

DURING LYMPHATIC REGENERATION, PRECURSORS FOR
MAJOR HISTOCOMPATIBILITY COMPLEX-RESTRICTED
CYTOTOXIC T CELLS APPEAR BEFORE
ALLOREACTIVE PRECURSORS

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Over 1% of murine T cells are devoted to recognizing cells from a different mouse strain (alloantigens) (1). This high frequency of alloreactive cells is in marked contrast to its obvious lack of biological significance. All other antigens (e.g., viruses, haptens, and minor histocompatibility antigens) are recognized on the cells' surface in association with the self major histocompatibility complex (MHC)¹ antigens (i.e., the phenomenon called MHC or self restriction; reviewed in ref. 2). Now there is increasing evidence that alloreactive and MHC-restricted T cell populations are overlapping, e.g., there are cell lines showing both MHC restriction and allospecificity (3). We studied these two forms of T cell recognition (using the cytotoxic T lymphocyte [CTL] response against allogeneic cells or haptenated syngeneic cells as models) during the regeneration after a sublethal dose of an immunosuppressive drug, cyclophosphamide (Cy). 8 d after a dose of 300 mg/kg Cy, the spleen contained cells that could give rise to only MHC-restricted CTL. A low alloresponse was first obtained 15 d after the Cy injection, and it was still defective after 6 wk. These data indicate that if these two types of T cell recognition are due to the same or overlapping cell populations, the different stages of maturation show differential capacity to give rise to allospecific vs. MHC-restricted CTL.

Materials and Methods

Mice. (C₅₇BL/6 × CBA)F₁ and BALB/c mice were obtained from the breeding unit of this department and used at an age of 2–4 mo.

Administration of Cy. Cy (Syklofosamid, Lääke Oy, Turku, Finland) was dissolved in sterile water immediately before use, and a sublethal dose (300 mg/kg) was injected intraperitoneally.

In Vitro Sensitizations and Cytotoxicity Assay. The method used to elicit and assay a cytotoxic T cell response has been previously described (4). Briefly, spleens from mice were removed and teased, and the resulting cell suspension was washed once in RPMI 1640 medium and then resuspended at 5×10^6 cells/ml in RPMI 1640 medium containing 10% fetal calf serum (FCS) (Gibco, Glasgow, Scotland) with 10 mM Hepes, glutamine, penicillin and streptomycin, and 5×10^{-5} M 2-mercaptoethanol. The responding cells were cocultured with mitomycin C-treated stimulator cells in 25-cm³ plastic tissue culture flasks. The stimulators were either allogeneic or 2,4,6 trinitrophenyl (TNP)-modified syngeneic cells. TNP modification was done according to Shearer (5). In some experiments, nylon wool (NW)-purified cells (6) were used as responders. After 5 d of incubation in a humidified, 5% CO₂ atmosphere, the cultures were harvested, washed once in RPMI 1640 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 were made. The various concentrations of attacking cells were then plated in triplicate in wells of a microtiter plate, and 2×10^4 to 4×10^4 ^{51}Cr -labeled target cells were added per well. The target cells were spleen cells that had been cultured 48–72 h in the presence of $4 \mu\text{g/ml}$ concanavalin A (Con A), labeled for 90 min with ^{51}Cr -sodium chromate, and then washed twice.

In anti-TNP cytotoxic responses, the targets were TNP-modified after ^{51}Cr -labeling. The attacker to target cell ratios normally used were 20:1, 10:1, 5:1, and 2.5:1. The plates were spun briefly and then incubated at 37°C in a 5% CO_2 atmosphere for 3 h before harvesting the supernatants for gamma counting. Maximum release was the amount of ^{51}Cr released from Triton-treated target cells (Rohm and Haas Co., Philadelphia, Pa.); spontaneous release was that released by target cells incubated in medium alone. Regression lines were calculated from the percent of corrected lysis at the four attacker to target cell ratios used, and from these lines the percent of corrected lysis at 10:1 attacker to target cells was taken. Only when the r^2 value for such regression lines lay between 0.9 and 1.0 was the percent of corrected lysis at 10:1 regarded as positive. The percent of lysis of nonhaptentated Con A-induced blasts (syngeneic to the responder) was always $<5\%$ with every FCS batch used, indicating that the response was specific for the hapten (and not directed against FCS-associated antigenic determinants). Each experiment reported was repeated at least three times, and concordant results were obtained. The values given are from representative experiments.

Production of Interleukin 2 (Il-2). 5×10^6 cells/ml SDK rat spleen cells were incubated for 48 h with $4 \mu\text{g/ml}$ Con A in RPMI 1640 medium supplemented as in CTL sensitization cultures. Cells and debris were removed by centrifugation, and the supernatant was filtered through a $0.2\text{-}\mu\text{m}$ filter and stored at -20°C . This supernatant was used as the source of Il-2 at a final concentration of 10–20%, and 0.05 M α -methylmannoside was used to block the residual Con A activity.

Results and Discussion

(CBA \times C57BL/6) F_1 mice were injected intraperitoneally with a sublethal dose (300 mg/kg) of Cy. 3 d after this, the number of spleen cells was reduced to $\sim 2 \times 10^6$, but at day 6 the regeneration had started ($\sim 20 \times 10^6$ cells/spleen). At day 8 the cellularity was back to normal. These data are in accordance with those of Kolb et al. (7), who also noted that the dense clusters of cells at the beginning of the regeneration were in the subcapsular space, suggesting that the cells originated from the migration of bone marrow cells rather than from the multiplication of the small pool of Cy-resistant spleen cells. We wanted to follow the appearance of CTL precursors into these spleens. Spleen cells derived from mice injected with Cy 8 d previously could not respond either to alloantigens (BALB/c spleen cells) or to TNP-coupled syngeneic spleen cells. When the requirement for T helper cells was bypassed by adding Il-2 to the culture (reviewed in ref. 8), a clear anti-TNP CTL response was obtained (Fig. 1, left), although the response against the alloantigen (BALB/c) was still negative (Fig. 1, right). These anti-TNP CTL were H-2 restricted, similar to those derived from a normal spleen: the lysis of TNP-coupled allogeneic targets (TNP-BALB) was much lower. The anti-TNP CTL derived from Cy-treated spleens showed a lower killing of nonhaptentated allogeneic targets (BALB/c) than CTL derived from a normal spleen. Nonhaptentated syngeneic targets were not killed, indicating that these cells are not autoreactive (data not shown). 15 d after the Cy injection, a low anti-TNP CTL response was obtained even without added Il-2, but Il-2 in culture increased the response to the same level as with normal spleen responders (Fig. 2, left). The alloresponse of the same population was low, and it was minimally enhanced by added Il-2 (Fig. 2, right). The cross-reactivities of the anti-TNP CTL derived from Cy-treated spleens were comparable to those of CTL derived from normal spleens (Fig. 2, left).

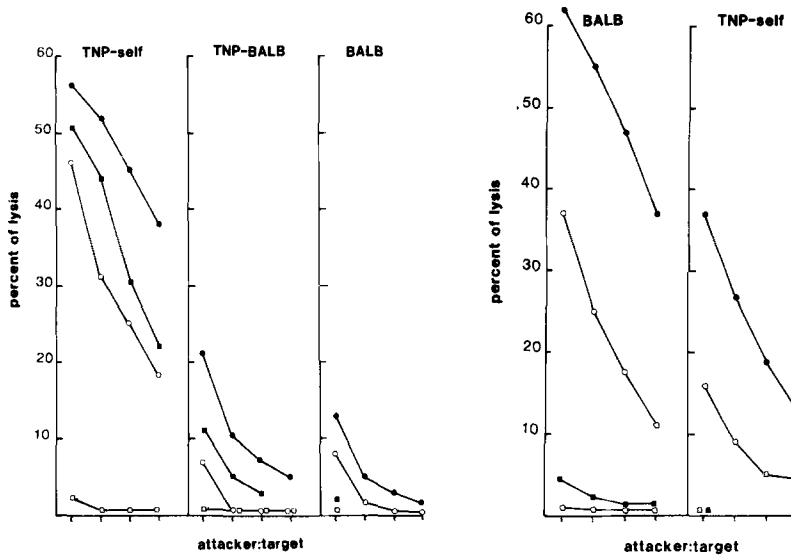


FIG. 1. The MHC-restricted (anti-TNP-self) (left) and allospecific (anti-BALB/c) (right) CTL responses by spleen cells derived from (C57BL/6 × CBA)_{F1} mice injected 8 d previously with a 300 mg/kg dose of Cy. The target used is indicated in the upper part of the panel. These responder spleen cells were derived either from normal mice (○●) or from Cy-treated mice (□■). The closed symbols represent cultures to which Il-2 was added. The attacker to target ratios were, from the left, 20:1, 10:1, 5:1, and 2.5:1.

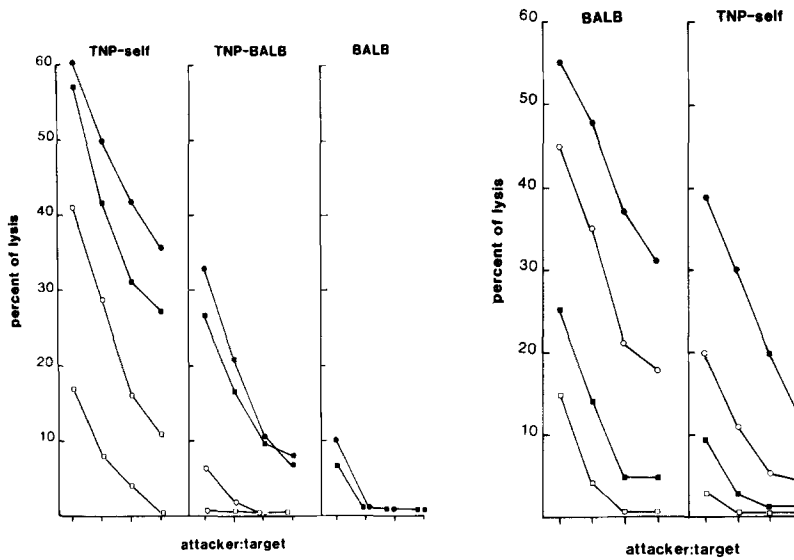


FIG. 2. The MHC-restricted (anti-TNP-self) (left) and allospecific (anti-BALB/c) (right) CTL responses by spleen cells derived from (C57 BL/6 × CBA)_{F1} mice injected 15 d previously with a 300 mg/kg dose of Cy. The attacker to target ratios were, from the left, 20:1, 10:1, 5:1, and 2.5:1. Normal mice (○●); Cy-treated mice (□■).

The conclusion from these data is obvious: the MHC-restricted CTL precursors appeared in the regenerating spleen before alloreactive precursors, so that the cell population in the spleen 8 d after Cy injection contains only precursors of MHC-restricted CTL. These precursors are not retained in the NW columns (Table I). NW

TABLE I
*CTL Responses of NW-purified Spleen Cells Derived from (C57 × CBA)F1
 Mice Injected 8 d Previously with 300 mg/kg Cy*

Responder culture	Percent of specific ⁵¹ Cr-release*	
	Alloresponse‡	Anti-TNP response§
20 × 10 ⁶ Normal spleen cells	44.0	19.4
20 × 10 ⁶ Cy spleen cells + II-2	-0.5	22.9
20 × 10 ⁶ NW spleen cells	53.1	21.1
20 × 10 ⁶ NW Cy spleen cells	-0.7	1.7
20 × 10 ⁶ NW Cy spleen cells + II-2	0.3	50.0

* Attacker to target ratio, 10:1.

‡ The cultures were stimulated with BALB/c spleen cells, and the cytotoxicity was tested using ⁵¹Cr-labeled, BALB/c-derived Con A blasts.

§ The cultures were stimulated with TNP-coupled syngeneic cells, and the cytotoxicity was tested using ⁵¹Cr-labeled, syngeneic TNP-coupled Con A blasts.

treatment made the anti-TNP CTL responses higher, confirming the reported presence of adherent suppressor cells in this population (9). The response of NW-treated cells against allogeneic cells was still entirely negative. Thus, it seems that the precursors for anti-TNP CTL in the regenerating spleen are different from the "post-thymic precursor cells" characterized by Stutman because it has been reported that this cell type is NW adherent (10). The possibility that the regenerating spleen would contain suppressor cells specifically inhibiting alloactions was excluded by showing that the regenerating cells added to the culture did not suppress the alloaction by normal spleen responders (data not shown).

These data are of importance in answering two open questions about the function of T cells: (a) studies with chimeric mice (11) have shown that the MHC genotype of the thymus determines the MHC restriction specificity of T cells (not the MHC genotype of the lymphatic precursors), but the mechanism and the exact stage of this "acquisition" of the restriction specificity is not known. We (12) and others (13) have previously demonstrated that thymusless (nude) mice are able to give rise to MHC-restricted CTL in vitro. This finding indicates a selective role for the thymus (i.e., the thymus selects cells recognizing thymic MHC antigens for further maturation from an already differentiated repertoire). The present data support this interpretation: the regenerating cells are possibly derived from the bone marrow, thus being "pre-thymic," and the anti-TNP CTL derived from this population are clearly MHC restricted (Fig. 1, left). We are currently investigating the surface characteristics of the regenerating population and also the regeneration in thymectomized mice to confirm the suggested origin of these cells.

(b) The second open question concerns the relationship between MHC-restricted and allospecific T cell responses. There is experimental evidence that these populations are overlapping: CTL stimulated with alloantigens also kill syngeneic cells carrying foreign antigens (14) and foreign antigen-specific CTL cross-react with alloantigens (15), and additionally, there are cell lines that show both alloantigen and foreign antigen plus self-MHC specificities (3). Now we found that the spleen, 8 d after Cy injection, contained cells that were able to give rise to MHC-restricted CTL only. These anti-TNP CTL responses showed a lower cross-reactivity towards allogeneic cells than CTL responses of normal spleen cells (Fig. 1, left). Because the concept about the common origin of these two types of CTL responses is so well supported

experimentally, we prefer to modify it rather than suggest that these responses are derived from different cell lineages. Our data are easily explained if we assume that the specificity of the CTL precursors is primarily towards non-MHC (foreign) antigens plus self MHC and that alloresponses are due to activation of the memory cell pool of restricted CTL. This model was originally suggested by Finberg et al. (15) to explain their data that showed that continuous stimulation of mice with Sendai virus induced a population with high alloreactivity. Our approach with the regenerating spleen (and with the use of I1-2 to bypass the need for T helper cells) has allowed us to study earlier stages during the T cell maturation where the precursors probably have not been in contact with environmental antigens, and this led to the finding of a cell population containing only precursors of MHC-restricted CTL.

These data might also be compatible with a recent hypothesis by W. Droege (16) that was based on mathematical probability models on the effects of different kinds of selective processes on the development of T cell repertoire. He suggests that there are two selection processes during the somatic development of T cell repertoire. First, the MHC antigens of an "inducer" cell are recognized by the immature T cells, and this forces the T cell system to recognize preferentially cell bound antigens and results in a high degree of MHC restriction and self reactivity. The subsequent second selection process serves the purpose of self tolerance induction (negative selection of clones with high affinity against self antigens), and this results also in a partial loss of self-MHC restriction and in a selective enrichment of alloreactive T cells.

Summary

Mice were injected with a sublethal dose of cyclophosphamide (Cy) (300 mg/kg), and the appearance of the capacity of the regenerating spleen to form cytotoxic T lymphocytes (CTL) in response against 2,4,6 trinitrophenyl-coupled syngeneic cells or against allogeneic cells was followed. It was found that 8 d after Cy injection, the spleen contained cells that could give rise to CTL, but only 2,4,6 trinitrophenyl-specific CTL responses could be obtained at this stage. A low alloresponse was first seen 2 wk after Cy injection.

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