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The capacity of microsomally-activated cyclophosphamide to induce immunosuppression *in vitro*

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Summary. Cyclophosphamide (CY) was activated *in vitro* with washed rat liver microsomes and co-factors. Pretreatment of mouse spleen cells *in vitro* with the activated drug abolished their capacity to give a primary antibody response to SRBC and levan on transfer to irradiated syngeneic recipients. However, responsiveness returned if challenge was delayed for 7 or more days after transfer. Part of this was shown to be of donor origin by an allotype marker. The treatment of normal spleen cells with activated CY *in vitro* also prevented B cells from regenerating their immunoglobulin receptors after capping with anti-immunoglobulin serum. The induction of suppression required contact between lymphocytes and activated CY for at least 30 min at 37° and did not appear following incubation for 1 h at 0°. Since the antibody response of drug-treated spleen cells to SRBC could not be restored with purified normal B or T cells, it is probable that B and T lymphocytes are both susceptible to suppression by activated CY *in vitro*.

Similar pretreatment abrogated the graft-versus-host (GVH) reactivity of spleen cells as measured by survival and in a popliteal lymph node assay. B cell chimerism in F_1 recipients of drug-treated parental spleen cells was demonstrated by the presence of congenic allotype markers. This suggests a possible approach for the attenuation of GVH disease which is associated with bone marrow transplantation in man.

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INTRODUCTION

The alkylating agent cyclophosphamide (CY) is known to exert a potent suppressive effect on immune responses when it is administered shortly before or after antigen (Aisenberg & Davis, 1968; Many & Schwartz, 1971; Bach, 1975). This immunosuppressive activity is not demonstrable *in vitro* since it is dependent upon microsomal activation of CY within the liver. A sequence of enzymatic and/or spontaneous changes leads to the formation of several intermediary and terminal metabolites some of which have been identified as cytotoxic in experimental tumour systems (Connors, Cox, Farmer, Foster & Jarman, 1974).

The fact that CY can be activated *in vitro* using isolated liver microsomes (Brock & Hohorst, 1963; Connors, Grover & McLoughlin, 1970) has been explored in the present study with regard to its immunosuppressive potential for lymphoid cell suspensions *in vitro*. The effect of CY-treatment on spleen cells was evaluated by: (1) their capacity to give a primary antibody response on cell transfer; (2) assaying for an antibody response *in vitro* and

(3) their graft-versus-host (GVH) reactivity. In addition, the potential of activated CY to impair surface Ig receptor regeneration, similar to that induced by CY *in vivo* was tested (Shand & Howard, 1978).

MATERIALS AND METHODS

Animals and their irradiation

Male and female mice between 3 and 6 months of age were used of the following strains bred in this laboratory: CBA/1ac, C57BL, CBA/Ig1b, (CBA/1ac × C57BL)F_1 and (CBA/Ig1b × C57BL)F_1. Breeding pairs of the congenic CBA/Ig1b strain were supplied originally by Dr H. S. Micklem, Department of Zoology, University of Edinburgh.

Mice were irradiated with either 600 or 900 rad from a ^{137}Cs source delivering approximately 20 rad/min at a focal distance of 60 cm.

Activation of cyclophosphamide in vitro

Liver microsomes from Cobb outbred rats were isolated and washed by ultracentrifugation using the method described by Grover & Sims (1968). Washed microsomes at a concentration equivalent to 1 g of original liver per ml were stored at −20° for periods up to 3 months without any apparent deterioration of activity.

CY was activated using the procedure described by Connors *et al.* (1970) with minor modifications. The following reagents were all prepared in Tris buffer pH 7·4 and incubated at 37° for 30 min.

Washed rat liver microsomes	3 ml
$MgCl_2$ at 10 mg/ml	1 ml
Nicotinamide adenine dinucleotide phosphate (Sigma) at 2·1 mg/ml	1 ml
D-Glucose-6-phosphate (Sigma) at 15·5 mg/ml	1 ml
Glucose-6-phosphate dehydrogenase (Sigma) at 10 units/ml	0·3 ml
Cyclophosphamide monohydrate (Koch-Light) at 5 mg/ml	1 ml
Tris buffer pH 7·4	2·7 ml

Dilutions of the activated drug were prepared in medium 199 and added to an equal volume of spleen suspension containing 1×10^8 cells/ml. The concentrations of activated CY used to treat cells were calculated from the 500 μg/ml level in the original activation mixture and no account was taken of possible differences in the efficiency of activation. The time and temperature of incubation are as specified in individual experiments. After treatment cells were washed once prior to use.

Immunological reactivity of treated cells

In vivo transfers. Treated cells (5×10^7) were transferred to lethally-irradiated (900 rad) syngeneic recipients and challenged with either 5×10^8 sheep red blood cells (SRBC) or 10 μg levan (LE) on the following day unless specified otherwise. Direct plaque forming cell (PFC) assays were performed 5 days later. LE was coupled to SRBC as described by Miranda (1972).

In vitro cultures. Treated spleen cells from mice primed 7 days previously with 5×10^6 SRBC were cultured in Marbrook chambers as described previously (Shand, 1976). Total (direct and indirect) PFC were counted after 4 days of incubation.

Estimation of GVH reactivity. For local GVH assays, treated parental spleen cells were tested in a popliteal lymph node (PLN) assay (Ford, Burr & Simonsen, 1970) by injecting 1×10^7 parental cells into the footpad of F_1 mice. In systemic GVH assays 1×10^8 treated parental spleen cells were injected i.v. into irradiated F_1 or allogeneic recipients and the incidence of lethal GVH disease recorded. The presence of chimerism in (CBA/Ig1b × C57BL)F_1 survivors of treated CBA/1ac spleen cells was established by immunodiffusion. Anti-Ig1b and anti-Ig1a sera were generously provided by Dr C. J. Elson, MRC Unit, University of Bristol.

Purification of cells

T and B lymphocyte populations were isolated from normal mouse spleen by affinity chromatography and anti-Thy 1·2 treatment as described previously (Shand, 1975).

Detection of immunoglobulin receptors on the surface of B cells

The procedure used to test for regeneration of surface Ig (SIg) after anti-Ig treatment has been reported previously (Shand & Howard, 1978). Briefly, spleen cells were capped with rhodamine-labelled rabbit-anti-mouse Ig, washed thoroughly and cultured in Marbrook chambers for 24 h. New SIg receptors were detected by an indirect staining

method utilizing the $F(ab')_2$ fragment of rabbit-anti-mouse Ig, followed by fluorescein-labelled sheep-anti-rabbit Ig. The latter conjugate was obtained from Wellcome Reagents Limited.

RESULTS

The effect of activated CY on lymphocytes *in vitro*

In an initial experiment, spleen cells from normal mice were treated with various concentrations of CY previously activated at 37° for 30 min. Drug-cell mixtures were incubated at 37° for 1 h, washed once and transferred to lethally-irradiated syngeneic recipients. Control suspensions were incubated with: (1) activating reagents in the absence of microsomes and (2) activating reagents without CY. The recipient animals were challenged with 5×10^8 SRBC on the following day and PFC assays performed after a further 5 days. Activated CY pretreatment was profoundly immunosuppressive which was not found when either microsomes or CY were omitted from the activation mixture (Table 1). A similar suppressive effect was seen: (1) when primed spleen cells were pretreated with activated CY and cultured *in vitro* (Table 2) and (2) when transferred drug-treated cells were challenged with LE (Table 3).

Table 1. The immunosuppressive effect of activated CY on normal mouse spleen cells *in vitro*

5×10^7 Spleen cells pretreated* with		Number of direct anti-SRBC PFC/spleen‡
Activated CY†	100 μg/ml	100 (2·00)
Activated CY	30 μg/ml	434 (2·64 ± 0·03)
Activated CY	10 μg/ml	2339 (3·37 ± 0·11)
Non-activated CY (minus microsomes)	100 μg/ml	5468 (3·74 ± 0·10)
Reagents only (minus CY)	(dilution equivalent to 100 μg/ml)	7230 (3·86 ± 0·09)
Medium only		5141 (3·71 ± 0·05)

*Spleen cells were incubated for 1 h at 37° washed once and transferred to 900 rad-irradiated syngeneic recipients.

†CY was activated at 37° for 30 min.

‡Geometric mean (log mean ± s.e.) (n = 4).

Table 2. The immune response of activated CY-treated spleen cells *in vitro*

2×10^7 Primed spleen cells pretreated* with		SRBC (3×10^6)	Number of total anti-SRBC PFC/culture‡
Activated CY †	100 μg/ml	+	407 ± 37
Activated CY	30 μg/ml	+	2554 ± 488
Activated CY	10 μg/ml	+	2227 ± 70
Non-activated CY (minus microsomes)	100 μg/ml	+	4240 ± 400
Reagents only (minus CY)	(dilution equivalent to 100 μg/ml)	+	6894 ± 2111
Medium only		+	4034 ± 1068
Medium only		—	1627 ± 240

*Spleen cells from CBA mice primed 7 days previously with 5×10^6 SRBC were incubated for 1 h at 37° and washed once prior to culture.

†CY was activated at 37° for 30 min.

‡Arithmetic mean ± s.e. (n = 3).

Table 3. The immune response of transferred spleen cells to levan after pretreatment with activated CY *in vitro*.

5×10^7 spleen cells preatreted* with	Number of anti-LE PFC/spleen‡
Activated CY†—50 μg/ml	202 (2·31 ± 0·14)
Medium only	2,014 (3·30 ± 0·10)

*Spleen cells were incubated for 1 h at 37° washed once and transferred to 900 rad-irradiated syngeneic recipients.
†CY was activated at 37° for 30 min.
‡Geometric means (log mean ± S.E.) (n = 4).

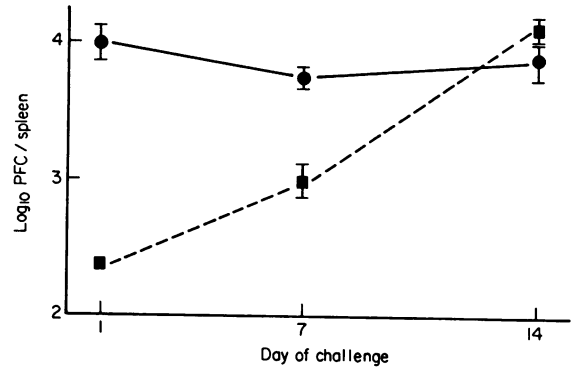

Figure 1. The anti-SRBC PFC response of 5×10^7 transferred spleen cells when challenged at various times after transfer. Cells were pretreated with either 50 μg/ml of activated CY (■) or medium (●) for 1 h at 37°. CY was activated with washed rat liver microsomes and cofactors for 30 min at 37°. Geometric mean ± s.e. (n = 4).

A further experiment was designed to ascertain whether a return of immunocompetence occurred in treated cells when antigen challenge was delayed, similar to that found after CY is administered *in vivo* (Bach, 1975). Groups of lethally-irradiated mice were injected with activated CY-treated syngeneic spleen cells and challenged at various times thereafter. Recovery of responsiveness was partial after 7 days and complete by 14 days (Fig. 1), somewhat slower than after CY *in vivo*. To establish conclusively that donor B cells were synthesizing immunoglobulin, drug-treated CBA/Ig1b spleen cells were transferred to CBA/1ac (Ig1a) lethally-irradiated recipients. Serum from recipient mice contained both allotypes 14 and 21 days after transfer (Table 4), confirming the recovery of donor-derived cells. Since the immune response shortly after transfer was totally inhibited, it seems likely that immunoglobulin synthesis by donor cells arose either from B cells or precursors which were not irreversibly inhibited.

Effect of time and temperature of incubation on activated CY-induced suppression *in vitro*

Normal spleen cells were treated for varying periods with 50 μg/ml of activated CY and transferred to lethally-irradiated syngeneic recipients. All groups were challenged with 5×10^8 SRBC the following day and direct PFC assays performed 5 days later. Figure 2 shows that significant suppression is not evident until cells have been exposed to the drug for 30 min and maximal suppression

Table 4. Demonstration of donor-derived Ig producing cells in lethally-irradiated CBA/1ac (Igla) recipients of CBA/Ig1b spleen cells pretreated with activated CY *in vitro*

CBA/Ig1b spleen cells pretreated* with	Immunoglobulin Host–Ig1a Day 14	Day 21	Allotype Donor–Ig1b Day 14	Day 21
Activated CY†–50 μg/ml	+	+	±	+
Activated CY–20 μg/ml	+	+	+	+
Medium only	+	+	±	+
No spleen cells‡	+	N.D.	—	N.D.

*CBA/Ig1b spleen cells were incubated for 1 h at 37° washed once and transferred to 900 rad-irradiated CBA/1ac recipients.
†CY was activated at 37° for 30 min.
‡Irradiated recipients which were not injected with spleen cells survived only until day 14.

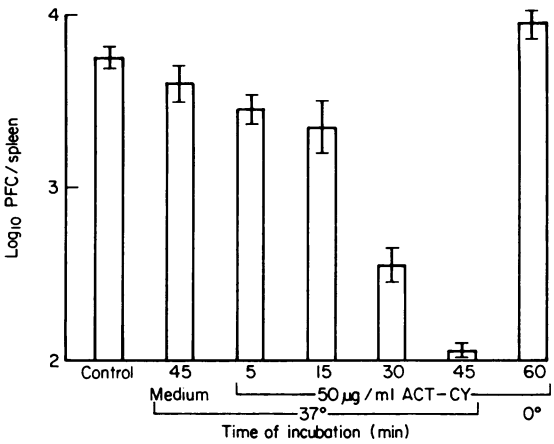

Figure 2. The anti-SRBC PFC response of 5×10^7 transferred spleen cells which were pretreated with 50 μg/ml of activated CY for varying periods of time at 37°. The control is the anti-SRBC PFC response of 5×10^7 spleen cells kept on ice until other treatments had been completed. The extreme right column represents the anti-SRBC PFC response of 5×10^7 spleen cells pretreated for 1 h at 0° with 50 μg/ml of activated CY. All groups were challenged 1 day after the transfer. CY was activated with washed rat liver microsomes and cofactors for 30 min at 37°. Geometric mean ± s.e. (n = 4).

was attained only after 45 min. When incubation was performed at 0° for 1 h no suppressive effect was detectable.

Supplementation of treated cells with purified B or T cells

To detect any possible selective involvement of B or T cells by this *in vitro* treatment, normal spleen cells were treated with two concentrations of the activated drug (50 and 10 μg/ml) and transferred to lethally-irradiated syngeneic recipients. Mice from each of these groups were injected also with T cells (purified by affinity chromatography) or B cells (purified with anti Thy 1·2 serum + complement).

Figure 3 shows that neither normal B nor T cells were able to restore the anti-SRBC response of spleen cells treated with 50 μg/ml of activated CY. This suggests that both B and T cells are equally susceptible to the activated drug *in vitro*. Furthermore, both B and T cells were able to restore the response of spleen cells treated with 10 μg/ml of activated CY, indicating that both cell types are limiting in cell suspensions treated with low concentrations of the drug. The data in Fig. 3 suggests that T cells might be more sensitive than B, since the restorative capacity of normal T cells was superior to that of B cells on suspensions treated with 10 μg/ml of activated CY. This point has not been investigated further.

Impaired B cell-receptor regeneration induced by activated CY *in vitro*

In a previous report (Shand & Howard, 1978) it was shown that B cells obtained from mice injected 20 h previously with 150 mg/kg CY, although they capped normally after anti-Ig treatment, were subsequently unable to regenerate their SIg receptors. The capacity of *in vitro* treatment with the activated form of the drug to produce a similar inhibitory effect was sought.

Spleen cells from normal mice were treated for 1 h at 37° with 50 μg/ml of activated CY. After one wash, the cells were incubated in a 1/40 dilution (medium 199 + 5% FCS) of rhodamine-labelled rabbit-anti-mouse Ig for 45 min at 37°. Controls were incubated in medium 199 + 5% FCS only. Treated suspensions were washed three times and 1×10^7 cells seeded into Marbrook chambers for overnight culture. A sample of each suspension was stained with a 1/20 dilution of a $F(ab')_2$ rabbit-anti-mouse Ig followed by a 1/20 dilution of fluorescein-labelled sheep-anti-rabbit Ig. This staining

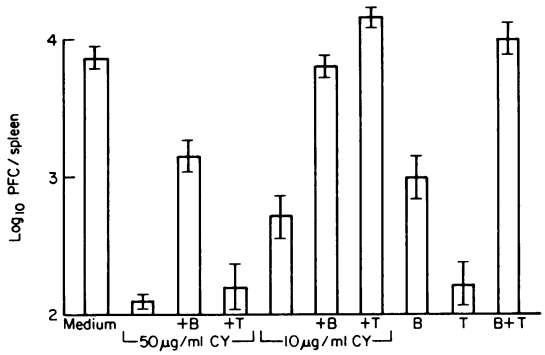

Figure 3. The anti-SRBC PFC response of 5×10^7 transferred spleen cells which were pretreated with either 50 or 10 μg/ml of activated CY and supplemented with purified normal B or T cells. All groups were challenged one day after the transfer. CY was activated with washed rat liver microsomes and cofactors for 30 min at 37°. Geometric mean ± s.e. (n = 4).

Table 5. Impairment of B cell receptor regeneration induced by pretreatment with activated CY *in vitro*

Spleen cells pretreated with	anti-Ig* treatment	% B cells staining with FITC only at 0 h	24 h
Activated CY–50 μg/ml	—	30·8	28·8 (51)
Activated CY–50 μg/ml	+	<1·0	2·5 (49)
Medium only	—	32·1	29·3 (53)
Medium only	+	<1·0	22·8 (50)

*Treated cells were incubated at 37° for 45 min in a 1/40 dilution of rhodamine-labelled rabbit-anti-mouse Ig. Cells were washed three times prior to culture.

Figures in parentheses represent viabilities as evaluated by trypan blue dye exclusion.

Table 6. Inhibition of PLN enlargement induced by pretreating donor spleen cells with activated CY *in vitro*

CBA spleen cells pretreated* with	PLN weight (mg)† in (CBA × C57BL)F1 recipients at day 7 Left	Right‡	PLN index
Activated CY – 100 μg/ml	1·87 × 1·15	1·03 × 1·07	1·82
Activated CY – 50 μg/ml	2·75 × 1·03	1·22 × 1·14	2·25
Activated CY – 25 μg/ml	3·11 × 1·21	1·19 × 1·24	2·61
Medium only	2·10 × 1·23	0·66 × 1·36	3·18

*CBA spleen cells were incubated for 1 h at 37° washed once and 1×10^7 cells injected into the left footpad of F_1 mice.

†Geometric mean × s.e. (n = 5),

‡All groups were injected into the right footpad with 1×10^7 syngeneic F_1 cells.

Table 7. Inhibition of systemic GVH reaction by activated CY-treatment of donor cells *in vitro*

Recipient	Irradiation (rad)	Spleen cells	Pretreated* with activated CY at (μg/ml)	MST†
(CBA × C57BL)F_1	600	CBA	100	>49‡
(CBA × C57BL)F_1	600	CBA	10	21·0 ± 2·0
(CBA × C57BL)F_1	600	CBA	Nil	14·75 ± 2·4
C57BL	900	CBA	50	19·0 ± 2·0
C57BL	900	CBA	Nil	7·7 ± 0·2
CBA	900	C57BL	50	13·8 ± 1·6
CBA	900	C57BL	Nil	7·3 ± 0·5

*Spleen cells were incubated at 37° for 1 h washed once and 1×10^8 cells injected i.v.

†Mean survival time ± s.e.

‡This group of mice was used for a PFC assay on day 49.

Table 8. Demonstration of donor-derived immunoglobulin synthesis in lethally-irradiated (CBA/Ig1b × C57BL)F_1 recipients of CBA/1ac (Ig1a) spleen cells pretreated with activated CY *in vitro*

CBA/1ac spleen cells pretreated* with	Deaths (at day 34)	Immunoglobulin Allotype at day 34	
		Host	Donor
Activated CY – 50 μg/ml	1/4	+ +	+
Activated CY – 20 μg/ml	0/4	+ +	+
Medium only	3/4	+ +	+

*CBA/1ac spleen cells were incubated at 37° for 1 h, washed once and 1×10^8 cells injected i.v. into 900 rad-irradiated (CBA/Ig1b × C57BL)F_1 recipients.

procedure was repeated on the following day when the cultures were harvested.

Table 5 shows that activated CY-treated B cells were unable to regenerate their SIg receptors after anti-Ig treatment. This effect cannot be ascribed to drug-induced cytotoxicity, since cell viabilities were similar in all experimental groups.

Effect of activated CY treatment *in vitro* on GVH reactivity

As a further indication of the influence of activated CY treatment *in vitro* on T cell function, we investigated whether drug-treated cells lost their ability to induce GVH reaction. Firstly, treated (50 μg/ml for 1 h at 37°) and untreated (incubated only) CBA/1ac spleen cells were compared in a PLN assay. Drug-treated parental cells produced significantly less node enlargement when injected into the footpad of (CBA/1ac × C57BL)F_1 recipients (Table 6). Secondly, the survival of (CBA/1ac × C57BL)F_1 recipients was significantly prolonged when the parental spleen cells injected had been pretreated with activated CY (Table 7). Thirdly, lethally-irradiated recipients of drug-treated allogeneic spleen cells also survived significantly longer (Table 7).

In a final experiment lethally-irradiated (CBA/Ig1b × C57BL)F_1 mice were injected i.v. with treated or untreated CBA/1ac (Ig1a) spleen cells to determine whether recipients of treated cells became chimeric or whether activated CY merely eliminated the entire donor cell population. The serum immunoglobulin consisted of both allotypes 34 days after transfer, thereby confirming the presence of donor-derived cells (Table 8).

DISCUSSION

The present results clearly establish that a potent immunosuppressive capacity is generated by microsomal-activation of CY *in vitro*. Mixtures from which either microsomes or CY had been omitted were devoid of this activity. Although *in vitro* activation of CY by isolated liver microsomes has been used extensively to investigate the metabolic pathway of CY (Brock & Hohorst, 1963; Connors *et al.*, 1974) and its effect on tumour cells (Connors *et al.*, 1970; Padgett & Colvin, 1972; Brock, 1976), this procedure has hitherto escaped the attention of cellular immunologists as a means of analysing the nature of CY-induced immunosuppression.

Induction kinetics revealed that: (1) a minimum exposure time was required between activated CY and spleen cells and (2) suppression was not evident when pretreatments were performed for 1 h at 0°. These results implicate a metabolic event involving the transport of metabolites from the extracellular fluid to the vicinity of nuclear DNA. Immunocompetence began to recover in transferred drug-treated spleen cells after 7 days, suggesting that B cells or their precursors (and presumably the immediate precursors of helper T cells) were not irreversibly inhibited. Although this recovery period is rather longer than the 3–4 days seen after CY is administered *in vivo* (Bach 1975) it is not known to what extent cell recruitment plays a role in the latter situation. Confirmation of immunoglobulin synthesis by cells of donor origin was obtained by utilizing congenic CBA/1ac (Ig1a) mice as recipients of CBA/Ig1b drug-treated spleen cells. Whether it is the stem cell, a later antecedent stage of lymphocyte differentiation or the mature lymphocyte

which recovers from the effect of alkylation is unknown, since the ability of activated CY-treated cells to produce 'colony forming units' *in vivo* was not ascertained. However, it is known that tumour cells are more sensitive than normal cells to the alkylating activity of CY both *in vivo* and *in vitro* (Brock, 1976).

One of the more striking changes induced by CY *in vivo* is the apparent selective depletion of B cells from thymic-independent areas of lymphoid tissue (Lerman & Weidanz, 1970; Turk & Poulter, 1972) even though both B and T cells from the spleen of CY-injected mice are functionally unresponsive (Bach, 1975). The present experiments confirm this latter finding, since the addition of normal B or T cells to 50 μg/ml drug-treated spleen suspensions did not restore their immunocompetence although it is recognized that the conditions of drug exposure '*in vitro*' may be different to those '*in vivo*'. It is possible that the depletion of B but not T cells, seen after CY is injected *in vivo*, is related to their differing recirculatory pathways, which might influence their contact with the activated form of the drug. Alternatively, T cells may be recruited into the spleen at a faster rate than B cells during the recovery phase following CY injections. The application of activated drug treatment to purified cell populations *in vitro* should enable us to ascertain the relative sensitivity to CY of various T cell subsets, particularly suppressor T cells.

A further distinctive sequel to the incubation of spleen cells with activated CY *in vitro* is the subsequent inability of B cells to regenerate their SIg receptors after anti-Ig treatment. This observation reproduces an effect reported previously for B cells recovered from the spleen of CY-injected mice (Shand & Howard, 1978), which was considered as a likely explanation for the unique ability of CY to promote tolerance to thymus-independent antigens. The efficient tolerance-promoting effect of CY for thymus-dependent antigens seems to be sited in T cells and is likely to involve a different mechanism (Many & Schwartz, 1970; Ramshaw, Bretscher & Parish, 1977). With the ability to treat cell suspensions *in vitro*, it should be possible to explore the tolerogenicity of T-dependent antigens for B cells during CY-induced suppression.

CY is one of the few immunosuppressive agents which has been used successfully to abrogate GVH disease in experimental animal models. Owens & Santos (1971) found that CY was particularly effective when administered to both donor and recipient and led to the establishment of stable chimerism. However, a major limitation to the use of CY in man is its toxicity. The present *in vitro* treatment protocol, whilst duplicating the beneficial effect of CY *in vivo* is devoid of toxic side-effects. This observation, plus the fact that recipients of drug-treated allogeneic spleen cells were shown to be chimeric without detectable GVH disease, offers a possible method for controlling GVH disease associated with bone marrow transplantation in man.

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