Brief Definitive Report

CYCLOPHOSPHAMIDE-SENSITIVE T LYMPHOCYTES SUPPRESS THE IN VIVO GENERATION OF ANTIGEN-SPECIFIC CYTOTOXIC T LYMPHOCYTES*

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Murine T lymphocytes sensitized in vitro against either allogeneic lymphocytes or syngeneic hapten-conjugated lymphocytes do differentiate into highly effective cytotoxic T lymphocytes (CTL) (1–3). In vivo immunization of T lymphocytes to the same antigens, however, results in the generation of only marginal cytotoxic activity (1, 4, 5). Recently we found that the weakness of in vivo generated cytotoxicity is not due to a failure of antigen-induced T-cell sensitization but rather due to suppression of the in vivo differentiation of sensitized CTL precursors into effective CTL (6). In keeping with this finding it was postulated that suppressor cells may regulate the in vivo differentiation of CTL.

We now report, that cyclophosphamide-sensitive T cells suppress the in vivo differentiation of antigen-specific CTL. Thus, pretreatment of mice with a single dose of cyclophosphamide (100 mg/kg) converts their state of low responsiveness to a state of high responsiveness.

Materials and Methods

Mice. CBA/J, C3H, BALB/c, and C3H nu/nu mice from Bomholtgaard, Ry, Denmark, 8–12 wk of age, were used.

Administration of Cyclophosphamide. Cyclophosphamide (Endoxan; Asta-Werke, Brackwede, Germany) was dissolved in sterile saline immediately before use and injected by the intraperitoneal (i.p.) route as a single dose in graded amounts at varying intervals before immunization.

In Vivo Induction of BALB/c or TNP-Specific Cytotoxic Lymphocytes. The method used has been described (5, 6). In brief, mice were injected with 2×10^7 allogeneic or TNP-conjugated syngeneic spleen cells into the hind foot pad. The TNP conjugation was performed as described (3, 7). In brief, splenic lymphocytes were incubated in phosphate-buffered saline (PBS) containing 10 mM trinitrobenzol sulfonic acid (TNBS) (Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.). 3 days later the draining lymph nodes were removed and the cells obtained were tested for cytotoxic activity against either ^{51}Cr-labeled P815 mastocytoma tumor cells or TNP-conjugated lipopolysaccharide (LPS)-induced syngeneic blast cells.

Preparation of TNP Targets. Replicate cultures of splenic lymphocytes (4×10^6) were cultured in the presence of 5 μg LPS (Difco Laboratories, Detroit, Mich.) over a period of 48 h. The cells were harvested, labeled with [^{51}Cr]chromate and incubated for 10 min at 37°C in PBS containing 10 mM TNBS. The cells were washed and used as targets (3, 7).

Cytotoxicity Assay. Variable numbers of viable lymph node (LN) cells were incubated for 3 h

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TABLE I

*The Effect of Pretreatment with Cyclophosphamide on the In Vivo Generation of TNP-Specific Cytotoxic T Cells**

Dose of cyclophosphamide administered i.p. at day 2	* Specific lysis of TNP-conjugated CBA targets			
	Cytotoxic activity of CBA LN cells at various intervals after immunization (days):			
	2	3	4	5
mg/kg				
0	2	9	4	−1
10	−2	5	3	0
50	3	7	25	7
100	5	29	42	11
200	1	12	10	13

* 2 Days after cyclophosphamide treatment, CBA mice were injected with 2×10^7 TNP-conjugated syngeneic spleen cells into the hind foot pad. At various intervals the draining LNs were removed and the cells were tested for cytotoxic activity against TNP-conjugated LPS-induced syngeneic blast cells at an effector to target cell ratio of 30:1. Assay time of the ^{51}Cr tests was 3 h, background lysis of the targets was less than 22%.

with a constant number of 2×10^4 ^{51}Cr-labeled target cells. Percent specific ^{51}Cr release was calculated (7).

Preparation of Cell Suspensions Used For Reconstitution Experiments. Cortison-resistant thymocytes were obtained from mice that were injected i.p. with 1.5 mg cortisone acetate (Ciba-Geigy, Wehr, Germany) 24 h earlier. Bone marrow cells and spleen cells were obtained according to standard procedures. Enrichment of splenic T cells was achieved by use of the nylon wool column technique as described (8). Enrichment of splenic B cells was done by the use of AKR anti-Thy 1.2 serum plus complement. The antiserum was raised and used as described previously (9).

Results

The data depicted in Table I show that CBA mice immunized in vivo by a local graft of TNP-conjugated syngeneic spleen cells (2×10^7 cells) do not generate a substantial amount of cytotoxic T effector cells (CTL) in the draining LN. This finding is in accordance with earlier reports (4, 6). Surprisingly however, CBA mice treated with cyclophosphamide 2 days before in vivo immunization with trinitrophenylated CBA cells did generate in vivo effectively TNP-specific CTL (Table I). Maximal specific cytolysis was observed 4 days after in vivo immunization. While cyclophosphamide at a dose of 100 mg/kg had an optimal augmenting effect, larger doses were considerably less efficient (Table I). Data given in Table II demonstrate that the cyclophosphamide-dependent augmenting effect on the capacity of mice to generate in vivo CTL is not only a feature of the "TNP model," but applies also to the in vivo generation of alloantigen-reactive CTL. As detailed in Table II, in the $H\text{-}2^k$ anti-$H\text{-}2^d$ system the optimal time point for the pretreatment with cyclophosphamide was 2 days before in vivo immunization. Drug application simultaneously with the alloimmunization had no augmenting effect on the differentiation of anti BALB/c CTL. Furthermore it was noted that the effect of cyclophosphamide decreased with an increasing time interval between drug administration and the in vivo immunization, 2 days being optimal.

The above results suggest that under normal in vivo conditions the stimulatory potential of both allogeneic lymphocytes and TNP-conjugated syngeneic lymphocytes is suppressed by some unknown mechanism that is sensitive to cyclophosphamide. To test whether this mechanism operates on the level of

TABLE II

*The Effect of Pretreatment with Cyclophosphamide of CBA Mice at Various Intervals on the In Vivo Generation of H-2^d Specific Cytotoxic T Cells**

Cyclophosphamide (100 mg/kg) administered i.p. at various intervals	Cytotoxic activity of CBA LN cells 3 days after immunization			
	% specific lysis of ^{51}Cr-labeled targets			
	P 815 (*H-2*^{d})		EL 4 (*H-2*^{b})	
	30:1	3:1	30:1	3:1
days				
0	6	−1	4	1
−2	52	24	11	−3
−4	26	13	6	2
−6	23	9	−2	0
−8	17	10	1	3

* CBA mice, after pretreatment with cyclophosphamide (100 mg/kg) at various intervals earlier, were injected with 2 × 10^7 BALB/c (*H-2*^{d}) spleen cells into the hind foot pad. 4 days later the draining LNs were removed and the cells were tested for cytotoxic activity against P815 (*H-2*^{d}) and EL 4 (*H-2*^{b}) target cells at an effector to target cell ratio of 50:1 and 5:1. Assay time of the ^{51}Cr test was 3 h, background lysis of the targets was less than 13%.

TABLE III

*Effect of Syngeneic Lymphocyte Transfer to Cyclophosphamide-Treated Mice on the Capacity to Generate in Vivo TNP-Specific CTL**

C3H cells used for i.v. reconstitution of cyclophosphamide-treated mice	Cytotoxic activity of C3H LN cells	
	% Specific lysis of TNP-conjugated C3H targets	
	30:1‡	5:1‡
No cells	45	21
Cortison-resistant (100 × 10^6) thymocytes	19	5
Bone marrow (20 × 10^6)	40	18
Spleen (50 × 10^6)	8	−1
Spleen, nylon (50 × 10^6) fiber passed	13	2
Spleen treated with (50 × 10^6) anti-Thy 1.2 plus C	41	17
nu/nu spleen (50 × 10^6)	43	20

* C3H mice were treated i.p. with one dose of cyclophosphamide (100 mg/kg). 48 h later, the mice were first injected intravenously with the cell populations listed and then injected with 2 × 10^7 TNP-conjugated C3H spleen cells into the hind foot pad. 4 days later the draining LNs were removed and the cells were tested for cytotoxic activity against TNP-conjugated LPS-induced syngeneic blast cells. Assay time of the ^{51}Cr test was 3 h, background lysis of the targets was less than 19%.

‡ Ratio effector cells to target cells.

lymphocytes, cyclophosphamide-treated mice, simultaneously with the immunization with TNP-conjugated stimulator spleen cells, were also "reconstituted" with grafts to various cell types derived from normal syngeneic mice. The results given in Table III indicate that B lymphocytes did not influence the differentiation of CTL in cyclophosphamide-treated mice. However, transfer of T cells from normal CBA mice abolished the cyclophosphamide-induced CTL-augmenting effect.

Discussion

The new aspect of the experiments described consists in the observation that cyclophosphamide treatment of mice before immunization with cell-bound antigens results in a strong augmentation of their capacity to generate in vivo

antigen-specific CTL. This refers to T-cell-mediated in vivo cytotoxic allograft responses, and in particular to *H-2*-restricted in vivo cytotoxic anti-hapten responses. In the latter system it has been proven difficult to induce in vivo CTL (4, 5). Therefore, from an operational point of view, cyclophosphamide treatment of mice results in a conversion of their in vivo state of low responsiveness into a state of high responsiveness. This conversion can again be reverted back to a state of low responsiveness by reconstituting the cyclophosphamide-treated mice with normal T cells, suggesting that the in vivo effect of cyclophosphamide operates on the level of T cells. From that it follows that in vivo cyclophosphamide-sensitive T cells suppress the in vivo generation of alloimmune and hapten-immune cytotoxic T-effector cells.

It is known that in vitro sensitization of murine T lymphocytes towards alloantigens or hapten-conjugated syngeneic lymphocytes results in the generation of highly effective CTL (1–3). Consequently the observed in vivo state of low responsiveness cannot be explained by a lack of antigen-reactive T cells with the appropriate specificities. Moreover, recently we noted that T cells of mice sensitized in vivo, but incapable to differentiate into CTL, differentiate in CTL after transfer in vitro (6). These results suggest that the observed state of in vivo low responsiveness is neither due to a lack of antigen recognition nor antigen processing through macrophages, but rather due to an inability of CTL precursors to differentiate in vivo into effective CTL. Since the results described here suggest that the in vivo blocked differentiation pathway of CTL can be overcome by pretreatment of mice with cyclophosphamide, and since this effect in turn can be neutralized by normal T cells, cyclophosphamide-sensitive suppressor T cells most likely block in vivo the final antigen-independent phase of the differentiation pathway of CTL.

The immune response augmenting effect of cyclophosphamide appears not to be restricted to the in vivo differentiation of CTL. For example, Lagrange et al. have found that cyclophosphamide-potentiated T-cell-dependent delayed-type hypersensitivity (DTH) to sheep erythrocytes (SRBC) (10). More recently Askenase et al. provided evidence that the augmenting effect of cyclophosphamide on DTH to SRBC is due to the inactivation of cyclophosphamide-sensitive suppressor T cells, which do not effect antibody responses (11). Furthermore Miller et al. have demonstrated in the DTH system (12), and Debré et al. in the T-helper cell system (13) that low responsiveness of mice to antigens, which are under *Ir*-gene control, can be overcome by cyclophosphamide. These reports together with the data presented here, favor the concept that the T-cell immune responsiveness in vivo against a variety of antigens is under the control of cyclophosphamide-sensitive suppressor T cells.

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