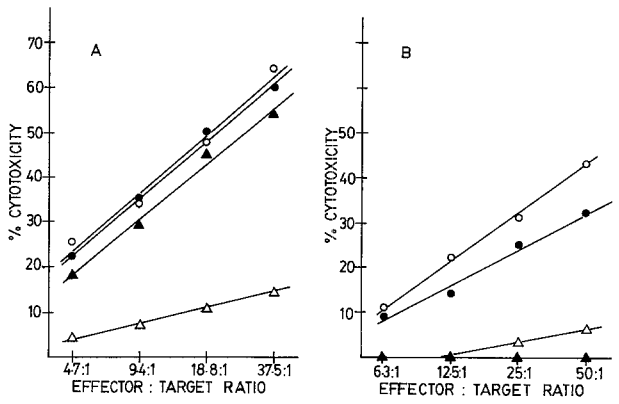

Fig. 2. A and B Restoration of cytotoxic responses with normal DBA/2 thymocytes. Spleen cells (3×10^5) from normal or CY-treated DBA/2 animals cultured with 3×10^5 irradiated C57Bl/6 spleen (**A**) or 1×10^4 mitomycin C-treated P815 (**B**) with or without 3×10^5 normal DBA/2 thymus for 4–5 days and assayed for cytotoxicity in a ^{51}Cr-release assay against EL4 (**A**) or P815 (**B**). Results shown as mean % cytotoxicity of three wells (SD < 10%). Data plotted for untreated animals without added thymocytes (O———O), and with added normal thymocytes (●———●) and for 100 mg/kg animal without added thymocytes (△———△), and with added normal thymocytes (▲———▲)

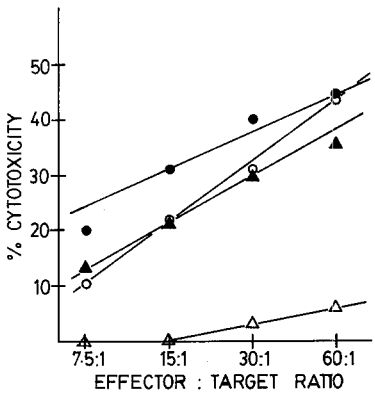

Fig. 3. Restoration of cytotoxic response with IL2. Spleen cells (3×10^5) from normal or CY-treated DBA/2 animals cultured with or without 5 U/ml IL2 and with 1×10^4 mitomycin C-treated P815 for 5 days and assayed for cytotoxicity against ^{51}Cr-labeled P815. Results shown as mean % cytotoxicity of three wells (SD < 10%). Data plotted for untreated mouse without added IL2 (O———O), and with IL2 added (●———●); and for treated mouse (100 mg CY/kg) without IL2 added (△———△), and with IL2 added (▲———▲)

cell population. Both the anti-tumour and the allogeneic response were effectively eliminated by doses of 100–200 mg/kg. The 150 and 200 mg/kg doses were not routinely used, since they resulted in very low yields of spleen cells and a low recovery of activated cells after 4- to 5-day culture (data not shown).

Effect of Cyclophosphamide on IL2 Production

Since IL2 has been implicated in the triggering of CTL [25], it was possible that the injection of CY eliminated an IL2-producing cell and this in turn prevented the generation of CTLs. We therefore tested cells from CY-treated animals for their ability to produce IL2 after Con A stimulation. Figure 1 shows that pretreatment with CY (50 or 100 mg/kg) did not influence IL2 production. This experiment was repeated using other doses of CY up to 200 mg/kg, but at no time was IL2 production reduced. Thus, it appears that CY affected either CTLp pools or amplifier cells important in CTLp activation.

Restoration of Cytotoxic Responses

It has been shown by Merluzzi's group [10, 11] that the CTL response to alloantigens of spleen cells from animals treated with high doses of CY (100–200 mg/kg) can be restored by the addition of normal thymocytes. In similar experiments, we attempted to restore the cytotoxic responses depleted by CY, by the addition of IL2 or normal DBA/2 thymocytes to cultures. Figure 2 shows that normal thymocytes restored the response to alloantigens but not the response to the syngeneic tumour.

The depression of the anti-tumour response by the addition of normal thymocytes was a consistent observation both in this work and in our studies on specific helper cells in the anti-tumour CTL response [6]. Although the cause of this effect has not been resolved, we postulate that it is the result of suppressor cells or their precursors in the normal thymocyte population. In contrast to the failure of normal thymocytes to restore the ability of spleen cells from CY-treated animals to generate a CTL response to P815, Fig. 3 shows that IL2 was sufficient to restore the anti-tumour response. These results indicate that the CTLp were not eliminated by CY treatment.

In order to determine more precisely the effect of treatment with CY on CTLp, we assayed for CTLp using a limiting dilution technique similar to that of Teh et al. [22]. Table 2 shows the results of such experiments. It can be seen that treatment with CY significantly decreased the precursor

Table 2. The effect of cyclophosphamide on CTL precursor frequency to a syngeneic tumour

Dose of cyclophosphamide (mg/kg body weight)	No. of responding cells[a]	% Specific lysis per culture[b]	No. of responding cultures[c]	CTLp's per 10^6 spleen cells[d]	Range	χ^2
0	12,800	91.6 ± 1.3	12/12	3,350	2,060–5,440	0.08
	6,400	87.9 ± 2.5	12/12	(1/299)		
	3,200	83.3 ± 4.6	12/12			
	1,600	52.5 ± 4.6	12/12			
	800	25.4 ± 3.9	11/12			
	400	12.4 ± 3.3	9/12			
50	12,800	87.7 ± 7.4	12/12	1,860	1,190–2,900	2.65
	6,400	81.4 ± 1.2	12/12	(1/537)		
	3,200	56.9 ± 5.8	12/12			
	1,600	32.4 ± 5.7	12/12			
	800	15.2 ± 3.0	10/12			
	400	5.3 ± 2.9	4/12			
100	12,800	49.2 ± 5.4	12/12	,282	180– 443	2.40
	6,400	22.0 ± 3.8	11/12	(1/3,540)		
	3,200	6.6 ± 2.3	5/12			
	1,600	2.1 ± 2.0	5/12			
	800	0	1/12			
	400	0	1/12			

[a] Responding cells from untreated or CY-treated animals were cultured with 640 mitomycin C-treated P815 per well for 5 days and then assayed for cytotoxicity against ^{51}Cr-labelled P815. Il2 present at 2.5 U/ml in all cultures

[b] Results expressed as mean ± SE for 12 wells

[c] Test cultures were scored as positive if their counts exceeded the 95% confidence limits of the spontaneous counts (mean spontaneous release + 2.2 × SD of mean)

[d] CTLp per 10^6 spleen responder cells calculated from plotting -ln [fraction non-responding cultures (Fo)] against number of responding cells. Number of cells/well giving a fraction of non-responding cultures (Fo) = 0.37 is an estimate of the frequency of CTLp. Range = 95% confidence limits. $\chi^2 < 6$ indicates consistent data. This experiment was repeated five times with concordant results

frequency in the syngeneic CTL response. Such effects can not be demonstrated readily in bulk cultures, presumably because the reduced number of CTLp expand in culture to larger average clone sizes, giving equivalent lytic activity to that generated from an untreated cell population.

Discussion

In our studies on specific helper cells involved in the generation of cytotoxic T cells to a syngeneic tumour [6], we attempted to remove the suppressor cells and naturally occurring helper cells present in normal spleen by pretreating responder animals with CY. There have been frequent reports [2, 5, 7, 18] of the use of animals so treated to study cytotoxic responses. Low doses of CY appear to selectively inactivate suppressor T cells [5, 8, 18, 19], while high doses depress or eliminate the generation of CTL [5, 10]. Merluzzi et al. have shown that doses of 100–200 mg/kg eliminate the allo response but that this response can be restored by adding normal thymocytes [10]. We therefore attempted a similar pretreatment of responder animals with CY before stimulation in vitro with syngeneic tumour cells. As a control, the response of these mice to C57Bl/6 alloantigens was also tested. In Table 1, we show that the response to both the syngeneic tumour, and alloantigens, was depressed or eliminated by pretreatment of DBA/2 mice with 100 mg CY/kg 24 h before cultures were initiated.

In agreement with the work of Merluzzi et al. [10], the addition of normal thymocytes restored the response to the alloantigens. Although in the experiments of Merluzzi et al. the thymocytes did not appear to provide CTLp for the allogeneic response, the work of Pilarski [14] clearly shows that this precursor population is present in normal thymus. We did not investigate the source of the CTLp in the anti H-2^b response, but proceeded to examine the effect of added thymocytes on the syngeneic anti-P815 response. We have found previously (data not shown) that in the presence of IL2, anti-P815 CTLp in the thymuses of DBA/2 mice can be activated. Interestingly, normal DBA/2 thymocytes could not restore the anti-tumour response (Fig. 2). This may be related to low numbers of helper cells for the anti-tumour response in the thymuses of unprimed animals.

The ability to restore the allo response by adding thymus suggested to Merluzzi that CY did not deplete the precursor pool but merely an amplifying thymocyte population [11]. The results of our limiting dilution experiments in Table 2 disagree with this interpretation of the data, and indicate that at least for the anti-tumour response, the precursor frequency was markedly reduced. This observed decrease in the anti-tumour CTLp frequency may be of prime importance when considering the use of cyclophosphamide in cancer chemotherapy. This alternative interpretation is supported by our observation that the addition of IL2 to cultures (Fig. 3) restores the anti-tumour response satisfactorily. Since the capacity of the cells to produce IL2 was unaffected by CY (Fig. 1), our data suggest that the treatment with CY blocks the normal process of generation of IL2, perhaps by elimination of a helper cell subclass [24] whose absence is sufficient in itself to depress or eliminate the CTL response. In any case, the addition of Con A can apparently bypass this block and permit the activation of IL2-producing cells.

In summary, our results indicate that the treatment of animals with high doses of CY introduced at least two lesions in the triggering sequence of CTL, one at the level of IL2

production and a second in the number of CTL precursors present. The nature of these lesions is currently under investigation in our laboratory.

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