

AUTOREACTIVE T CELL CLONES SPECIFIC FOR CLASS I AND CLASS II HLA ANTIGENS ISOLATED FROM A HUMAN CHIMERA

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The establishment of tolerance is a prerequisite to obtain successful transplantation and to achieve a stable chimerism. Despite the numerous studies undertaken in animal bone marrow chimeras (1–3), the mechanisms underlying the induction and maintenance of transplantation tolerance are still unclear. The elucidation of these mechanisms is essential not only to provide a way to induce tolerance to allografts, but also to gain understanding of the discrimination between self and non-self.

Patients suffering from severe combined immunodeficiency (SCID)¹ are naturally devoid of T cells. Because of the absence of specific T cell-mediated immune responses, transplants of allogeneic immature lymphoid cells are relatively easily accepted. However, the development of graft-versus-host disease (GVHD) by the newly implanted donor immune system exposes every host tissue to immunologic attack.

Recently we described a SCID patient in whom HLA-incompatible fetal liver and thymus transplantation (FLTT) has been successfully used (4, 5). The HLA typing performed over the years revealed a stable engraftment of T cells from the transplant, whereas the B cells and monocytes remained of host origin (6). The follow up is 10 yr now.

Despite the complete HLA mismatch between donor-derived T lymphocytes and recipient cells, no signs of acute or chronic GVHD have been observed. Furthermore, donor-derived T lymphocytes appeared to be able to cooperate with host cells since normal *in vivo* and *in vitro* antibody responses against thymus-dependent antigens such as tetanus toxoid (TT) were obtained (6).

These observations suggested that the fetal lymphocytes, progressively differentiating in an allogeneic environment, acquired tolerance towards the host HLA determinants and became able to recognize them as restriction elements in response to foreign antigens. To better define the mechanisms underlying this process of self-education and acquisition of tolerance, we established a series of T cell clones from the PBL of the patient.

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¹ Abbreviations used in this paper: FLTT, fetal liver and thymus transplantation; GVHD, graft-versus-host disease; SCID, severe combined immunodeficiency; TT, tetanus toxoid.

In the present paper, we describe eight selected T cell clones that reacted specifically with the class I or class II HLA antigens of the recipient cells *in vitro*. Both proliferative and cytotoxic responses were observed, indicating that the establishment of transplantation tolerance observed in this stable human chimera is not due to the elimination of host-reactive T cells from the repertoire. In addition, to our knowledge this is the first demonstration of autocytotoxic cells directed against class I HLA antigens in human.

Materials and Methods

Patient. The infant S.P. had a family history of SCID and was brought up in a strict isolation from birth. At the age of 1 and 5 mo, he received FLTT from two different HLA incompatible donors, 13 and 10 wk old (gestational age), respectively. The clinical course of the patient before and after transplantations has been reported in detail previously (4).

The patient is 11 yr old now, he shows a normal evolutive growth, and is in apparent good health. The clinical course of occasional minor upper respiratory infections occurring over the years had been mild and not different from that observed in normal children. Despite a normal total lymphocyte count, he has a persistent inverted CD4⁺/CD8⁺ ratio ranging from 0.56 to 0.78. He has a regular immunization history with TT and received the last booster injection 4 yr ago.

Establishment and Culture of T Cell Clones. Autoreactive T cell clones were obtained during the process of generating TT-specific T cell clones from patient's PBL. 10⁶ PBL were suspended in 1 ml Yssel's medium (7) supplemented with 1% human AB⁺ serum (heat inactivated, 30 min at 56°C) and were stimulated with TT (Calbiochem-Behring Corp, La Jolla, CA) at a concentration of 25 µg/ml.

After incubation at 37°C in 5% CO₂ for 5 d, these TT-activated cells were resuspended in medium containing 20 IU rIL-2 per milliliter (kindly provided by Dr. R. Kastelein, DNAX Research Institute, Palo Alto, CA). 10 d after the onset of the culture the cell sample was restimulated with a feeder cell mixture consisting of 10⁶ irradiated (4,000 rad) allogeneic lymphocytes per milliliter, 10⁵ irradiated (5,000 rad) cells per milliliter of the patient's EBV-transformed B cell line (SPS), and 0.1 µg per milliliter purified PHA (Wellcome Diagnostics, Beckenham, Kent, UK).

Another 12 d later, the cells were cloned by limiting dilution at a concentration of one cell per three wells in a Titertek 96-well round-bottomed plate (Flow Laboratories, Irvine, Scotland) in the presence of the feeder cell mixture described above and TT. After 14 d, proliferating T cell cultures were transferred into 24-well tissue culture plates (Linbro, Flow Laboratories) and restimulated with the feeder cell mixture. The clones were further expanded in medium containing IL-2. 10 d after the last stimulation the clones were screened for specificity and functional activity as described below.

Cell Lines. The EBV-transformed cell lines of the patient (SPS), his father (UD93), and his mother (UD94) were originated from infection of fresh PBL with EBV obtained from the marmoset lymphoblastoid cell line B 95-8. All cell lines were cultured in Yssel's medium supplemented with 1% human AB⁺ serum.

HLA Typing. HLA typing was carried out on the T cell clones and EBV-transformed B cell lines using a cytotoxicity assay previously described (8).

Proliferation Assays. 2 × 10⁴ T cells that had been rested for 10–12 d after addition of the feeder mixture were mixed with 2 × 10³ irradiated (5,000 rad) stimulator cells in the presence or absence of antigens in 200 µl Yssel's medium. After 3 d of incubation, 1 µCi (37 kBq)[³H]TdR (New England Nuclear, Dreieich, Federal Republic of Germany) was added to each well. 4 h later, the cells were harvested onto glass fiber strips using a semi-automated cell harvester and the amount of incorporated [³H]TdR was assessed by liquid scintillation counting. The results are expressed as the mean of triplicate cultures ± the standard deviation. The effect of mAbs on the proliferative capacity was determined by adding varied amounts of mAb to the responder cell-stimulator cell mixtures at the onset of the cultures.

Cytotoxicity Assays. Cytotoxic activity was determined using a ^{51}Cr -release assay. Effector cells were mixed with either 10^3 (EBV cell lines) or 10^4 (PBL) of ^{51}Cr -labeled target cells in 200 μl Iscove medium (Gibco, Glasgow, Scotland) with 0.25% BSA in U-shaped wells of a microtiter plate, spun down at 50 g , and incubated for 4 h at 37°C in a humidified atmosphere of 5% CO_2 . The supernatants were harvested using a Skatron supernatant collection system (Skatron, Lier, Norway) and were counted in a gamma counter. Each effector-to-target cell interaction was measured in triplicate and the data are presented as percent specific ^{51}Cr release, which is determined as follows: ^{51}Cr release = $100 \times [(\text{experimental release} - \text{SR})/(\text{MR} - \text{SR})]$. The maximum release (MR) was determined after incubation of the cells in 1% Triton X-100 and the spontaneous release (SR) by measuring the release of target cells in medium only. To determine the capacity of mAbs to affect the cytotoxic reaction, target cells were preincubated with mAb for 15 min at room temperature. The cells were then mixed with effector cells and the ^{51}Cr -release assay was carried out as described above.

Monoclonal Antibodies. The anti-CD2 mAb used in this study was CLB-T11 (kindly provided by Dr. R. Van Lier, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service). The anti-CD4 mAb RIV6 was a kind gift of Dr. Kreeftenberg (R.I.V. Bilthoven, The Netherlands). The anti-CD8 mAb SPV-T8 and the anti-CD3 mAb SPV-T3b have been described before (9). The mAb W6/32, which detects a common determinant on all class I HLA molecules, was obtained from Sera Lab (Crawley Down, U.K.). The antibody SPV-L3 reacts with a monomorphic determinant on HLA-DQ molecules (9). The mAb Q5/13 was a kind gift from Dr. S. Ferrone (Medical College, Valhalla, NY) and detects a determinant common to HLA-DR and HLA-DP molecules (10).

Fluorescence Analysis. 10^5 cells were added per well of a V-bottomed microtiter plate and washed once with PBS containing 0.02 mM NaN_3 and 1% BSA. The cells were then incubated with mAbs for 30 min at 4°C. After two washes in PBS/azide/BSA, the cells were incubated with a 1:40 dilution of FITC-labeled F (ab'_2) fragments of a goat anti-mouse antibody (Bioart, Meudon, France) and incubated for 30 min at 4°C. After three washes, the cells were transferred to FACS tubes (Becton Dickinson & Co., Oxnard, CA) and analyzed on a FACS 440 (Becton Dickinson & Co.).

Results

HLA Typing. The HLA typing of parental PBL and patient's PBL before transplantation is shown in Table I. HLA typing of the patient's PBL, carried out by conventional cytotoxicity assay during the follow up after transplant, revealed the progressive engraftment of cells from the second donor (HLA-A1,2; C7; B8,18; DR3,9), while the cells from the first donor (HLA-A2,11; C4; B62,27; DR1,8), present at low numbers at the beginning, progressively became undetectable (4, 6).

Furthermore, the HLA typing, performed 7 yr after transplantation on separated T and B cell populations and monocytes (Table I), indicated that all the B lymphocytes and monocytes remained of host origin, whereas the T lymphocytes were donor derived (6).

HLA typing of the T cell clones and the EBV-transformed B cell line obtained from the patient's PBL confirmed this complete split chimerism. All the auto-reactive T cell clones have the phenotype of the second donor: HLA-A1,2; C7; B8,18; DR3,9, whereas the EBV-transformed B cell line SPS has the recipient phenotype: HLA-A33,3; C6,2; B14,47; DR4,5. It has to be mentioned that the recent HLA typing on T cell clones differs for DR locus from initial typing carried out 6 yr ago on patient's PBL (HLA-DR 3,9 instead of HLA-DR1,7).

Screening and Characterization of Autoreactive T Cell Clones. In the course of the screening performed as described in Materials and Methods, 15 of 50 clones

TABLE I
HLA Typing of the FLTT Subject and His Parents

| Subject | HLA locus | HLA locus | HLA locus | HLA locus |
|----------------------|------------|-----------|-------------|------------|
| | A | C | B | DR |
| Father* | | | | |
| a | 1 | W4 | 17 | 7 |
| b | W33 | W6 | 14 | 4 |
| Mother* | | | | |
| c | 3 | W2 | W47 | 5 |
| d | W26 | W4 | 12 | 1 |
| Patient* | | | | |
| b | W33 | W6 | 14 | 4 |
| c | 3 | W2 | W47 | 5 |
| Patient [†] | | | | |
| T cells donor | 1-2 | W7 | 8-18 | 3-9 |
| B cells host | W33-3 | W6 | 14-W47 | 4-5 |
| Monocytes host | W33-3 | | 14-W47 | 4-5 |

* HLA genotypes of the family

[†] HLA phenotype of patient 7 y after the transplant. HLA typing was carried out on enriched cell populations.

were obtained that proliferated in response to SPS (Table II). The majority of these clones were also cytotoxic for SPS. The proliferative response and the cytotoxic activity were not enhanced when TT was added to the culture (not shown). Phenotyping of these autoreactive clones showed that all the 15 clones were CD2⁺, CD3⁺, 6 were CD4⁺, and 9 were CD8⁺ (Table II).

The clones SP-A3, SP-A10, SP-B23, SP-B33, SP-C5, SP-C6, SP-D47, and SP-D68, which displayed the highest proliferative and cytotoxic activity against SPS, were selected for further characterization.

To determine whether the responses of these clones to SPS (recipient cells) were specific, we examined the capacity of allogeneic EBV-transformed B cell lines to induce proliferation. In addition, the cytotoxic activity of the clones against allogeneic target cells was tested. In Table III it is shown that the T cell clones were specifically reacting with stimulator/target cells sharing HLA-A3 (NOB) or HLA-DR4 (JY) antigens with SPS, whereas they did not react with the cell lines HSY, IAD and QBL which share no HLA determinants with SPS.

Specificity Study. The nature of the self-determinants recognized by the autoreactive T cell clones of donor origin was examined by blocking studies with mAbs directed against class I and II HLA antigens.

The proliferation of the three CD4⁺ clones (SP-A3, SP-B23, SP-B33) in response to SPS was blocked by the anti-HLA-DR, DP mAb Q5/13. In contrast, mAbs against HLA-DQ (SPV-L3) and HLA-A, B, C (W6/32) were ineffective (Table IV). The inhibition by Q5/13 was specific since this mAb was unable to affect the proliferative activity of the CD8⁺ class I MHC-specific T cell clone SP-C5 (Table IV). Comparable results were obtained for the T cell clones SP-A3 and SP-B23 in the cytotoxicity assay (Table V), since the anti-HLA-DR mAb blocked their cytotoxic reactivity against SPS whereas the mAbs against HLA-DQ and class I HLA antigens were ineffective. These findings indicate that the

TABLE II
*Proliferative and Cytotoxic Responses of Autoreactive T Cell Clones
 against the Host-derived EBV-transformed B Cell Line (SPS)*

| T cell clones | Phenotype* | Proliferation [‡] | Cytotoxicity [§] |
|---------------|------------------|-------------------------------------|-----------------------------|
| | | (³ H]TdR incorporation) | (⁵¹ Cr release) |
| | | <i>cpm</i> × 10 ⁻³ | % |
| SP-A3 | CD4 ⁺ | 80.3 | 25 |
| SP-A8 | CD8 ⁺ | 5.3 | 27 |
| SP-A10 | CD8 ⁺ | 12.3 | 37 |
| SP-B18 | CD4 ⁺ | 8.5 | 32 |
| SP-B23 | CD4 ⁺ | 83.2 | 35 |
| SP-B24 | CD4 ⁺ | 15.0 | 0 |
| SP-B30 | CD8 ⁺ | 14.4 | 28 |
| SP-B33 | CD4 ⁺ | 78.0 | 0 |
| SP-B35 | CD8 ⁺ | 13.8 | 0 |
| SP-B37 | CD4 ⁺ | 12.7 | 0 |
| SP-C5 | CD8 ⁺ | 8.2 | 63 |
| SP-C6 | CD8 ⁺ | 7.9 | 50 |
| SP-C12 | CD8 ⁺ | 5.0 | 40 |
| SP-D47 | CD8 ⁺ | 8.3 | 51 |
| SP-D68 | CD8 ⁺ | 12.9 | 60 |

* All the clones were CD2⁺ and CD3⁺.

[‡] Responder/stimulator cell ratio 1:1. In all cases the SD was < 10% of the total cpm and the proliferation of T cells in the absence of stimulator cells was < 0.5 × 10⁻³ cpm

[§] Effector/target cell ratio 10:1.

TABLE III
*Proliferative Response and Cytotoxic Activity of Autoreactive T Cell Clones against Allogeneic
 EBV-transformed B Cell Lines*

| T cell clones | Stimulator/target cells | | | | | | | | | |
|-------------------|----------------------------|--------------------|---------------------|-------------|-----------------------------|-------|-------------------------------|-------|------------------------------------|-------|
| | NOB [A3 B7 DR2 DQ2]* | | JY [A2 B7 DR4,6] | | QBL [A26 B18 DR3 DQ3] | | HSY [A1,19 B8,13 DR3,7] | | IAD [A1,2 B13,17 DR7,13 DQ1] | |
| | cpm [‡] | Lysis [§] | cpm | Lysis | cpm | Lysis | cpm | Lysis | cpm | Lysis |
| ×10 ⁻³ | | % | ×10 ⁻³ | % | ×10 ⁻³ | % | ×10 ⁻³ | % | ×10 ⁻³ | % |
| SP-A3 | 0.1 | 0 | <u>70.8</u> | <u>18.5</u> | 0.1 | 8.1 | 0.02 | 3.3 | 0.05 | 2.4 |
| SP-A10 | 1.3 | 2.3 | 0.8 | 0 | 1.3 | 10.1 | 0.4 | 4.1 | 0 | 3.4 |
| SP-B23 | 1.8 | 8.3 | <u>75.3</u> | <u>67.7</u> | 0 | 5.8 | 0.02 | 0 | 0 | 6.5 |
| SP-B33 | 3.8 | 0 | <u>13.0</u> | 1.7 | 0.3 | 5.5 | 1.2 | 6.4 | 0.6 | 3.2 |
| SP-C5 | <u>11.0[‡]</u> | <u>67.8</u> | 0.07 | 2.7 | 0.1 | 1.8 | 0.02 | 4 | 0.3 | 6.2 |
| SP-C6 | 1.0 | 0 | 0.2 | 10.3 | 0.8 | 4.2 | 2.2 | 6 | 0.2 | 7.5 |
| SP-D47 | 0.5 | 10 | 0 | 8.2 | 0 | 1.9 | 0.4 | 9.1 | .08 | 4.3 |
| SP-D68 | <u>69.1</u> | <u>60.8</u> | 0.3 | 5 | 0.7 | 1.5 | 0.1 | 0 | 2.1 | 8 |

* HLA phenotype of the allogeneic EBV-transformed cell lines.

[‡] Values represent the mean [³H]TdR incorporation at a responder/stimulator cell ratio of 1:1.

[§] Values represent the mean percent of ⁵¹Cr release at an E/T cell ratio of 10:1.

[‡] Underlining indicates positive reactivity.

TABLE IV
Effect of Anti-HLA mAbs on the Proliferative Responses of T Cell Clones against SPS

| mAb added* | Percent inhibition of proliferation with responder T cell clones: | | | |
|--|---|----------------|----------------|---------------|
| | SP-A3 | SP-B23 | SP-B33 | SP-C5 |
| W6/32 [anti HLA-A, B, C] | 6 | 11 | 0 | <u>92</u> |
| SPV-L3 [anti HLA-DQ] | 3 | 0 | 0 | 0 |
| Q5/13 [anti HLA-DR, DP] | <u>90</u> | <u>100</u> | <u>65</u> | 0 |
| Proliferative response in the absence of mAbs (cpm $\times 10^{-3}$) [†] | 28.5 \pm 2.5 | 44.2 \pm 3.3 | 78.0 \pm 3.4 | 4.0 \pm 0.5 |

Responder/stimulator cell ratio was 1:1

* mAbs were used at a final dilution of 1:200 of ascites fluid

[†] Values represent the mean [³H]TdR incorporation \pm SD.

CD4⁺ autoreactive clones derived from the donor T cells (HLA-DR 3,9) recognize HLA-DR 4 or HLA-DR 5 determinants on the recipient cells.

The cytotoxic activity of the five CD8⁺ clones (SP-A10, SP-C5, SP-C6, SP-D47, SP-D68) against SPS was blocked by anti-HLA-A, B, C mAb (W6/32), but not by anti-HLA-DQ (SPV-L3) and HLA-DR (Q5/13) mAbs (Table V). As expected, W6/32 did not inhibit the cytotoxic activity of T cell clones SP-A3 and SP-B23, which are class II MHC specific (Table V). Moreover the proliferative response of the CD8⁺ clone SP-C5 to SPS was inhibited by W6/32 (Table IV). These data demonstrate that the CD8⁺ T cell clones (HLA-A1,2; C7; B8,18) recognize HLA class I determinants (HLA-A33,3; C6,2; B14,47) of the recipient that resulted in cytotoxic as well as proliferative responses.

Family Study. To get more information on the fine specificity of these autoreactive T cell clones, we tested the proliferative responses of the CD4⁺ clones and the cytotoxic activity of the CD8⁺ clones against the parental EBV-transformed B cell lines. The response against patient's and parental fresh PBL and PHA blasts was determined in parallel, to exclude a reactivity either to foreign antigens introduced by the in vitro culture or to EBV antigens.

TABLE V
Effect of Anti-HLA mAbs on the Cytotoxic Activity of T Cell Clones against SPS

| mAb added [‡] | Percent inhibition of cytotoxicity with effector T cell clones:* | | | | | | |
|--------------------------------------|--|-----------|------------|-----------|-----------|------------|-----------|
| | SP-A10 | SP-C5 | SP-C6 | SP-D47 | SP-D68 | SP-A3 | SP-B23 |
| W6/32 [anti HLA-A, B, C] | <u>100</u> | <u>91</u> | <u>100</u> | <u>77</u> | <u>98</u> | 0 | 0 |
| SPV-L3 [anti HLA-DQ] | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Q5/13 [anti HLA-DR,DP] | 0 | 0 | 0 | 0 | 0 | <u>100</u> | <u>87</u> |
| Percent lysis in the absence of mAbs | 36 | 44 | 50 | 53 | 55 | 14 | 39 |

* E/T cell ratio of 10:1.

[‡] mAbs were used at a final dilution of 1:200 of ascites fluid.

TABLE VI
Family Study of ($CD4^+$, $CD8^-$) Autoreactive Proliferative T Cell Clones

| Stimulator cells* | $[^3H]TdR$ incorporation with responder T cell clones: | | |
|--------------------|--|--------------------------------------|----------------------------------|
| | SP-A3 | SP-B23 | SP-B33 |
| EBV cell lines | | <i>cpm</i> $\times 10^{-3}^\ddagger$ | |
| "Autologous" (SPS) | <u>28.6 \pm 3.5</u> | <u>44.2 \pm 3.3</u> | <u>78.0 \pm 3.4</u> |
| Mother (UD94) | 0.1 | 0.6 | 1.3 |
| Father (UD93) | <u>20.3 \pm 1.5</u> | <u>40.8 \pm 5.9</u> | <u>63.7 \pm 2.3</u> |
| PBL | | | |
| Mother | 0.7 | 0.3 | ND |
| Father | <u>3.3 \pm 1.1</u> | <u>5.3 \pm 0.4</u> | ND |

* Responder/stimulator cell ratio was 1:1. In all cases, spontaneous $[^3H]TdR$ incorporation of T cells in the absence of stimulator cells was $< 0.7 \times 10^{-3}$ cpm.

‡ Results are \pm SD.

In Table VI it is shown that the $CD4^+$ class II HLA-specific T cell clones SP-A3 and SP-B23 both proliferated in response to the patient's EBV cell line SPS (HLA-DR 4,5), but not in response to his PHA-stimulated T lymphoblasts (HLA-DR 3,9) (not shown). Furthermore, they proliferated in the presence of the father's EBV cell line UD93 and fresh PBL, sharing the HLA-DR4 antigen with the patient. No responses to the mother's EBV cell line UD94 and fresh PBL that share the HLA-DR5 antigen with the patient were observed. Clone SP-B33 proliferated in response to SPS and UD93 sharing HLA-DR4. These results combined with the proliferation induced by JY (Table III) and the blocking experiments with the mAbs (Table IV) suggest that all three $CD4^+$ clones recognize an epitope present on HLA-DR4.

In Table VII it is shown that the $CD8^+$ clones were cytotoxic for SPS and

TABLE VII
Family Study of ($CD4^-$, $CD8^+$) Autoreactive Cytotoxic T Cell Clones

| Target cells* | Percent ^{51}Cr release with effector T cell clones: | | | | |
|--------------------|--|-----------|-----------|-----------|-----------|
| | SP-A10 | SP-C5 | SP-C6 | SP-D47 | SP-D68 |
| EBV cell lines | | | | | |
| "Autologous" (SPS) | <u>37</u> | <u>30</u> | <u>40</u> | <u>41</u> | <u>34</u> |
| Mother (UD94) | <u>40</u> | <u>48</u> | 5 | 4 | <u>47</u> |
| Father (UD93) | 7 | 4 | <u>68</u> | <u>63</u> | 0 |
| PHA Blasts | | | | | |
| Patient | ND | 0 | 0 | 3 | 0 |
| Mother | ND | <u>26</u> | 0 | 9 | <u>42</u> |
| Father | ND | 0 | <u>34</u> | <u>28</u> | 0 |
| PBL | | | | | |
| Patient | ND | <u>19</u> | <u>20</u> | <u>18</u> | <u>23</u> |
| Mother | <u>86</u> | <u>74</u> | 0 | 0 | <u>17</u> |
| Father | 0 | 0 | <u>16</u> | <u>21</u> | 0 |

* E/T ratio was 10:1.

fresh PBL of the patient that contain B cells and monocytes of the recipient. In contrast, the PHA blasts were not lysed, since they are entirely of donor origin and consequently have the same HLA determinants as the T cell clones. Furthermore, the clones SP-A10, SP-C5, and SP-D68 display cytotoxic activity against the mother's EBV cell line UD94, PBL, and PHA blasts (Table VII), suggesting that they interact with either the class I HLA antigens A3, C2, or B47 antigens shared with the mother. Since we demonstrated that clones SP-C5 and SP-D68 are also cytotoxic towards the EBV cell line NOB (Table III), which expresses the HLA-A3 determinant, we conclude that these clones are specific for HLA-A3. Clones SP-C6 and SP-D47 were cytotoxic against the EBV cell line UD93, PBL, and PHA blasts of the father, suggesting that they are specific for either the class I HLA antigens A33, C6, or B14 antigen shared with the father.

Discussion

We studied a patient suffering from SCID who has been successfully transplanted with two subsequent HLA-mismatched FLTT >10 yr ago. This transplantation resulted in the progressive differentiation of donor lymphoid cells in the HLA-incompatible host environment (4, 5).

In this child we observed the stable engraftment of only T cells from the second transplant, while the fetal cells from the first donor, present at low concentrations shortly after transplantation, became progressively undetectable in peripheral blood. The B cells and monocytes remained exclusively of host origin (6).

Despite this situation of stable split chimerism with a complete HLA mismatch between donor-derived T lymphocytes and recipient cells, no signs of acute or chronic GVHD were observed and full immunological reconstitution has been achieved. Recently, we demonstrated that the T cells of donor origin were able to cooperate with recipient non-T cells across the allogeneic barrier (6, Roncarolo, M. G., et al., manuscript submitted for publication).

Therefore, this patient provides an unique model to gain insight in the understanding of education or selection of self recognition resulting in acquisition of tolerance. For this reason, a study of clonal level of patient's PBL has been performed.

A series of T cell clones specifically reacting against recipient cells were obtained from the PBL of the patient. All these autoreactive clones had proliferative activity and 11 of 15 were also cytotoxic.

The specificity of these T cell clones was documented by their reactivity with the patient's EBV-transformed B cell line, with the parental haploidentical EBV cell lines and with EBV cell lines from unrelated subjects sharing the relevant HLA antigens. No reactivity with EBV cell lines bearing nonshared HLA antigens was observed. The clones displayed cytotoxic activity against the parents' PHA blasts, demonstrating that they were not directed against viral antigens present on the EBV transformed B cell lines. Furthermore, the observation that cytotoxic/proliferative activity was detected also against fresh PBL exclude the possibility that the T cell clones recognize foreign antigens introduced by *in vitro* culture.

Most strikingly in this study we demonstrate that all the CD8⁺ autoreactive T cell clones recognized the class I HLA determinants of the recipient. Autoreactive T cells and T cell clones have been described in patients suffering from autoimmune diseases or in situations where autoaggressive processes occur (11–13). In addition, autoreactive T cells and T cell clones have been generated *in vitro* from autologous mixed lymphocyte cultures carried out with lymphocytes of healthy donors (14), normal T cells activated by autologous antigen specific T cell clones (15), and from T cell cultures activated by soluble antigens (16, 17). However, in contrast to the clones obtained in our study, all autoreactive clones isolated either from normal donors or from patients suffering from autoimmune diseases described thus far, were either specifically directed against class II HLA determinants or their activities were class II HLA restricted (11–17).

Class II HLA determinants are only expressed at low levels on a limited number of cell types. Therefore, it has been proposed that even if class II HLA specific autoreactive T lymphocytes do exist *in vivo*, they would only affect the limited number of cell types bearing sufficient class II HLA antigens (14, 16). In addition, such clones have been considered to represent a normal regulatory mechanism involving class II HLA molecules (15–17).

From our patient we isolated autoreactive T cell clones recognizing class I HLA antigens which are ubiquitous and highly expressed in the organism. Consequently, if these autocytotoxic cells are operational *in vivo* they would constantly attack every cell and tissue of the host.

Comparable T cell clones directed against either the class I or class II HLA antigens of recipient or donor have been isolated *in vivo* from graft-infiltrating lymphoid cells of transplanted patients in whom acute fatal GVHD or irreversible graft rejection occurred (18). However, in the patient described here, these class I HLA-specific autocytotoxic cells or their precursors could exist *in vivo* >10 yr after the transplant without any apparent pathological manifestations. Therefore, the possibility of a peripheral autoregulatory suppressor mechanism, as has been described for some animal models (2, 19–22), could be put forward. In particular, it could be that cells are present *in vivo* that are either able to prevent the differentiation of autoreactive precursor cells or to inactivate the autoaggressive effector cells. Further investigations are in progress to determine this suppressor mechanism. These studies are becoming more complicated now, since we have observed that a proportion of TT-specific T cell clones, recently isolated from patient's PBL after an *in vivo* booster with TT, expressed the HLA phenotype of the first donor (Roncarolo, M. G., et al., manuscript submitted for publication). This finding indicates that, in spite of the fact that T cells from the first transplant were not detectable in PBL by conventional HLA typing, these cells are circulating and can become functional. Thus we must envisage not only an induction of tolerance of the T cells derived from the two transplants towards the non-T cells of the patient, but also towards each other. Perhaps tolerance between these two completely mismatched mature populations of transplanted T cells involves veto cells that can be responsible for the mutual *in vivo* tolerization (23, 24). In contrast, the T cells of donor origin reacting *in vitro* against the HLA antigens of the recipient cells could not be inactivated *in vivo* through

a veto mechanism since the recipient CTL that have to veto donor-anti-recipient CTL are completely absent.

It is still unclear whether the thymus repopulates and a normal thymic education takes place in SCID children transplanted with FLTT. Furthermore, it is not known whether the transplanted fetal liver cells migrate and are educated in the host thymus or that they are educated in the donor's fetal thymus that may have developed at the site of infusion (25).

Studies performed in human and animal HLA incompatible bone marrow transplantation models suggested that intrathymic cells imprint the self-recognition pattern of engrafted HLA-incompatible T cells and thus self tolerance (26, 27). Therefore, in our patient the induction of tolerance could have occurred through a mechanism of clonal deletion in the host thymus (28). However, the isolation from peripheral blood of various T cell clones of donor origin specific for the recipient HLA determinants excludes this possibility and demonstrates that at least no complete elimination of host-reactive cells in the thymus took place.

It is noteworthy that the patient has an inverted ratio of CD4⁺/CD8⁺ cells and that a high spontaneous [³H]TdR incorporation by PBL was measured (4, 6). These observations suggest the presence of ongoing activation processes involving CD8⁺ T cells. Whether this reflects the maintenance of an active peripheral suppressor mechanism remains to be determined.

Summary

T cell clones of donor origin that specifically react with recipient cells were obtained from a SCID patient successfully reconstituted by allogeneic fetal liver and thymus transplantation performed 10 yr ago. The majority of these clones displayed both cytotoxic and proliferative responses towards PBL and an EBV-transformed B cell line derived from the patient. In addition, these T cell clones had proliferative and cytotoxic responses towards the parental PBL, EBV cell lines, and PHA blasts. Blocking studies with anti-class I and anti-class II HLA mAbs indicated that the activity of the CD4⁺ T cell clones was specifically directed against class II HLA antigens of the recipient. On the other hand, the cytotoxic and proliferative responses of the CD8⁺ T cell clones were specific for class I HLA antigens which are ubiquitously expressed on the recipient cells.

Thus, the establishment of transplantation tolerance observed in this stable human chimera is not due to the elimination of host-reactive T cells from the repertoire and suggests the presence of a peripheral autoregulatory suppressor mechanism.

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