

## CLOINED LYT-2<sup>+</sup> CYTOLYTIC T LYMPHOCYTES DESTROY ALLOGENEIC TISSUE IN VIVO

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The once widely held notion that acute allograft rejection is mediated by cytolytic T lymphocytes (CTL) (1, 2)<sup>1</sup> recently has been called into question (3). In the mouse, Loveland and colleagues (4, 5) demonstrated by adoptive transfer that T lymphocyte subsets enriched for Lyt-1<sup>+</sup>,2<sup>-</sup> cells, and therefore thought to lack CTL precursors, were as effective in restoring allograft immunity as were unseparated T lymphocytes. In the rat, Dallman et al. (6) demonstrated that subsets enriched for T helper lymphocytes (OX8<sup>-</sup>, W3/25<sup>+</sup>) could restore allograft immunity, but subsets enriched for CTL (OX8<sup>+</sup>, W3/25<sup>-</sup>) could not. The simplest interpretation of these observations is that CTL are neither necessary nor sufficient for allograft rejection. However, several considerations suggest that such an interpretation may be invalid. First, Dallman et al. (6) found significant numbers of OX8<sup>+</sup> cells in rejecting allografts of recipients reconstituted only with OX8<sup>-</sup> lymphocytes. As noted by the investigators, the presence of the OX8<sup>+</sup> must be adequately explained before ruling out CTL as important effector cells in allograft rejection. Second, it is questionable whether adoptive transfer of the CTL subset, which is also likely to include T suppressor lymphocytes, is a fair evaluation of CTL function in allograft rejection since regulatory forces may be strongly shifted in favor of nonreactivity. Finally, but perhaps most importantly, the prediction of T lymphocyte function on the basis of cell surface differentiation antigens appears to be more complex than previously supposed (7-9). For example, unlike "typical" Lyt-1<sup>+</sup>,2<sup>+</sup> CTL, some mouse CTL reactive with class II histocompatibility antigens express the Lyt-1<sup>+</sup>,2<sup>-</sup> phenotype (8). This could be particularly important in allograft rejection where a CTL response to class II antigens of donor vascular-endothelial cells may represent a critical determinant of the rejection process (10-12). Hence, it seems doubtful that any rigid conclusions concerning the relevance of CTL to allograft rejection can be deduced from the study of adoptively transferred lymphocytes that have

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<sup>1</sup> Abbreviations used in this paper: BSS/FCS, balanced salt solution containing 10% fetal calf serum; CTL, cytolytic T lymphocytes; EC, epidermal cells; FACS, fluorescence-activated cell sorter; IL-2, interleukin 2; NK, natural killer; PBS, phosphate-buffered saline.

been selected solely on the basis of cell surface differentiation antigens. In order to avoid such ambiguities, we studied the capacity of well-defined cloned CTL to mediate destruction of allogeneic tissue *in vivo* in terms of immunologic specificity, dose dependence, host cell recruitment, and the histologic events associated with *in vivo* reactions. Some of our findings have been reported in preliminary form (13).

### Materials and Methods

*Derivation of CTL Clones and Subclones.* The derivation and characterization of the parental CTL clone 21-4 from C3H/He mice immunized with CBA/J epidermal cells (EC) and its propagation with interleukin 2 (IL-2) and alloantigen has been described (14). The 21-4 CTL clone recognizes a tissue-restricted non-H-2 alloantigen of EC designated Epa-1 and is restricted by H-2K<sup>k</sup> products (14). Four subclones of 21-4 (21-4.24, 21-4.29, 21-4.33, and 21-4.85) were derived by micromanipulation of individual cells (15). In brief,  $1 \times 10^4$  21-4 CTL were placed in a 60-mm dish containing 4 ml of IL-2 media and several fourfold dilutions were delivered into replicate dishes. The dishes were examined under an inverted phase contrast microscope to locate individual cells which then were removed with a fine-bore glass micropipette and transferred to a fresh dish containing media alone. The new dish was examined to confirm the presence of a single cell which was then transferred to a 0.2-ml microtiter well containing IL-2 and "feeder cells" (irradiated allogeneic CBA EC plus irradiated syngeneic spleen cells). After 1–2 wk of culture, the contents of wells with actively growing cells were transferred to 2-ml tissue culture wells containing IL-2 media and fresh feeder cells. Cytolytic activity was determined in 3-h <sup>51</sup>Cr-release assays, as previously described (14).

*Fluorescence-activated Cell Sorter (FACS) Analysis.* The CTL lines were propagated as described above and then depleted of feeder cells by Ficoll-diatrizoate sedimentation (LSM, Litton Bionetics, Kensington, MD). FACS analysis was conducted after staining the cells with monoclonal anti-mouse Lyt-1, Lyt-2, and Thy-1 Biotin-conjugated antibodies (nos. 1341, 1351, and 1331, respectively; Becton Dickinson, Mountain View, CA) and with Biotin-conjugated monoclonal antibody 10-2.16 (anti-Ia.17 obtained originally from Oi and Herzenberg, Stanford, CA and maintained locally by Dr. C. S. David) as a negative control. Cells were washed in phosphate-buffered saline (PBS) with 0.2% BSA and 0.05% NaN<sub>3</sub> (PBS buffer), aliquoted to  $2 \times 10^6$  cells per tube and incubated with 30  $\mu$ g of antibody-Biotin conjugate for 30 min on ice. After washing 3 times with PBS buffer, the supernatant fluids were discarded and the cells were incubated with 30  $\mu$ g of Avidin-FITC (no. BA-101, E-Y Labs, San Mateo, CA) in PBS buffer for 30 min on ice. After 3 more washes,  $10^4$  cells were analyzed with a Becton Dickinson FACS IV.

*Evaluation of Cloned CTL Function In Vivo.* The capacity of 21-4 CTL and its four subclones to mediate tissue destruction *in vivo* was determined using the immune lymphocyte transfer tests (16). Cloned CTL were harvested from culture and feeder cells removed by Ficoll-diatrizoate sedimentation. Interface cells were collected and washed 4 times in balanced salt solution containing 10% fetal calf serum (BSS/FCS) and the desired number of CTL ( $1-10 \times 10^6$ ) were resuspended in 30  $\mu$ l of BSS/FCS. The CTL were injected intradermally with a 30-gauge needle on a Hamilton syringe into the shaved lateral-thoracic region of the test host and the injection sites were observed daily for development of skin lesions. Some mice were sacrificed at various intervals after injection and the skin and subcutis encompassing the injection site was excised and processed for 1- $\mu$ m thick, Epon-embedded, Giemsa-stained sections and transmission electron microscopy as previously described (10).

### Results and Discussion

We first injected  $10^7$  Epa-1-specific, H-2K<sup>k</sup>-restricted clone 21-4 CTL into syngeneic C3H control hosts or semiallogeneic (C3H  $\times$  CBA)F<sub>1</sub> hosts that express

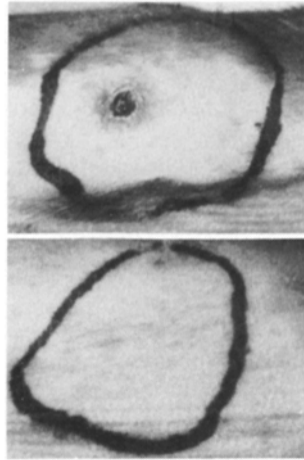


FIGURE 1. Gross appearance of the skin of a (C3H  $\times$  CBA) $F_1$  host (*top*) and a syngeneic C3H/He host (*bottom*) 5 d after intradermal injection of  $10^7$  21-4 CTL.

TABLE I  
*Specificity of Tissue Destruction by 21-4 CTL In Vivo*

Host*	No. tested	No. with skin lesions <sup>†</sup>	Epa-1 <sup>‡</sup>	H-2 K I D
(C3H $\times$ CBA) $F_1$	29	29	+	k k k
AKR	3	3	+	k k k
B10.BR	5	5	+	k k k
CBA	3	3	+	k k k
RF	3	3	+	k k k
C3H/He	18	0	-	k k k
B10.A	6	6	+	k k/d d
B10.OL	6	0	+	d d k
B10.MBR	6	0	+	b k q
A/J	6	0	-	k k/d d
C57BL/6	3	0	+	b b b

\* All hosts received  $10^7$  21-4 CTL intradermally.

<sup>†</sup> Skin lesions in susceptible hosts invariably progressed to ulceration within 5 d, whereas in nonsusceptible hosts no discernible lesions were observed at any time.

<sup>‡</sup> Only one allelic form of Epa-1 has so far been defined; hence hosts are designated Epa-1<sup>+</sup> or Epa-1<sup>-</sup>.

Epa-1 and H-2K<sup>k</sup>. No discernible reaction was grossly apparent in any syngeneic host at any time, whereas every  $F_1$  host developed a progressive skin lesion characterized by edema and erythema (day 1), induration (days 2-3), and ulceration (days 3-5; see Fig. 1). In more than 45 tests, we never have experienced a discrepancy from these observations (see Table I).

We analyzed the pathogenesis of 21-4 CTL-mediated lesions by light and electron microscopy. 21-4 CTL were easily identified in 1- $\mu$ m sections of skin reactions by virtue of their prominent cytoplasm and numerous cytoplasmic granules (Fig. 2, *a* and *c*). By ultrastructure (Fig. 2*b*), 21-4 CTL cytoplasmic

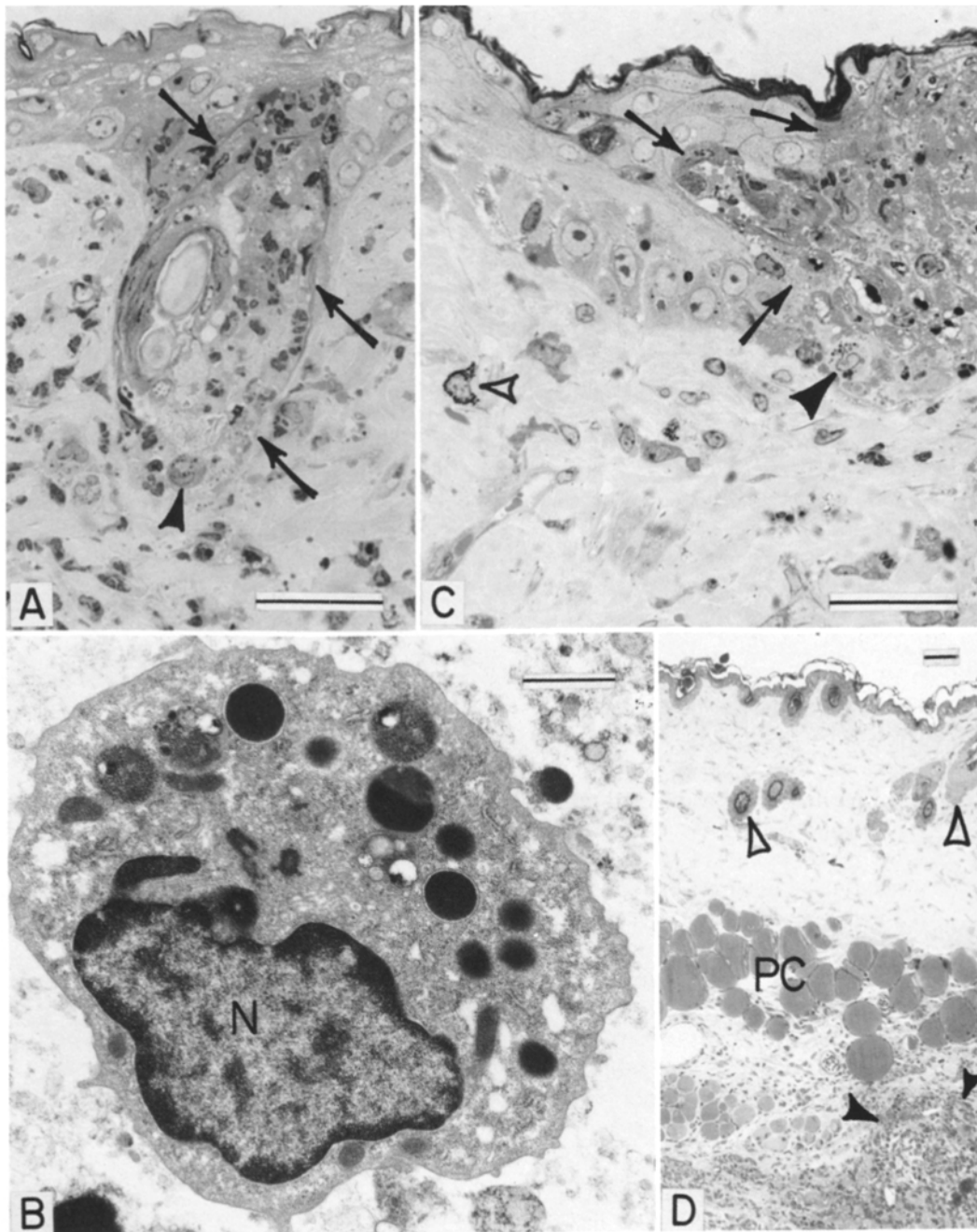
granules resembled those of other cloned mouse leukocytes that express natural killer (NK)-like activity (17–19) or suppressor (19) or cytolytic (20) T cell function. Like cloned cells expressing NK-lysis or suppressor T cell function (19), 21-4 CTL also exhibited large deposits of cytoplasmic glycogen when processed for electron microscopy by the osmium tetroxide-potassium ferrocyanide technique (19). In (C3H × CBA)<sub>F</sub><sub>1</sub> mice, injection of 21-4 CTL was followed by marked dermal edema, infiltration of dermis, and epidermis by 21-4 CTL, and necrosis of epidermal structures. Hair follicles were damaged particularly early and exhibited extensive necrosis by day 1 after injection (Fig. 2*a*). Necrosis involved large areas of the epidermis by day 2 (Fig. 2*c*) and virtually the full-thickness of the dermis by day 3. In addition to 21-4 CTL, injection sites in (C3H × CBA)<sub>F</sub><sub>1</sub> hosts also exhibited infiltration by host leukocytes. These included neutrophils, which were particularly prominent in and around necrotic structures (e.g. Fig. 2*a* and *c*), as well as occasional small mononuclear cells (e.g. Fig. 2*c*), presumably representing recruited host lymphocytes. A few macrophages, identifiable by their abundant cytoplasm with large phagolysosomes encompassing cellular and nuclear debris, were present in the deep dermis in the vicinity of the initial injection site at day 2 and increased in number by day 4. By contrast, light and electron microscopy revealed few, if any, macrophages in the upper dermis or epidermis.

In contrast to susceptible (C3H × CBA)<sub>F</sub><sub>1</sub> mice, C3H (control) hosts developed no cutaneous necrosis. Instead, 21-4 CTL remained confined to the injection site (Fig. 2*d*) where they were identifiable by light or electron microscopy for at least 4 d, strongly suggesting that the migration of 21-4 CTL into epidermal structures is triggered by exposure to specific alloantigen (Epa-1).

Having established that 21-4 CTL did evoke allogeneic tissue destruction *in vivo*, we next evaluated the immunologic specificity of the reaction by employing a panel of hosts selected for various expression of Epa-1 and H-2 antigens. As shown in Table I, only hosts expressing both Epa-1 and H-2K<sup>b</sup> were susceptible to the destructive effects of 21-4 CTL. It should be noted that in many instances the nonsusceptible hosts were fully allogeneic to 21-4 CTL but no gross evidence of a host-vs.-clone response was observed at the injection site. The strain distribution of susceptible hosts exactly paralleled the susceptibility of host EC to lysis *in vitro* by clone 21-4 CTL (14). Hence, the alloantigenic (Epa-1) and H-2 restriction (H-2K<sup>b</sup>) specificity of 21-4 CTL *in vivo* was identical to that recorded *in vitro*.

We then analyzed the dose dependence of tissue destruction by 21-4 CTL. As shown in Table II, all (C3H × CBA)<sub>F</sub><sub>1</sub> hosts receiving 5–10 × 10<sup>6</sup> 21-4 CTL developed lesions within 3 d. By contrast, hosts receiving 2.5 × 10<sup>6</sup> had lesions by day 5, and only 1 of 3 hosts receiving 1 × 10<sup>6</sup> CTL developed any ulceration. It should be noted that 21-4 CTL require an exogenous source of IL-2 for long-term survival and for growth in response to alloantigen (14). Therefore, the minimum number of 21-4 CTL required for consistent lesion formation is probably less than 2.5 × 10<sup>6</sup> cells because exogenous IL-2 was not provided in these experiments.

We next investigated whether radiosensitive host leukocytes were necessary participants in the development of the lesions, as would be expected if the lesions



**FIGURE 2.** Light and electron microscopic findings in skin injected with clone 21-4 CTL. (a) By day 1, hair follicles (arrows) of F<sub>1</sub> mice exhibited necrosis and infiltration by clone 21-4 CTL and other leukocytes, predominantly neutrophils. Clone 21-4 CTL (solid arrowhead) were large, with an eccentrically located, often lobulated or reniform nucleus and prominent dark cytoplasmic granules. (b) Electron micrograph of a typical clone 21-4 CTL 1 d after injection into an F<sub>1</sub> mouse. Clone 21-4 CTL in control (C3H) injection sites or prepared for electron microscopy before their injection in vivo had a similar appearance by ultrastructure. Clone 21-4 CTL contain large, membrane-bound cytoplasmic granules, often distributed in greatest number on one side of the nucleus. The contents of some granules appear homogeneously electron dense; others contain variable amounts of electron dense particles or small vesicular structures. N = nucleus. (c) By day 2, large regions of F<sub>1</sub> epidermis were necrotic (arrows) and infiltrated by clone 21-4 CTL (solid arrowhead) as well as neutrophils and mononuclear cells apparently devoid of prominent cytoplasmic granules. In contrast to clone 21-4 CTL, dermal mast cells (open arrowhead) had a less abundant cytoplasm with smaller and more numerous granules. (d) Control skin 3 d after injection of clone 21-4 CTL, which remained confined to the injection site (solid arrowheads) beneath the panniculus carnosus (PC). The dermis, epidermis, and hair follicles (open arrow-heads) appear normal. Scale bars: a, c, and d, 50  $\mu$ m. b, 1  $\mu$ m, Epon-embedded, Giemsa-stained sections.

TABLE II  
Dose Dependence of Tissue Destruction by 21-4 CTL

21-4 CTL cell dose	Fraction with lesions*				
	Days postinjection				
	1	2	3	4	5
$1 \times 10^6$	0/3	0/3	1/3	1/3	1/3
$2.5 \times 10^6$	0/3	1/3	2/3	2/3	3/3
$5 \times 10^6$	0/3	1/3	3/3	3/3	3/3
$10 \times 10^6$	0/3	0/3	3/3	3/3	3/3

\* A lesion was defined as ulceration at the site of injection.

TABLE III  
Radiosensitive Host Leukocytes Are Not Required for Tissue Destruction by Cloned CTL

Host*	No. tested	No. with skin lesions
(C3H $\times$ CBA)F <sub>1</sub>	8	8
C3H/He	4	0

\* Hosts were exposed to 850 rad of whole-body x irradiation 24 h before intradermal injection of  $10^7$  21-4 CTL. All hosts died 7-15 d after irradiation.

were mediated by a delayed-type hypersensitivity mechanism (21). To address this possibility, (C3H  $\times$  CBA)F<sub>1</sub> hosts were lethally irradiated (850 rads) and given an intradermal injection of  $10^7$  21-4 CTL 24 h later. As shown in Table III, lethal irradiation of F<sub>1</sub> hosts did not abrogate the capacity of 21-4 CTL to mediate tissue destruction. In fact, the severity and tempo of the reactions in irradiated F<sub>1</sub> hosts was somewhat increased.

To provide further evidence that the *in vivo* reactions were unambiguously attributable to CTL, we subcloned 21-4 CTL by micromanipulation of single cells. Four subclones were derived and all proved to have identical *in vitro* cytolytic specificity as 21-4 CTL (Table IV). When tested *in vivo*, all four subclones ( $5 \times 10^6$  CTL per host) uniformly produced ulcerating lesions in (C3H  $\times$  CBA)F<sub>1</sub> hosts ( $n = 3$  for each subclone), whereas no lesions were produced in syngeneic C3H hosts ( $n = 3$  for each subclone).

We investigated the cell-surface phenotype of parental and subcloned 21-4 CTL by FACS analysis with monoclonal Thy-1, Lyt-1, and Lyt-2 antibodies. In three such analyses, all cells of the parent and subclones were found to stain distinctly for Thy-1 and Lyt-2. However, as exemplified by the FACS plots in Fig. 3, on two of three occasions a small fraction of the cells (5-9%) of parental and subcloned 21-4 CTL also stained distinctly for Lyt-1. We have not elucidated the reason for the low variable Lyt-1 expression, but it is in accord with our previous results obtained with parental clone 21-4 CTL (14) as well as the recent results of Palladino et al. (22), who detected low levels of Lyt-1 antigens on all the Lyt-2<sup>+</sup> CTL clones they examined. Hence, although the majority of 21-4 CTL express the typical Thy-1<sup>+</sup>, Lyt-1<sup>-</sup>, 2<sup>+</sup> phenotype, a more accurate description of the cloned population is Thy-1<sup>+</sup>, Lyt-1<sup>+/-</sup>, 2<sup>+</sup> to reflect the variable Lyt-

TABLE IV  
The Cytolytic Specificity of Clone 21-4 and Four of its Subclones  
In Vitro

Clone or subclone	Percent specific lysis of EC targets*			
	CBA	B10.A	B10.OL	C3H
21-4	76.4	23.8	-1.2	-0.7
21-4.24	78.5	28.4	-1.9	-1.5
21-4.29	71.9	25.9	-1.0	-0.8
21-4.33	67.1	24.9	2.4	1.4
21-4.85	64.1	31.3	-0.3	2.5

\* Percent specific lysis was determined in a 3-h  $^{51}\text{Cr}$ -release assay as previously described (14). Specific lysis of all spleen cell targets was  $<6.3\%$  and the spontaneous  $^{51}\text{Cr}$  release was always  $<15.7\%$ . The effector-to-target ratio was 16:1. For Epa-1 and H-2 genotypes of the target cell donors see Table I.

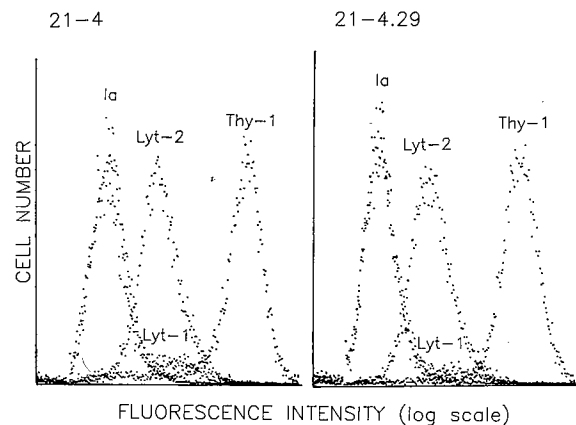


FIGURE 3. FACS analysis of clones 21-4 and 21-4.29 (see text for technical details). The histograms shown are typical of the results obtained on two of three occasions where reactivity with Lyt-1 monoclonal antibody was observed, as indicated by the small Lyt-1 peak. However, all the cells reacted with Lyt-2 and Thy-1 monoclonal antibodies on all three occasions.

l expression.

The results of the present study prove that Lyt-2<sup>+</sup> CTL are capable of mediating immunologically specific destruction of allogeneic tissue in vivo in a dose-dependent fashion. Thus, they support the findings of Engers et al. (23) who demonstrated that intravenous injection of cloned Lyt-2<sup>+</sup>, H-2<sup>d</sup>-reactive CTL induced rejection of P815 (H-2<sup>d</sup>) tumor cells in the peritoneal cavity of immunosuppressed hosts. In addition, our studies extend the observations of Enger et al. (23) by clearly demonstrating the in vivo immunologic specificity of the CTL and localizing their presence to the site of allogeneic tissue destruction. For clarity of interpretation certain differences between our study and that of Engers et al. should be emphasized. The Lyt-2<sup>+</sup> CTL clones we used require an exogenous source of IL-2 for proliferation in response to alloantigen in vitro and presumably in vivo (14, M. E. Snider and J. D. Tyler, unpublished observations). In contrast, the cloned Lyt-2<sup>+</sup> CTL used by Engers et al. are capable of producing their own IL-2 and it was speculated that this property may be

especially suitable to the in vivo function of cloned CTL. IL-2 was not provided in our experiments and it is improbable that significant amounts of IL-2 were carried over from culture to in vivo sites in our studies because the CTL were thoroughly washed before intradermal injection. Therefore, it would appear that IL-2 is not essential for the effector function of Lyt-2<sup>+</sup> CTL in vivo. On the other hand, if the intravenous route of injection is used, as in the study by Engers et al., a source of IL-2 may be required to permit sufficient numbers of viable CTL to survive, migrate to, and effect destruction of allogeneic tissue at a distant site.

The precise mechanism by which the cloned CTL destroy allogeneic tissue remains unknown, but it would seem probable that direct cytolysis is involved. In addition, indirect mechanisms dependent on lymphokines produced by cloned CTL (23–25) or on blood vessel injury (26) may also play a role. In this regard, our results suggest that indirect mechanisms, if involved, do not require the participation of radiosensitive leukocytes.

Our data taken together with the frequent detection of alloimmune CTL in recipients of allografts and in allografts themselves (27–35) indicate that these T lymphocytes constitute an important barrier to successful transplantation. However, our results should not be construed as disputing a role in transplantation immunity for other functionally distinct T lymphocytes such as those mediating classic delayed-type hypersensitivity reactions (36–38). In our view, the relative importance and precise contribution of different effector mechanisms may be addressed best by evaluating the efficacy of functionally distinct T lymphocyte clones, transferred alone or in combination, to mediate rejection of a tissue allograft.

### Summary

The long-accepted notion that alloimmune cytolytic T cells (CTL) mediate transplantation immunity has recently been called into question. In order to ascertain directly whether alloimmune CTL can mediate destruction of foreign tissue, we tested the ability of mouse CTL expanded as cloned populations in vitro to destroy allogeneic skin in vivo. The results of these studies prove unequivocally that cloned Lyt-2<sup>+</sup> CTL can perform this task in an immunologically specific, H-2-restricted, and dose-dependent fashion.

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