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Direct evidence for clonal destruction of allo-reactive T cells in the mice treated with cyclophosphamide after allo-priming

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SUMMARY

It has previously been reported that a single i.p. injection of 200 mg/kg cyclophosphamide (CP) 2 days after priming with 10^8 donor spleen cells (SC) leads to donor-specific skin allograft tolerance in H-2 compatible, multiminor antigen incompatible murine strain combinations. It is speculated that the i.v. injection of donor cells may result in synchronized proliferation of donor-reactive host T cells and subsequently administered CP may specifically destroy these proliferating T cells in the periphery. Although this unique action of CP is considered to be a principal mechanism in this method, direct evidence has not yet been obtained. In the present article, this *in vivo* destructive effect of CP is clearly demonstrated by assessing detailed kinetics of host-derived blastoid T cells and donor (Mls-1^a)-reactive Vβ6$^+$ T cells in the model system of C3H mice rendered tolerant to AKR. Frequencies of the blastoid cells and Vβ6$^+$ cells, which increased as a result of AKR priming, decreased rapidly with the administration of CP. C3H mice, which received AKR SC alone, also exhibited partial deletion of Vβ6$^+$ T cells, but both tempo and magnitude of decrease in the frequency of Vβ6$^+$ cells were quite different from those of the C3H mice given AKR SC and CP, which showed more rapid and profound elimination of Vβ6$^+$ T cells. In accordance with these kinetic studies, *in vitro* proliferative response to Mls-1^a antigens was greatly impaired in mice treated with SC and CP, whereas a low but appreciable response was detected in mice given SC alone. Furthermore, skin graft tolerance was not obtained in mice treated with SC alone, rather such mice rejected donor skin graft in an accelerated fashion, in contrast to the induction of profound skin graft tolerance in CP-treated mice. Thus, CP-induced clonal destruction of antigen-stimulated-and-then-proliferating T cells actually works in our tolerance-inducing method and is obviously distinct from the peripheral clonal deletion seen after Mls priming alone.

INTRODUCTION

Cyclophosphamide (CP) is one of the popular anti-mitotic drugs and is known to selectively kill proliferating cells by the formation of cross-links between strands of DNA.[1] Making use of this unique feature of CP, a method of allo-tolerance induction in adult mice has previously been reported that comprises i.v. injection of 10^8 allogeneic spleen cells (SC) followed, usually 2 days later, by administration of 200 mg/kg of CP via the i.p. route. Using this protocol, a minimal degree of mixed chimerism associated with a long-lasting skin allograft tolerance in H-2 haplotype-identical strain combinations in mice has regularly been established.[2–4] Furthermore, combining this method with the administration of anti-Thy-1 or anti-CD4 monoclonal antibodies (mAb), Mayumi and Good obtained a long-lasting allograft tolerance even across the fully allogeneic (entire H-2 plus multiminor H antigens) barriers.[5]

As to tolerance induction to allogeneic antigens, many attempts have been made experimentally. These include neonatally induced tolerance,[6,7] irradiation-induced tolerance,[8–10] tolerance induced by means of immunosuppressive agents, such as cyclosporin A or mAb against CD3, CD4 or other accessory molecules,[11–14] and donor-specific transfusion (DST)-induced tolerance.[15–17] To explain the induction process of T-cell tolerance, several mechanisms have been proposed to be involved in each system, i.e. clonal deletion of donor-reactive T cells, clonal anergy of them and induction of suppressor T cells.[3,13,18–20] In considering the possible mechanisms of CP-induced tolerance, the most characteristic one is *in vivo* action of CP to destroy selectively donor-reactive host T cells that proliferate immediately after administration of donor SC. This *in vivo* effect of CP has been called CP-induced clonal destruction and it is proposed that this mechanism also works to destroy host-reactive donor T cells which can cause graft-versus-host disease.[21] So far, this

Abbreviations: CP, cyclophosphamide; MST, mean survival time; SC, spleen cells.

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destructive effect of CP has been shown by assessing T cells bearing Vβ6 reactive to Mls-1[a] or Vβ3 reactive to Mls-2[a] antigen in Mls-incompatible strain combinations.

Recently, it has been revealed that mature T cells which encounter superantigens in the periphery are induced not only to anergy but also to apoptosis. Kawabe and Ochi[22] and Rellahan *et al.*[23] demonstrated that mice injected with SEB, a bacteria-derived superantigen that strongly activates Vβ8$^+$ T cells, showed a decrease in the frequency of Vβ8$^+$ T cells in association with DNA fragmentation. Furthermore, Webb *et al.*[24] reported that injection of AKR (Mls-1[a]) SC to B10.BR (Mls-1[b]) mice by itself leads to specific elimination of donor-reactive Vβ6$^+$ T cells. Taking these lines of evidence into consideration, it is still unclear whether CP actually destroys Mls-1[a]-reactive host T cells *in vivo* in Mls-incompatible strain combinations as described in previous reports.

In the present article, in order to address this issue more directly, an investigation was made of the detailed kinetics of donor-reactive host T cells, which were represented by host-derived blastoid cells or Mls-1[a]-reactive Vβ6$^+$ T cells in the periphery, early after CP treatment in a model system of C3H (Mls-1[b]) mice rendered CP-induced tolerant to AKR (Mls-1[a]) skin allografts. These results clearly indicate that *in vivo* administration of CP 2 days after injection of donor SC results in the active elimination of donor-reactive host T cells in the periphery and this early occurring clonal destruction was important to achieve skin allograft tolerance.

MATERIALS AND METHODS

Mice

Inbred C3H/HeSlc (C3H; H-2[k], Mls-1[b]), B10.BR SgSnSlc (B10.BR; H-2[k], Mls-1[b]), C57BL/6 (B6; H-2[b], Mls-1[b]) mice were obtained from the Japan SLC Inc. (Shizuoka, Japan). Inbred AKR/JSea (AKR; H-2[k], Mls-1[a]) mice were obtained from Seiwa Experimental Animal Center (Nakatsu, Japan). Female mice, from 8 to 10 weeks old, were used.

Cell preparation

Mice were killed by decapitation. The spleen and lymph nodes (LN) (axillary, inguinal and mesenteric) were collected and kept on ice in RPMI-1640 medium supplemented with 10% heat-inactivated foetal calf serum (FCS). They were disrupted in the medium by pressing their fragments between two glass slides. Cell suspensions were filtered through cotton gauze and washed three times in the medium. Viable nucleated cells were counted using trypan blue dye exclusion in a standard way. In some experiments, numbers of mononuclear cells in spleen and LN were counted using Turk stain solution.

Drug and tolerance induction

CP (Endoxan, Shionogi, Tokyo, Japan) in phosphate-buffered saline (PBS) (20 mg/ml) was injected i.p. at a dose of 200 mg/kg. The day of CP injection is called day 0. Recipient mice were primed (on day −2) intravenously with 10^8 spleen cells of donor mice in 0·5 ml of RPMI-1640 medium and were given 200 mg/kg of CP i.p. 2 days later (on day 0).

Skin grafting

Using the procedure reported previously,[25] skin grafting was carried out usually on day 14. Briefly, square full-thickness skin grafts (1 cm^2) were prepared from the trunk skin of donors. Graft beds (1 cm^2) were prepared on the right lateral thoracic wall of the recipients. The graft was fixed to the graft bed with eight interrupted sutures of 5-0 silk thread. The first inspection was carried out 7 days after grafting, followed by daily inspections thereafter. Grafts were defined as rejected at the time of complete sloughing or when they formed a dry scab.

Flow microfluorometry

Undiluted culture supernatant of the rat hybridoma, 44-22-1 or KJ-16, was used as a mAb that recognizes Vβ6 or Vβ8 T-cell receptor (TcR) gene product respectively, followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG (Tago Inc., Burlingame, CA). Fluorescence-positive cells were measured by means of a FACScan (Becton Dickinson, Mountain View, CA). Dead cells were gated out by means of forward and sideward scatter, and fluorescence histograms (representing 10^4–10^5 viable cells) were accumulated on a logarithmic scale.

For three-colour analysis, a cell suspension was incubated with FITC-conjugated mAb, phycoerythrin (PE)-conjugated mAb, and biotin-conjugated mAb. Cells bound with biotin-conjugated mAb were revealed by another incubation with RED613-streptavidin.

T-cell proliferation assays

For mAb-induced proliferation, the purified mAb was diluted to the indicated concentration in PBS and 30 μl was added per round-bottom microtitre well. Plates were incubated for 4–6 hr at 37° and then washed three times with PBS before use. 2×10^5 LN cells in 200 μl of RPMI-1640 were added per well. After 48 hr, cultures were pulsed with 1 μCi of [^{3}H]thymidine and harvested 8–12 hr later.

For mixed lymphocyte cultures, 2×10^5 LN cells were incubated with 5×10^5 irradiated (2000 rads) spleen cells in 96-well flat-bottom microtitre plates for 2, 3 and 4 days, and were pulsed on each last day with 1 μCi of [^{3}H]thymidine and harvested 8–12 hr later. Values represent the arithmetic means of triplicate cultures. SD was generally $< 10\%$ of the mean.

Histology

Spleens were fixed in 10% buffered formalin, embedded in paraffin and cut at 5 μm. Sections were stained with haematoxylin and eosin.

Statistics

The statistical significance of the data was determined by Student's *t*-test when the data seemed to be parametric. A *P*-value of less than 0·05 was considered to be statistically significant.

RESULTS

CP treatment caused immediate reduction of host blastoid T cells generated early after priming with Mls-incompatible allogeneic SC *in vivo*

C3H/He (H-2[k], Mls-1[b], Thy-1.2) mice were injected i.v. with 10^8 AKR/J (H-2[k], Mls-1[a], Thy-1.1) SC, and some mice were followed by treatment with 200 mg/kg of CP i.p. 2 days later (on day 0). Spleens and LN of the recipient mice were subsequently removed and tested for the kinetics of total mononuclear cell

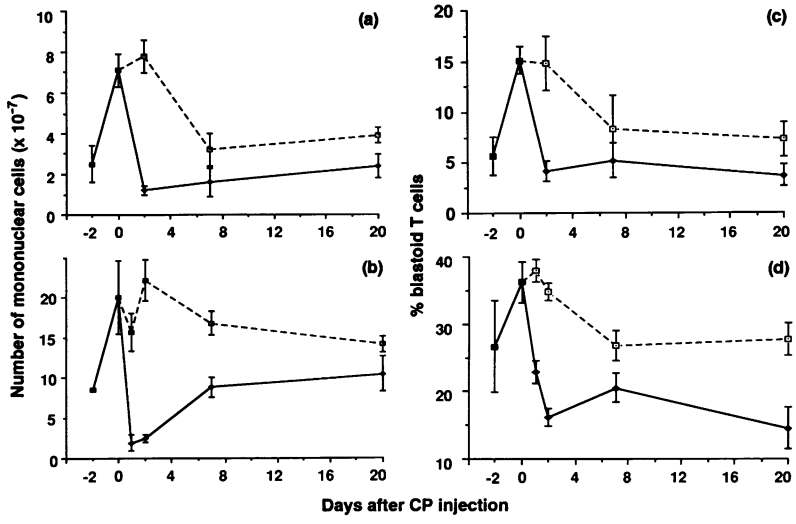

Figure 1. *In vivo* administration of CP after allo-priming caused a rapid decrease in both numbers of mononuclear cells and per cent blastoid cells in the periphery. C3H mice were injected i.v. with AKR SC on day −2 and injected i.p. with CP on day 0. Mice were killed at various intervals after these treatments to examine mononuclear cell numbers (a, b) and proportion of blastoid cells among Thy-1.2$^+$ T cells (c, d) in LN (a, c) and spleen (b, d). Data on day −2 represent those of untreated mice. Mice treated with SC alone (⊡); mice treated with SC and CP (◆). Each point represents the mean ± SD of at least four mice.

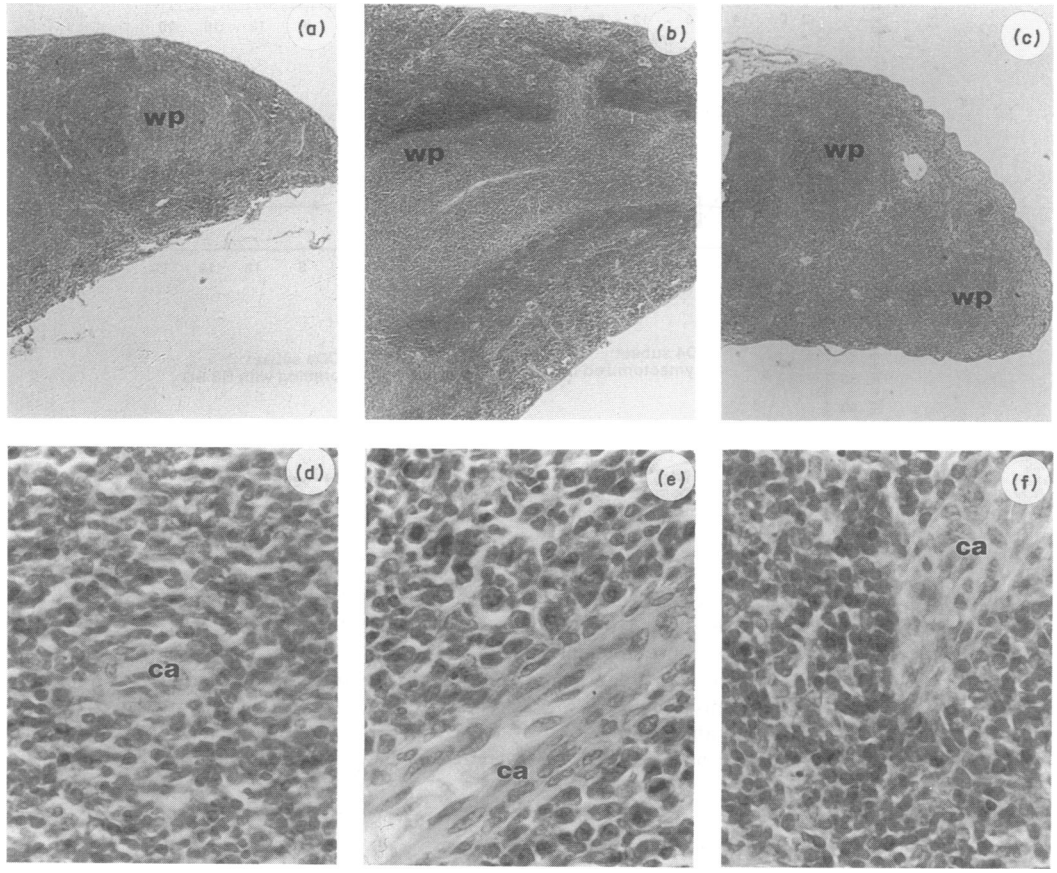

Figure 2. Photomicrograph of the spleen from untreated mice (a, d), mice treated with SC alone (b, e) and treated with SC plus CP (c, f). C3H mice were injected i.v. with AKR SC on day −2 and injected i.p. with CP on day 0. Histological examinations were performed on day 2. Low-power views (original magnification × 10: a–c) showed massive splenomegaly associated with enlarged white pulp in the AKR-primed mice (b) and atrophy in the mice given AKR SC plus CP (c). High-power views of the white pulp (original magnification × 160: d–f) showed obvious blastogenesis in the AKR-primed mice (e). Blastoid cells were rarely seen in mice given AKR SC plus CP (f). wp, white pulp; ca, central arteriole.

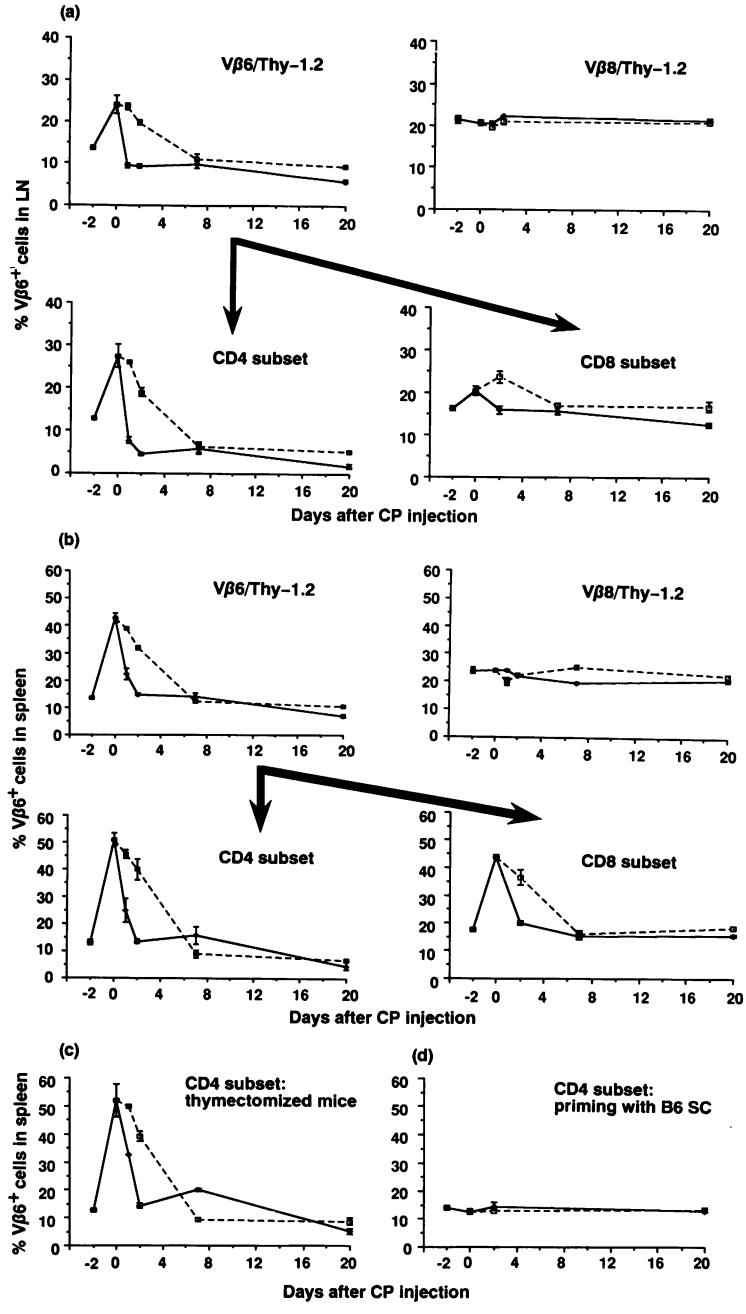

Figure 3. CP treatment after AKR priming caused rapid and profound elimination of $V\beta6^+$ T cells, not affecting the frequency of $V\beta8^+$ T cells. C3H mice were injected i.v. with AKR SC on day −2 and injected i.p. with CP on day 0. LN cells (a) and SC (b–d) from the recipient mice were studied by three-colour flow cytometric analysis for expression of Vβ6 or Vβ8, Thy-1.2 and CD4 or CD8 at the same intervals as in Fig. 1. In (c) mice were thymectomized 10 days before AKR SC injection. In (d) B6 SC (third party) were used for priming Ag. Vβ6/Thy-1.2 or Vβ8/Thy-1.2 means the proportions of $V\beta6^+$ Thy-1.2^+ or $V\beta8^+$ Thy-1.2^+ cells among Thy-1.2^+ cells. Analysis gate was further set on $CD4^+$ (or $CD8^+$) cells and the proportion of $V\beta6^+$ $CD4^+$ (or $V\beta6^+$ $CD8^+$) cells among $CD4^+$ (or $CD8^+$) cells was analysed. Mice treated with SC alone (□); mice treated with SC and CP (◆). Each point represents the mean ± SD of at least four mice.

numbers and proportions of enlarged cells (blastoid T cells) among recipient derived Thy-1.2^+ cells.

As shown in Fig. 1, numbers of mononuclear cells in both spleens and LN were remarkably increased 2 days after injection of AKR SC (on day 0), and then gradually increased in AKR-primed, CP-untreated mice (SC alone mice). When the recipient mice were treated with CP on day 0, numbers of spleen and LN cells immediately decreased and reached minimal levels by day 2. In parallel with the kinetics of mononuclear cell numbers, injection of AKR SC gave rise to a blastogenic response of recipient T cells and the proportions of blastoid T cells reached maximal levels on day 0, and then gradually declined to return

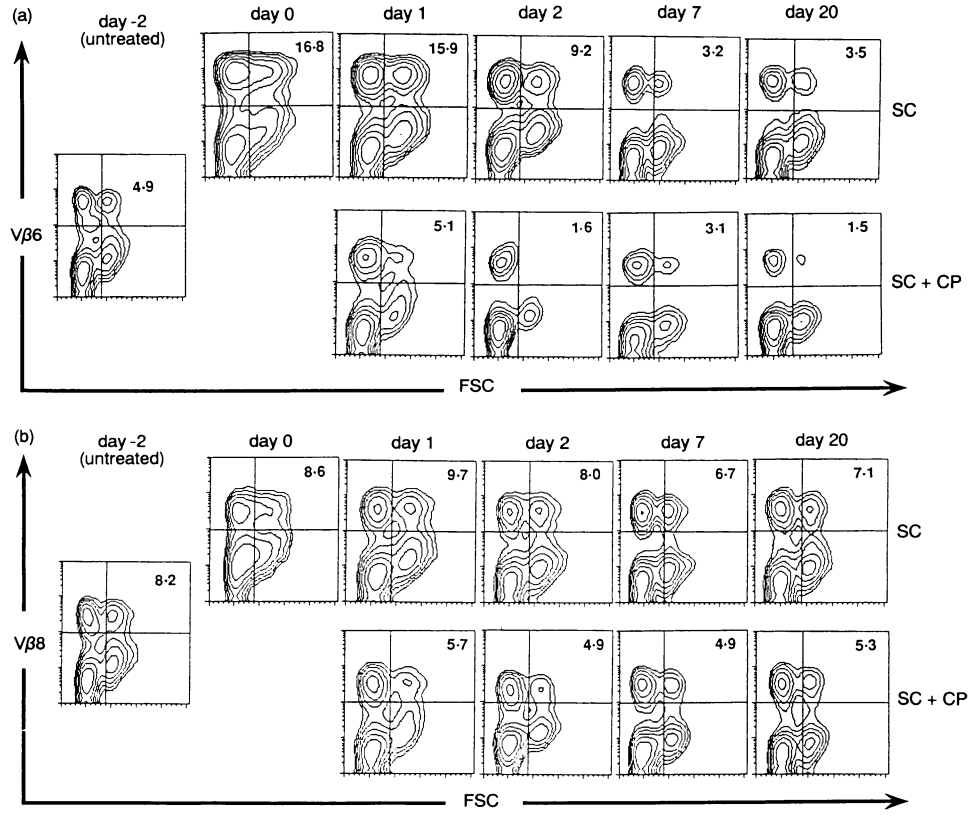

Figure 4. Representative two-dimensional profiles of forward light scatter (horizontal) versus fluorescence intensity (vertical) of the SC from the same mice as used in Fig. 3. Cells were first selected by gating on the basis of Thy-1.2 fluorescence, and the number shown within the panel indicates per cent blastoid Vβ6$^+$ cells (a) or Vβ8$^+$ cells (b) among Thy-1.2$^+$ cells.

to normal levels by day 7 in SC alone mice. On the other hand, CP treatment following injection of AKR SC caused immediate reduction of the per cent blastoid T cells in both spleens and LN.

This effect of CP to destroy blastoid cells generated after allo-priming was also seen in histological examination (Fig. 2). Four days after injection of AKR SC (on day 2), recipient spleens, especially their white pulp, were significantly swollen and contained a lot of enlarged blastoid cells with pleomorphic size and shape. In the mice treated with SC plus CP, the white pulp as well as the whole spleen were atrophic and the blastoid cells almost completely disappeared on day 2.

Kinetics of Vβ6$^+$ T cells in the recipient spleen and LN

In previous studies,[3] it has been reported that in C3H mice treated with AKR SC in combination with CP, Vβ6$^+$CD4$^+$ but not Vβ6$^+$ CD8$^+$ LN cells were selectively eliminated on day 14. At this time, however, clonal elimination of Vβ6$^+$ T cells are thought to take place even in the mice given AKR SC alone as has been demonstrated by Webb *et al.*[24] To verify further the effect of CP to actively eliminate donor-reactive host T cells, the detailed kinetics of peripheral Vβ6$^+$ T cells in mice given SC and CP was compared with that in mice given SC alone. Figure 3a shows the time-course of the frequencies of Vβ6$^+$ and Vβ8$^+$ T cells in LN as percentages among Thy-1.2$^+$, Thy-1.2$^+$ CD4$^+$, or Thy-1.2$^+$CD8$^+$ cells. In agreement with other recent reports,[24,26] 2 days after AKR priming (on day 0), the level of Vβ6$^+$/Thy-1.2$^+$ T cells in the recipient LN was markedly elevated to reach 28% (compared with about 13% in the untreated control). When these mice were also administered CP at that time, the proportion of Vβ6$^+$ T cells fell dramatically, below the level of the untreated control by day 1 and then gradually declined to reach 2% by day 20. On the other hand, in C3H mice treated with AKR SC alone, the frequency of Vβ6$^+$ T cells maintained high levels until day 2 (4 days after AKR priming), and then subsequently declined to settle down below the level of the untreated control on day 7 and after (5–6%). This phenomenon was thought to be donor specific because the proportion of Vβ8$^+$ T cells was almost unchanged. Specificity was also confirmed by the fact that injection of B6 SC (third party) and subsequent treatment with CP evoked no changes in the frequency of Vβ6$^+$ cells in C3H mice (Fig. 3d). Both proliferative and destructive responses of the Vβ6$^+$ cells were much more pronounced when Thy-1.2$^+$ cells were further gated on CD4$^+$ cells. Although the frequency of Vβ6$^+$ cells among the CD8 subset also increased after AKR priming, and decreased rapidly with CP treatment, the degrees of such changes were less than those in the CD4 subset (fourfold increase in the CD4 subset and 2·5-fold increase in the CD8 subset), and Vβ6$^+$ CD8$^+$ T cells remained at the percentage near the control level on day 20 in both groups of mice. Kinetic studies of SC (Fig. 3b) showed patterns similar to those of LN cells except that, unexpectedly, a

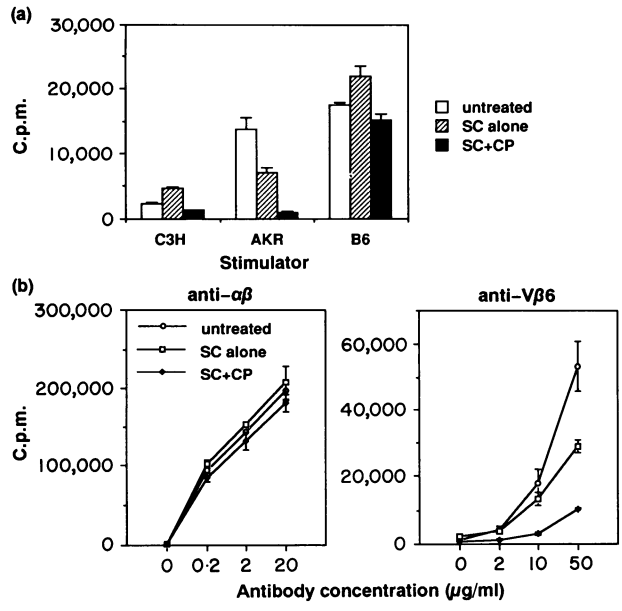

Figure 5. *In vitro* unresponsiveness of LN cells from C3H mice made tolerant of AKR. C3H mice were injected with AKR SC on day −2 and injected i.p. with CP on day 0. Proliferative responses were studied on day 20. (a) 2×10^5 LN cells from untreated mice (□), C3H mice that had received AKR SC alone (▨), or C3H mice that had been treated with AKR SC and CP (■), were incubated with 5×10^5 2000 rads-irradiated C3H, AKR, B6 SC for 2, 3 and 4 days. Representative data of 3-day mixed lymphocyte response (MLR) are shown. (b) 2×10^5 LN cells from the same mice as used in (a) were also incubated with anti-αβ or anti-Vβ6 mAb at the indicated concentrations for 2 days. Untreated mice (○); mice treated with SC alone (⊡); mice treated with SC and CP (◆).

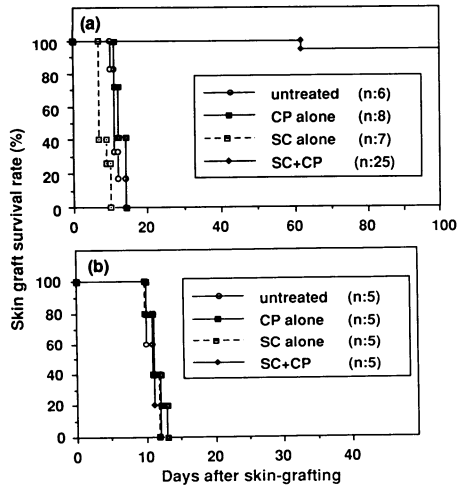

Figure 6. Accelerated rejection of AKR skin graft in the mice given AKR SC alone. AKR (a) or B10.BR (third party, b) skin grafting were performed on day 14 on untreated mice (○), mice given CP alone (■), mice given SC alone (⊡), or mice given SC and CP (◆). Mean survival time (MST) of AKR skin grafted to untreated mice was 11·5 days. In the mice given AKR SC alone, MST of AKR skin grafts was 8·1 days ($P < 0{\cdot}05$ compared with untreated mice given AKR skin grafts) and that of B10.BR skin grafts was 11·2 days.

reduced but appreciable number of Vβ6$^+$ CD4$^+$ T cells persisted in the spleens of mice given SC plus CP on day 7 (15·7% as compared with 13·1% in untreated mice). The proportion of these residual Vβ6$^+$ T cells in the spleens from CP-treated mice then gradually decreased to about 4% among CD4$^+$ T cells on day 20 (compared with 7·0% in SC alone mice).

When C3H mice were thymectomized beforehand, almost the same time-course of the per cent Vβ6$^+$/CD4$^+$ cells was obtained in both mice given SC in combination with CP and mice given SC alone, so the contribution of the thymus was clearly excluded (Fig. 3c).

Kinetics of blastoid cells among Vβ6$^+$ T cells in recipient spleens

To characterize further the residual Vβ6$^+$ T cells in spleens of two types of recipients, a time-course of blastoid cells among Vβ6$^+$ T cells was examined. Blast formation of Vβ6$^+$ T cells was obvious on day 0 (2 days after AKR priming), and in CP-untreated mice, a large proportion of blastoid cells remained on day 2. In contrast, injection of CP on day 0 resulted in almost complete elimination of Vβ6-expressing blastoid cells on day 2 (Fig. 4a). Vβ8$^+$ T cells, as a control of Vβ6$^+$ T cells, may also show some blastogenic response after AKR priming, since CP also diminished them slightly but noticeably (Fig. 4b). These partial changes may reflect either T cells which respond to minor H antigens or Mls-1^a-reactive Vβ8.1$^+$ T cells. Collectively, these results clearly indicated that administered CP actively destroyed Ag-stimulated-and-then-proliferating cells *in vivo* and this phenomenon was apparently distinct from peripheral clonal deletion seen in mice given SC alone.

Functional differences between CP-treated and CP-untreated mice previously primed with AKR SC

To evaluate functional differences between the remaining T cells from the C3H mice given AKR SC plus CP and those from C3H mice given AKR SC alone on day 20, *in vitro* proliferative response of the two was examined. In accordance with previous reports,[24,27] i.v. injection of AKR SC partially reduced anti-AKR proliferative response of LN cells but an appreciable level of the response remained. On the other hand, anti-AKR response of LN cells from the mice treated with SC and CP was completely abrogated in association with normal response to B6 (Fig. 5a). It was the same case when responder cells were stimulated with anti-TcR mAb. Namely, proliferative response to anti-Vβ6 mAb was obviously impaired in the C3H mice given AKR SC plus CP whereas low but definite response was seen in mice given SC alone, and response to anti-αβ mAb was equally high in each of the groups (Fig. 5b).

Accelerated rejection of donor skin grafts in mice pretreated with donor SC alone

As reported in previous studies, C3H mice injected with AKR SC in combination with CP treatment accepted skin grafts of the donor type specifically for >100 days (Fig. 6).[3] In contrast, pretreatment with AKR SC without CP resulted in accelerated rejection of the donor skin grafts. This type of accelerated rejection was donor specific because third-party B10BR skin grafts were rejected in normal fashion in AKR-primed mice

(Fig. 6b). These results indicate that injection of AKR SC results in the induction of memory cells for donor transplantation antigens despite the fact that both partial deletion and anergy of donor-Mls-1^a-reactive T cells occurred.

DISCUSSION

So far, CP injection immediately after antigenic stimulation has been shown to produce tolerance to a variety of antigens (e.g. sheep red blood cells, equine-anti-mouse-thymocyte globulin, equine γ globulin, mycobacteria or allo-skin) in adult mice or rats.[28–33] When considering the common feature of alkylating agents, it has been assumed that the underlying mechanism involved in CP-induced tolerance is to destroy selectively proliferating cells which have been activated by prior priming; however, none of the experiments have yet demonstrated direct *in vivo* evidence of this phenomenon. The present data clearly provide evidence of CP-induced clonal destruction of donor-reactive host T cells in the tolerance-inducing method. Injection of allo-SC resulted in an immediate increase in number of mononuclear cells in the recipient spleen and LN, which reflected the massive blastogenesis of host-derived (Thy-1.2$^+$) T cells (Fig. 1). This blastogenic response of C3H-derived T cells did not seem to be the consequence of a two-way reaction (AKR$\rightleftharpoons$C3H), because priming with (AKR $\times$ C3H) F_1 SC or heavily irradiated AKR SC, neither of which was considered to have the capacity to respond to C3H, also resulted in massive blastogenesis of C3H-derived T cells (data not shown). When mice were administered CP 2 days after allo-priming, the proportion of blastoid T cells decreased immediately in parallel with a decrease in the number of mononuclear cells. Thus, although non-blastoid T cells might also be killed by CP, blastoid T cells were shown to be far more sensitive to CP. The susceptibility of the blastoid cells to CP was also confirmed by histological examination which demonstrated that priming with AKR SC caused splenomegaly associated with massive blastogenesis of lymphoid cells in the white pulp in C3H mice and subsequent CP treatment resulted in almost complete elimination of the blastoid cells. In addition, by employing the superantigen system, examination could be made of the detailed kinetics of the host-derived Vβ6$^+$ T cells, which are strongly correlated with reactivity to Mls-1^a antigens, in C3H mice treated with AKR SC alone or AKR SC followed by CP. As has been recently reported,[24,26] the proportion of Vβ6$^+$/CD4$^+$ T cells rapidly increased after Mls-1^a priming and, without CP treatment, then gradually decreased to reach plateau levels below the control by day 7, whereas with CP treatment, the proportion of Vβ6$^+$/CD4$^+$ T cells fell much more sharply between days 0 and 2, and further declined progressively after day 7. Thus, peripheral clonal elimination of Vβ6$^+$ T cells caused by CP treatment following AKR priming is clearly distinguishable from that caused by AKR priming alone. Furthermore, the kinetic studies of blastoid cells within Vβ6$^+$ T cells revealed that the blastoid Vβ6$^+$ T cells of host origin were almost completely eliminated by day 0 (2 days after CP injection) (Fig. 4). Taken together, these data directly indicate that the administration of CP results in the active destruction of antigen-stimulated-and-then-proliferating T cells *in vivo*. The specific effect of CP on proliferating cells was further supported by the fact that when cyclosporin A was administered along with donor SC, subsequent administration of CP could not effectively destroy donor-reactive T cells and, as a result, skin allograft tolerance could not be obtained (K. Nomoto, M. Eto, Y. Yanaga, Y. Nishimura, T. Maeda and K. Nomoto, submitted for publication).

It is intriguing that the residual Vβ6$^+$ CD4$^+$ T cells persisting, particularly in the spleen, on day 7 were then progressively eliminated in mice treated with SC and CP (Fig. 3). No decrease in the frequency of the Vβ6$^+$ CD4$^+$ T cells at this relatively late phase was observed in the mice given SC alone. The direct effect of CP was unlikely to persist at this phase because clearance of CP and its activation products in humans or mice is very rapid; the mean plasma half-life period has been shown to be about 6–7 hr.[34,35] As this phenomenon was also seen in thymectomized mice, the contribution of dilutional effect by renewing T cells from the thymus seems to be excluded. It has previously been reported that mixed haematopoietic chimerism was detected in the periphery from the beginning of tolerance induction,[21] so the existence of donor cells may cause the peripheral clonal deletion of the remaining donor-reactive T cells. The importance of the peripheral chimerism to maintain tolerance is now under investigation using the tolerance-inducing method.

In agreement with recent reports,[36,37] Vβ6$^+$ CD8$^+$ T cells also responded, although the magnitude was lower than that of Vβ6$^+$ CD4$^+$ T cells, to Mls-1^a antigens *in vivo* (Fig. 3). Administration of CP also caused a rapid decrease in the frequency of Vβ6$^+$ CD8$^+$ cells, suggesting that CP also affected these proliferating Vβ6$^+$ CD8$^+$ T cells. However, in contrast to the CD4 subset, a substantial proportion of Vβ6$^+$ CD8$^+$ T cells still remained on day 20, supporting previous results.[3] This may be due to a quantitative difference in the preceding proliferative responses; relatively weak response of CD8$^+$ T cells to AKR priming may result in only a marginal destruction by CP. Another possibility is that persisting donor-derived cells in the periphery of mice given SC and CP may induce peripheral clonal deletion of only Vβ6$^+$ CD4$^+$, but not Vβ6$^+$ CD8$^+$, T cells. This possibility is supported by the present and recent reports which demonstrate that the peripheral clonal deletion induced by *in vivo* priming with superantigen, such as Mls-1^a or SEB, is restricted to the CD4 subset.[22,26,38] Furthermore, Jones *et al.*[39] showed that induction of *in vivo* tolerance to Mls-1^a was dependent on EαEβ molecules of class II major histocompatibility (MHC) antigens, and that *in vivo* exposure of mature T cells to Mls-1^a antigens after terminating anti-Eα mAb treatment leads to clonal deletion of Vβ6$^+$ CD4$^+$ T cells in the periphery.

At face value, treatment with AKR SC alone and AKR SC in combination with CP lead to a similar tolerant state, in that clonal elimination of Vβ6$^+$ T cells and *in vitro* unresponsiveness to both AKR SC and anti-Vβ6 mAb could be induced, although the degrees were different, in the two types of recipients. Previously, it was demonstrated that there was discrepancy between clonal anergy to Mls-1^a antigen and skin allograft tolerance from the fact that in DBA-primed BALB/c mice, which failed to mount an *in vitro* proliferative response to DBA, the survival of the skin grafts from DBA was not prolonged at all.[40] Here, despite the fact that not only clonal anergy but also clonal deletion of Vβ6$^+$ T cells could be induced partially in mice given AKR SC alone, subsequently grafted AKR skins were rejected in an accelerated fashion. These results suggest that the tolerance induced in C3H mice treated with AKR SC may be limited to the Mls antigen and tolerance to the transplantation

antigen may not be induced by that treatment. Thus, the injection of AKR SC only acted for priming on C3H mice, whereas the following treatment with CP is considered to lead recipient mice not to a 'memory' state but to a 'tolerant' state to AKR by means of the destruction of most of the donor-reactive T cells and subsequent induction of the mixed chimerism.

In previous studies, it was proposed that clonal anergy of donor-reactive T cells and generation of donor-specific $CD8^+$ suppressor T cells also contribute to maintaining allo-tolerance induced by this method, especially at the late stage of tolerance.[20,40,41] From the present study, CP-induced clonal destruction of donor-reactive T cells is considered to be of importance in terms of the first step to induce these sequential mechanisms in a non-lethal way. Recently, it was revealed that application of CP-induced tolerance to renal transplantation in rats clearly prolonged the graft survival across the fully allogeneic barriers (M. Eto and K. Nomoto, unpublished data). The CP-induced clonal destruction system, in which B cells have also been demonstrated to be involved,[28-32,42] may provide a new approach not only to clinical transplantation but also to autoimmune diseases in which target antigens for autoreactive cells are able to be identified.

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