

DIFFERENTIAL CYCLOPHOSPHAMIDE SENSITIVITY
OF PRECURSOR CELLS IN ALLOGENEIC
AND H-2 RESTRICTED CYTOTOXIC RESPONSES

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Cyclophosphamide (Cy) is an alkylating agent which acts on actively dividing cells. It has been widely used as immunosuppressive drug in clinical medicine (1). Cy has preferential toxicity for B cells compared to T cells, but it is clear that T cells also are affected (2-4). It has been reported that Cy can also have immunoenhancing effects which are probably a result of the inactivation of suppressor T cells or their precursors. Cy-sensitive suppressor T cells have been demonstrated both in cell-mediated and humoral immune responses (5-10). Other T-cell functions, like cytotoxic T-cell responses against allogeneic cells as well as delayed type hypersensitivity reactions, are relatively resistant to Cy (8, 10, 11). There is now increasing evidence that cytotoxic T cells are also generated after exposure to major histocompatibility complex (MHC)-identical cells expressing either viral antigens, chemically added haptens, or minor histocompatibility antigens (12-15). Efficient lysis is obtained only when the cytotoxic T cells and targets are syngeneic at the MHC. This kind of MHC-restricted cytotoxicity may play an important role e.g., in the control of tumour development and viral diseases. The cellular basis in this response is still largely unknown. The experiments reported here show that MHC-restricted cytotoxic T-cell responses are more sensitive to Cy than allogeneic responses. Two types of murine MHC (H-2)-restricted cytotoxic responses were used: the in vitro primary response to trinitrophenyl (TNP)-coupled syngeneic cells (13, 16) and the in vitro cytotoxic response of female cells to male spleen cells (H-Y antigen), in which response in vivo priming is required (14).

Materials and Methods

Animals and Immunizations. C57BL/6 and CBA mice were obtained from the breeding unit of this department. C57BL/6 female mice were primed to the male-specific antigen (H-Y) by the intraperitoneal injection of 1×10^7 viable syngeneic male spleen cells 3 wk before use.

Administration of Cyclophosphamide. Cyclophosphamide (Syklofosamid, Lääke O.Y., Turku, Finland) was dissolved in sterile water immediately before use and graded amounts were injected intraperitoneally 2 d before starting the in vitro spleen cell cultivation.

In Vitro Sensitizations and Cytotoxic Assay. The method used to elicit and assay a cytotoxic T-cell response has been previously described (17). Briefly, spleens from female mice (unprimed or primed to the H-Y antigen) were removed and teased, and the resulting cell suspension was washed once in RPMI medium and then resuspended at 5×10^6 cells/ml in RPMI medium containing 10% fetal calf serum (FCS) (Gibco, Glasgow, Scotland) with 10 mM *N*-2-hydroxyethylpiperazine-*N'*-ethane sulfonic acid (Hepes), glutamine, antibiotics, and 5×10^{-5} M 2-mercaptoethanol. The responding cells were cocultured with an equal number of Mitomycin-C treated stimulator cells in 25-cm² plastic tissue culture flasks. The stimulator cells were either allogeneic, syngeneic male, or TNP-modified. TNP-modification was done according to Shearer

TABLE I
Effect of In Vivo Cy Pretreatment on In Vitro Cytotoxic Responses to Allogeneic Cells, TNP-Modified Syngeneic Spleen Cells or to Syngeneic Male Spleen Cells

Donor of responder spleen cells	Dose of Cy* mg/kg	In vitro stimulators	Target‡	Corrected % lysis (SE)§
C57BL/6	0	CBA	CBA	37.9 (2.8)
	20	CBA	CBA	40.1 (2.9)
	100	CBA	CBA	37.0 (1.9)
	200	CBA	CBA	35.5 (1.4)
C57BL/6	0	C57BL/6-TNP	C57BL/6-TNP	35.4 (4.8)
	20	C57BL/6-TNP	C57BL/6-TNP	17.6 (2.7)
	100	C57BL/6-TNP	C57BL/6-TNP	10.5 (2.0)
	200	C57BL/6-TNP	C57BL/6-TNP	-3.2 (0.2)
C57BL/6 ♀ (primed 3 wk previously with male cells)	0	C57BL/6 ♂	C57BL/6 ♂	42.4 (4.1)
			C57BL/6 ♀	-0.3 (0.6)
	20	C57BL/6 ♂	C57BL/6 ♂	25.6 (1.6)
			C57BL/6 ♀	-2.1 (0.2)
	100	C57BL/6 ♂	C57BL/6 ♂	0.0 (1.0)
			C57BL/6 ♀	-2.1 (1.0)
	200	C57BL/6 ♂	C57BL/6 ♂	-1.5 (0.1)
			C57BL/6 ♀	-2.8 (2.3)

* Cy was injected intraperitoneally 2 d before starting the in vitro culture.

‡ ⁵¹Cr-labeled, concanavalin A-induced lymphoblasts.

§ Attacker: target = 10:1.

(16). After 5 d of incubation in a humidified, 10% CO₂ atmosphere, the cultures were harvested, washed once in RPMI medium, and then resuspended in Eagle's minimal essential medium with 10% FCS and 10 mM Hepes. The cell concentration was adjusted and three doubling dilutions made. The various concentrations of attacking cells were then plated in triplicate in wells of microtiter plate and 2-4 × 10⁴ ⁵¹Cr-labeled target cells were added per well. The target cells were spleen cells which had been cultured 48-72 h in the presence of 4 μg/ml concanavalin A, labeled for 90 min with ⁵¹Cr-sodium chromate and then washed twice. In anti-TNP cytotoxic responses the targets were TNP-modified after ⁵¹Cr-labeling. The attacker: target cell ratios normally used were 16:1, 8:1, 4:1, and 2:1. The plates were spun briefly and then incubated at 37°C in a 10% CO₂ atmosphere for 3 h before harvesting the supernates for gamma counting. Maximum-release was the amount of ⁵¹Cr released from Triton-treated target cells (Rohm & Haas Co., Philadelphia, Pa.); spontaneous release was that released by target cells incubated in medium alone. The corrected percent lysis was computed according to the formula of Wunderlich et al. (18). Regression lines were calculated from the percent of corrected lysis at the four attacker:target cell ratios used, and from these lines the percent of corrected lysis at 10:1 attacker:target was taken. Only when the r² value for such regression lines lay between 0.9 and 1.0 was the percent of corrected lysis at 10:1 regarded as positive. Each experiment reported in the table was repeated at least three times and concordant results were obtained. The values given are from representative experiments.

Results

The data depicted in Table I show that spleen cells from C57BL/6 (H-2^b) mice responded in vitro to allogeneic stimulator cells (CBA, H-2^k) regardless of the Cy pretreatment of the responder cell donor. But Cy pretreatment had a clear effect on the cytotoxic response of C57BL/6 mice to TNP-coupled syngeneic spleen cells. Already with the lowest dose tested (20 mg/kg) a clear decrease in cytotoxicity was noticed. The anti-H-Y cytotoxic response was similarly sensitive to Cy. In this

experiment the responder female mice were primed *in vivo* with syngeneic male cells and Cy was given 2 d before *in vitro* restimulation with male cells.

Discussion

The present studies examined the effect of Cy on two types of T-cell cytotoxicity, namely the allogeneic response and the H-2-restricted cytotoxic responses. The former was shown to be relatively resistant whereas H-2-restricted cytotoxicity was readily depressed with a low dose of Cy.

It is unclear whether the differential effect of Cy on these (and other) T-cell functions is due to a selective depletion of some T-cell subpopulations (e.g. cytotoxic T-cell precursors and/or helper cells for H-2-restricted responses) or to an alteration of function in a given T-cell population. The former alternative may be supported by the studies with Ly antigen markers: the generation of cytotoxic T cells to TNP-modified syngeneic cells required the presence of Ly 1⁺2⁺3⁺ cells, whereas alloreactive prekiller activity mainly resided in the Ly 1⁻2⁺3⁺ subclass (19). Ly 1⁺2⁺3⁺ cells comprise \cong 50% of peripheral T cells. They are immature and appear first in ontogeny and are sensitive to adult thymectomy (19). Therefore, some of these cells, e.g. precursor cells for some suppressor T cells and for H-2-restricted cytotoxic cells, might be rapidly dividing and thus, sensitive to Cy.

One explanation for the differential Cy sensitivities of allogeneic and H-2 restricted cytotoxic responses may be that there are lower numbers of precursors for H-2 restricted responses and Cy simply inhibits the clone expansion. Fischer et al. (20) have demonstrated that the frequency of cytotoxic cell precursors reactive to TNP-modified syngeneic cells are one-half of the frequency of alloreactive precursors and it was also noticed that one precursor cell undergoes three to four cell divisions to produce at least 8–16 cytotoxic lymphocytes after antigenic stimulation. Thus, the difference in cell proliferation in anti-TNP and in allogeneic responses is small and speaks against the possibility mentioned above.

In anti-H-Y cytotoxic response Cy has its effect on primed cells, inhibiting their response to restimulation. This indicates that not only the precursor cells but also memory cells in H-2 restricted responses are Cy sensitive.

Horton et al.¹ have recently demonstrated that anti-Ly-6 antiserum reveals heterogeneity among the precursors of cytotoxic T cells in a similar way as Cy: allogeneic precursors are Ly 6⁻, whereas the generation of cytotoxic cells to hapten-modified syngeneic cells is partially abrogated by serum pretreatment. The effector cells in both type of responses were Ly 6⁺.

Differential sensitivity of these two types of T-cell cytotoxicity to Cy (maybe also to other immunosuppressive drugs?) may have some clinical implications: in some cases suppression of MHC restricted responses might be desirable (autoimmune diseases) but in transplant recipients, the drug-induced decrease in allograft response can totally abolish the MHC-restricted response and this may result in the increase of virus infections and virus-induced tumours.

Summary

Spleen cells from cyclophosphamide-treated mice responded *in vitro* to allogeneic stimulator cells but not to TNP-coupled syngeneic spleen cells. Similarly, cells from

¹ M. A. Horton, P. C. L. Beverly, and E. Simpson. Expression of Ly-6 alloantigen during differentiation of cytotoxic T cells. Manuscript submitted for publication.

female mice, primed in vivo with syngeneic male cells, could not respond in vitro to male spleen cell stimulation if the mice were pretreated with cyclophosphamide. These results suggest that the precursor cells for H-2-restricted cytotoxic responses belong to a different T-cell subpopulation than the precursor cells for allogeneic responses.

I would like to thank Ms. Barbro Bång for the excellent technical assistance, Dr. Elizabeth Simpson for critical revision of the manuscript, and Emil Aaltonen Foundation for financial support.

Received for publication 7 September 1978.

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