

T-SUPPRESSOR CELLS SENSITIVE TO  
CYCLOPHOSPHAMIDE AND TO ITS IN VITRO  
ACTIVE DERIVATIVE 4-HYDROPEROXYCYCLOPHOSPHAMIDE  
CONTROL THE MITOGENIC RESPONSE OF  
MURINE SPLENIC B CELLS TO DEXTRAN SULFATE  
A Direct Proof for Different Sensitivities of Lymphocyte  
Subsets to Cyclophosphamide

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The alkylating agent cyclophosphamide (Cy) alters (suppresses or enhances) immune responses in vivo (1-5) but does not act on lymphocyte functions in vitro. The lack of in vitro effects of Cy is a result of the fact that Cy has to be metabolized into the active compounds which are formed by a sequence of reactions catalyzed by microsomal enzymes in the liver (6). It has been shown that Cy activated by liver microsomes in vitro acts as an immunosuppressive in vitro (7). 4-hydroperoxycyclophosphamide (4HP-Cy) is a relatively stable derivative of Cy which is spontaneously converted in aqueous solution into 4-hydroxycyclophosphamide, the naturally occurring, but very unstable, metabolite of Cy. Recently, it has been reported (6) that 4HP-Cy acts like activated Cy immunosuppressive in vitro. In this report, we present results which show that (a) lymphocyte functions, as analyzed by their capacity to respond to mitogens in vitro can be influenced (enhanced or suppressed) by pretreatment of the cell donors in vivo by Cy or by treatment of the cells in vitro with 4HP-Cy, and that (b) the enhancement of the mitogenic response of dextran sulfate-reactive B lymphocytes by Cy or 4HP-Cy is a result of functional inactivation of T-suppressor cells by these drugs, and that (c) B lymphocytes are less sensitive to Cy or 4HP-Cy action than this type of T-suppressor cell.

**Materials and Methods**

*Animals.* Female C57BL/6J, (C57BL/6J × DBA 2)F<sub>1</sub>, BALB/c, and athymic nude (BALB/c) mice were used at 8-10 wk of age. The animals were obtained from Dr. K. Friis, Gl. Bomholdgard Ltd., Ry, Denmark.

*Chemicals and Reagents.* Cy and 4HP-Cy were generously provided by the Asta-Werke, Brockwede, Federal Republic of Germany. Dextran sulfate (DS), mol wt  $5 \times 10^5$  from Serva Feinbiochemica, Heidelberg, Federal Republic of Germany; lipopolysaccharide (LPS) from *Escherichia coli* 0 55:B 5 from Difco Laboratories, Detroit, Mich.; concanavalin A (Con A) from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.; monoclonal anti-Thy-1.2 antibody from OLAC Ltd., Blackthorn, Bicester, Oxon, England; guinea pig complement

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(C) from Behring-Werke AG, Marburg/Lahn, Federal Republic of Germany; fetal calf serum (FCS), batch No. 104 from Seromed, Munich, Federal Republic of Germany; and RPMI-1640 medium from Microbiological Associates, Walkersville, Md. were used.

*Cell Suspensions.* Spleen cell and thymocyte suspensions were prepared in serum-free culture medium, as described earlier (8).

*Anti-Theta Serum and Complement Treatment.*  $1 \times 10^7$  spleen cells were incubated at room temperature for 30 min with anti-Thy-1.2 antiserum (final dilution 1:3,000). The cells were washed, resuspended at a concentration of  $10^7 \cdot \text{ml}^{-1}$  in agarose-adsorbed C (final dilution 1:6) and kept for 60 min at  $37^\circ\text{C}$ . The cells were washed and resuspended at a concentration of  $2 \times 10^6$  viable cells (as determined by the trypan blue dye exclusion test) per ml in serum-supplemented culture medium.

*Treatment of the Cells by 4HP-Cy.*  $10^7$  cells  $\cdot \text{ml}^{-1}$  (suspended in serum-free culture medium) were incubated with various doses of 4HP-Cy ( $0.1$ – $10 \mu\text{g} \cdot \text{ml}^{-1}$ ) for 1 h at  $37^\circ\text{C}$  in an atmosphere of 5%  $\text{CO}_2$  in air. As determined by trypan blue dye exclusion test, 4HP-Cy treatment did not change viability of the cells.

*Cell Cultures and Determination of [ $^3\text{H}$ ]Thymidine ([ $^3\text{H}$ ]TdR) Uptake by the Cells.* Conditions for cell cultures and for determination of [ $^3\text{H}$ ]TdR uptake by the cells has been described in detail earlier (9). Briefly, cells were cultured in triplicates in a total vol of 0.2 ml ( $2 \times 10^6$  cells  $\cdot \text{ml}^{-1}$ ) in microtiter plates (M24AR, Greiner, Nürtingen, Federal Republic of Germany) in an atmosphere of 5%  $\text{CO}_2$  in air at  $37^\circ\text{C}$  for 2 or 4 d in RPMI-1640 medium supplemented with 5% FCS,  $5 \times 10^{-5}$  M 2-mercaptoethanol, 2 mM L-glutamine  $\cdot \text{ml}^{-1}$ ; 100 U penicillin  $\cdot \text{ml}^{-1}$ , and 100  $\mu\text{g} \cdot \text{ml}^{-1}$  streptomycin. 0.1  $\mu\text{Ci}$  of [ $^3\text{H}$ ]TdR (sp act: 2 Ci  $\cdot \text{mM}^{-1}$ , Radiochemical Centre, Amersham, England) was added to the cultures for the last 4 h of the incubation period. Cells were collected on glass-fiber filters using a Skatron multiple cell culture collector (Flow Laboratories, Rockville, Md.). Incorporation of [ $^3\text{H}$ ]TdR into the nuclear DNA was then determined as described previously (9).

The results are expressed as  $\Delta$  counts/minute per culture (counts per minute in mitogen-treated culture – counts per minute in control cultures) or as stimulation indices (counts per minute in mitogen-treated cultures per counts per minute in control culture). Each value represents the arithmetic mean of  $\Delta$  counts per minute detected in triplicate cultures, SE was  $<10\%$ .

## Results and Discussion

Administration of Cy, as a rule, results in either immunosuppression, or immune tolerance (1). However, Cy injected several days before antigen administration paradoxically enhances T-cell-mediated, e.g., delayed type of hypersensitivity (DTH) (2), as well as humoral, e.g., to a synthetic antigen (3), immune responses. Suppression of antibody formation, as well as enhancement of DTH induction by the drug has been originally attributed to preferential elimination of short-lived B lymphocytes in the lymphoid organs (2, 10). Recently, the enhancing effect of Cy on DTH (4), as well as on humoral immune responses (3), has been attributed to elimination of T-suppressor cells. Along these lines, we presented results indicating that Cy-sensitive T-suppressor cells control the generation, as well as the expression of activity of autoreactive T cells in vivo (5). The results suggested that T-suppressor cells (or their precursors) should be more sensitive to Cy treatment in vivo than B lymphocytes or other T-cell subsets mediating various immunological activities.

A direct proof for these suggestions was lacking until now, because Cy does not act in vitro on lymphocytes. Recently, Shand and Howard (6) showed that Cy activated with microsomal enzymes in vitro, as well as derivatives of Cy, e.g., 4HP-Cy, can be used for in vitro studies. They reported that B, as well as T lymphocytes treated in vitro with either activated Cy or with 4HP-Cy transiently lost their capacity to give immune responses (6, 7).

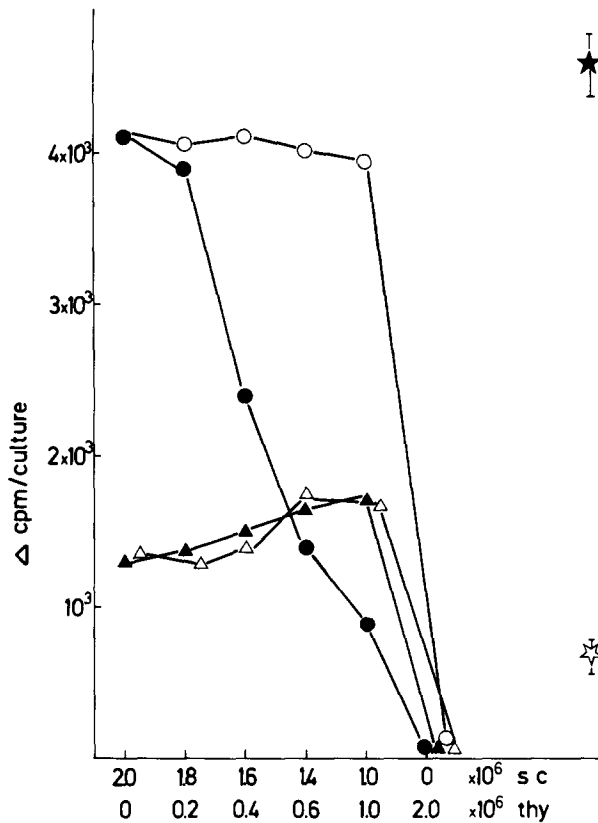


FIG. 1. The selective inhibition by 4HP-Cy-sensitive thymocytes of the DS response of spleen cells derived from Cy-injected mice. C57BL mice were injected with  $125 \text{ mg} \cdot \text{kg}^{-1}$  Cy s.c. 7 d later, the spleen cells of these animals and of controls were cultured with either DS ( $50 \mu\text{g} \cdot \text{ml}^{-1}$ ) or LPS ( $25 \mu\text{g} \cdot \text{ml}^{-1}$ ). Spleen cells of Cy-pretreated mice were mixed with various numbers of syngeneic 4HP-Cy-pretreated thymocytes ( $10^7$  thymocytes incubated with  $0.3 \mu\text{g} \cdot \text{ml}^{-1}$  4H-Cy for 1 h at  $37^\circ\text{C}$  and washed twice with medium) or control thymocytes (incubated for 1 h with medium) in presence and absence of DS or LPS. After 2 d, the cultures were pulsed for 4 h with  $[^3\text{H}]\text{Tdr}$  and the  $[^3\text{H}]\text{Tdr}$  uptake determined as described in Material and Methods. The results are expressed as  $\Delta$  counts/minute per culture. Each value represents the arithmetic mean of  $\Delta$  counts per minute detected in three individual cultures; se:  $<10\%$ . sc, spleen cells; thy, thymocytes; (☆) DS response of normal spleen cells; (★) LPS response of normal spleen cells; (●) DS response of spleen cells derived from Cy-injected mice cultured alone or mixed with thymocytes; (▲) LPS response of spleen cells derived from Cy-injected mice cultured alone or mixed with thymocytes; (○) DS response of spleen cells derived from Cy-injected mice cultured with 4HP-Cy-treated thymocytes; (△) LPS response of spleen cells derived from Cy-injected mice cultured with 4HP-Cy-treated thymocytes.

In this work, we have evaluated the mitogenic response in vitro of spleen cells derived from either Cy-pretreated mice or of spleen cells of normal mice treated in vitro with 4HP-Cy.

Treatment of mice with  $125 \text{ mg} \cdot \text{kg}^{-1}$  Cy results in a drastic depletion of the spleen of both B and T lymphocytes. 7 d after this treatment, the mice immunized with sheep erythrocytes gave a normal or even a slightly enhanced plaque-forming cell response, indicating recovery of the capacity of spleen cells to give humoral immune responses (data not shown).

Spleen cells derived from the same Cy-pretreated mice show, however, dramatic

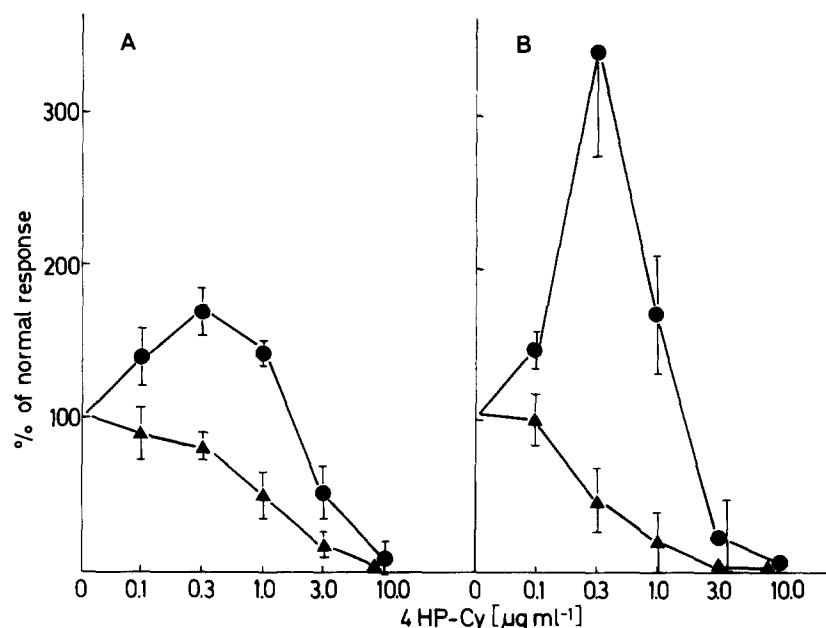


FIG. 2. Dose-dependent alterations (enhancement and suppression) of the DS response and suppression of the LPS response by 4HP-Cy treatment of spleen cells. (C57BL  $\times$  DBA2) $F_1$  spleen cells ( $10^7 \cdot \text{ml}^{-1}$ ) suspended in serum-free medium were incubated for 1 h at  $37^\circ\text{C}$  with various doses of 4HP-Cy. The cells were then washed twice with medium and incubated ( $2 \times 10^6 \text{ cells} \cdot \text{ml}^{-1}$ ) with either DS ( $50 \mu\text{g} \cdot \text{ml}^{-1}$ ) or LPS ( $25 \mu\text{g} \cdot \text{ml}^{-1}$ ) for 2 (A) or for 4 d (B). The [ $^3\text{H}$ ]Tdr uptake was then determined as described in Material and Methods. The results are expressed as percentage of the mitogenic response of the 4HP-Cy-treated cells as compared to nontreated control cells = 100%. The values represent the mean of the three individual experiments, each performed in triplicates. The SE are shown by the vertical bars. (●) Cells cultured with DS; (▲) cells cultured with LPS.

changes in their capacity to respond to two different B-cell mitogens (Fig. 1). As compared to controls, the response was enhanced to DS (up to eightfold) and diminished to LPS (up to 50%). The response of the cells to Con A was unchanged. Five individual experiments gave essentially identical results.

If one accepts the concept that DS and LPS act on different subsets of B lymphocytes (11), one of the possible explanations of this phenomenon could be that after damage of the spleen by Cy, less-mature DS-reactive B cells may repopulate the spleen earlier than more-mature LPS-reactive B cells. Alternatively, both types of B lymphocytes should be sensitive to Cy, but in contrast to the LPS-reactive cells, the DS-reactive cells should be under the negative control of Cy-sensitive suppressor cells. These suppressor cells (T-suppressor cells?) should be more sensitive to Cy action than B lymphocytes and may repopulate the spleen later than the DS-reactive and the LPS-reactive cells. If so, the net effect of Cy treatment would be an enhanced response to DS and a diminished response to LPS.

The addition of graded number of thymocytes decreases the enhanced response of the cells to DS, but even slightly enhanced the response of the cells to LPS (Fig. 1). Pretreatment of the thymocytes with presumably nontoxic doses of 4HP-Cy ( $1 \mu\text{g}$  per  $10^7$  cells) abrogated the capacity of thymocytes to suppress the enhanced DS response but did not significantly change the effect of thymocytes on the LPS response.

TABLE I  
*Diminished Mitogenic Response to DS and to LPS by 4HP-Cy Using T-Cell-depleted Spleen Cells*

Source of spleen cells	4HP-Cy treatment	Mitogenic response to		
		DS	LPS	Con A
BALB/c	–	825 (4)	4,553 (22)	9,070 (43)
	+	5,050 (16)	1,250 (4)	9,500 (25)
Anti-Thy-1.2 + C-treated BALB/c	–	2,660 (11)	4,320 (15)	NS
	+	970 (4)	1,170 (4)	NS
Athyric (BALB/c) nude	–	2,920 (12)	3,760 (16)	NS
	+	780 (3)	910 (4)	NS

$2 \times 10^6$ .ml<sup>-1</sup> spleen cells or spleen cells pretreated with anti-Thy-1.2 serum and C or spleen cells of nude mice were incubated with 0.3  $\mu$ g.ml<sup>-1</sup> of 4HP-Cy for 1 h, washed two times, and cultured with 50  $\mu$ g.ml<sup>-1</sup> LPS or 2  $\mu$ g.ml<sup>-1</sup> Con A for 4 d. Each value represents the arithmetic mean of [<sup>3</sup>H]Tdr uptake detected in triplicate cultures. The results are expressed as  $\Delta$  counts/minute per culture. Stimulation indices (s.i.) are in parentheses. The discrepancies between  $\Delta$  counts per minute and s.i. show the (up to threefold) enhanced background proliferation of the BALB/c cells as a result of 4HP-Cy treatment detectable in the majority of the experiments (6 out of 7). NS, no significant  $\Delta$  counts per minute cpm could be detected.

These results indicated, but did not directly prove the negative control by Cy-sensitive T lymphocytes on the proliferation of the DS-reactive B-lymphocyte subset. Experiments performed *in vitro* using 4HP-Cy—an *in vitro* active derivative of Cy—clearly demonstrate the validity of this suggestion.

Spleen cells of normal mice pretreated with small doses of 4HP-Cy showed an enhanced response to DS, whereas pretreatment of the cells with high doses of this drug diminished the response of the cells to DS. In contrast, the LPS-reactivity of the cells was either unchanged or diminished by small and drastically diminished by high doses of 4HP-Cy (Fig. 2).

If one assumes the inactivation by 4HP-Cy of T-suppressor cells is responsible for the enhanced DS response, no such effects should be detectable using spleen cells depleted of T cells or lacking functionally active T cells. As shown previously (8), and as seen in Table I, anti-theta serum-and-C-treated spleen cells or spleen cells of nude mice give a higher response to DS than normal spleen cells. The LPS reactivity was unchanged. As expected, the response to DS and to LPS was diminished by treatment of T-cell-depleted spleen cells with 4HP-Cy. The same dose of 4HP-Cy enhanced the response of normal spleen cells to DS. In addition, these results ruled out the possibility of a selective and direct potentiation effect of Cy, or of its active metabolites on DS-reactive B cells.

In conclusion, our results demonstrate directly (a) that lymphocytes can generally be functionally inactivated by Cy or by its active metabolites, (b) that, as expected from indirect *in vivo* experiments, T-suppressor cells (or their precursors) are more sensitive to Cy than B lymphocytes, and (c) suggest that the DS-reactive, but not the LPS-reactive, B-lymphocyte subset is under the negative control of T lymphocytes.

### Summary

As measured by [<sup>3</sup>H]thymidine uptake, spleen cells of mice injected 7 d previously with a single dose of cyclophosphamide (Cy) (125 mg.kg<sup>-1</sup>) gave an enhanced response to dextran sulfate (DS), a diminished response to lipopolysaccharide (LPS), and a normal response to concanavalin A. Addition of syngeneic thymocytes to spleen cells inhibited the enhanced response of the cells to DS and slightly enhanced their

response to LPS. Pretreatment of thymocytes by 4-hydroxyperoxycyclophosphamide (4HP-Cy) in vitro (an in vitro active derivative of Cy) abrogated the effect of thymocytes on the DS response but not on the LPS response. Pretreatment of spleen cells by small doses of 4HP-Cy ( $0.1\text{--}1.0\ \mu\text{g}\cdot\text{ml}^{-1}$ ) in vitro enhanced the capacity of the cells to respond to DS but either did not affect, or even diminished their capacity to respond to LPS. The enhancement of the DS response by 4HP-Cy treatment could not be detected using spleen cells depleted of T cells or lacking functioning T cells. 4HP-Cy doses  $>3\ \mu\text{g}\cdot\text{ml}^{-1}$  diminished or abolished the capacity of the spleen cells to respond to LPS as well as their capacity to respond to DS.

The results show (a) that in contrast to the LPS-reactive B-lymphocyte subset, the proliferative capacity of DS-reactive subset is negatively controlled by a Cy- and 4HP-Cy-sensitive T-cell subset and (b) that these T-suppressor cells are more sensitive to Cy and 4HP-Cy (to their respective active alkylating metabolites) than B lymphocytes and T cells carrying other immunological functions.

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