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A single [$^3$H]thymidine-based limiting dilution analysis to determine HTLp and CTLp frequencies for bone marrow donor selection

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Summary:

Histocompatibility between recipient and donor is a critical factor in allogeneic BMT which, to a large extent, determines the incidence of GVHD after BMT. Functional histocompatibility assays, such as the helper T lymphocyte precursor frequency assay (HTLp) and the cytotoxic T lymphocyte precursor frequency assay (CTLp), have proved to be helpful tools in facilitating donor selection procedures. However, a major drawback of these assays is that they are laborious and require large numbers of cells. We therefore adapted a [$^3$H]thymidine-based assay, the ‘JAM’ test, as a read-out for CTLp frequencies, to replace the more cumbersome $^{51}$Cr-release assay. Furthermore, we applied an experimental setup that enables the assessment of HTLp and CTLp frequencies from a single limiting dilution assay to reduce the number of cells needed. The newly developed assay is relatively easy to perform and has the advantage that different subsets of T cells can be quantified in a single ongoing alloreaction. When the combined assay was applied in unrelated donor selection it proved to be a sensitive method that enables differentiation in suitability of distinct donors for a single patient. Therefore, the combined HTLp/CTLp assay appears to be a practical and sensitive method for identifying functional histocompatibility in related and unrelated donor/recipient combinations.

Keywords: helper T lymphocyte; cytotoxic T lymphocyte; [$^3$H]thymidine; limiting dilution analysis.

In allogeneic BMT it is important to strive for histocompatibility between donor and recipient in order to reduce the incidence of GVHD after BMT. To this effect, minimal matching criteria have been established, i.e. serological identity at HLA-A and B-loci and molecular identity at DRB and DQB loci. However, a vast number of patients still develop GVHD, most likely due to mismatches in minor antigens and/or as yet undetected HLA-differences. Functional in vitro histocompatibility assays such as the mixed lymphocyte reaction (MLR), helper T lymphocyte precursor frequency assay (HTLp), and cytotoxic T lymphocyte precursor frequency assay (CTLp) can be helpful tools in addition to the standard matching procedures.

While it is generally assumed that the MLR is a poor predictor for the development of GVHD, there is no consensus exists about the relevance of HTLp and CTLp frequencies in donor selection. Concerning the HTLp frequency assay, high frequencies were found to correlate with an increased risk for the development of GVHD in unrelated donor BMT and HLA-identical sibling BMT, although this is not consistently found. The CTLp frequency assay appeared insufficiently sensitive to have a predictive value in HLA-identical sibling BMT. In unrelated donor BMT high CTLp frequencies were associated with an increased risk for the development of GVHD although these results are also not unequivocal.

Although it is clear that both IL-2 secreting and cytotoxic T cells play a role in GVHD, it is surprising that until now only one group has determined both HTLp and CTLp frequencies in a single study. They concluded that both HTLp and CTLp frequencies were informative, but that the advantage of the HTLp assay was that it was less labour intensive, more sensitive to class II mismatches, and capable of detecting minor antigen mismatches.

Since the data concerning the relevance of HTLp and CTLp frequency assays in BMT donor selection are not unequivocal and mostly based on studies of small groups of recipient–donor combinations, additional research is required to determine the relevance of these functional assays in BMT donor selection between unrelated individuals as well as within families.

A major drawback in performing these assays is that they are laborious and require a large number of cells to determine both HTLp and CTLp frequencies. Furthermore, to study the relevance of HTLp and CTLp frequency assays in BMT donor selection between unrelated individuals as well as within families, the assays used will preferentially need to be sufficiently sensitive to detect both minor antigen mismatches and major (HLA) mismatches. Thus, we designed a simplified protocol that would meet these requirements. We first developed an experimental setup that enables the assessment of HTLp and CTLp frequencies from a single limiting dilution assay (LDA), aiming at a reduction of the amount of cells needed. Secondly, we adapted a [$^3$H]ThD-R-based assay, the ‘JAM’ test, as a read-out for CTLp frequencies. This relatively easy to perform assay served as an alternative for the more cumbersome $^{51}$Cr-release assay. This approach led to the development of a complete [$^3$H]ThD-R-based method for determination of both HTLp and CTLp frequencies from a single LDA.
Materials and methods

Cells

PBMC were isolated by density centrifugation (Lymphoprep; Nycomed, Oslo, Norway) either from buffy coats from healthy blood donors or from blood samples drawn from patients waiting for an allogeneic BMT, as well as from their genotypically HLA-identical siblings or unrelated donors. Cells were frozen and stored in liquid nitrogen until use.

HLA typing

HLA typing for HLA A, B, DR and DQ was performed using the standard microcytotoxicity assay. Additional oligotyping for class II using the PCR-SSO technique was performed in several combinations. Apparent HLA identity between BMT patients and their HLA-identical sibling donors was confirmed by defining haplotype segregation patterns based on additional family typings. For two patients and their six potential (unrelated) donors class I typing was extended by using the class I SSP ARMS-PCR typing kit from the 12th International Histocompatibility Workshop (IHW). HLA-A2 subtyping was performed using the HLA-A*02 SSP ARMS-PCR subtyping kit from the 12th IHW. Subtyping of B*12 was performed using a nested ARMS-PCR adapted from De Luca et al., with additional primers to increase the resolution, and modified to run under the conditions of the 12th IHW protocols for HLA-A*02 subtyping.

Limiting dilution assay (LDA)

The limiting dilution was set up in RPMI-1640 with glutamax (Gibco, Paisley, UK) supplemented with pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, 10% FCS (Gibco) and 20% supernatant of the IL-2-producing cell line MLA-144 (ATCC, TIB 201) as a source of IL-2, at 37°C in a humified atmosphere containing 5% CO₂. Before use the CTL-p cells were washed five times with PBS and then resuspended in culture medium without IL-2. The HTLp assay was performed as described by Schwarer et al., with minor modifications. Briefly, a limiting dilution was set up as described above. On day 3, the plates were irradiated (40 Gy) and 1 x 10⁴ CTL-p cells in 50 µl culture medium were added to each well. After 24 h incubation at 37°C in a humified atmosphere containing 5% CO₂, 0.5 µCi [³H]TdR was added to each well and the plates were harvested the following day using a Micromate 196 harvesting device (Canberra, Meriden, CT, USA) and counted in the Packard Matrix 96 Direct Beta counter (Canberra). A well was scored positive if the amount of [³H]TdR incorporation exceeded the mean plus three times the standard deviation of the wells containing irradiated stimulator cells only.

CTLp frequency assay

The CTLp assay was performed as described by Roosnek et al., with minor modifications. Briefly an LDA was set up as described above and on days 3 and 6, fresh medium containing IL-2 (Proleukin) was added to a final concentration of 15 U/ml. On day 10 cells were resuspended, 150 µl of the suspension was transferred to new plates and a JAM test or ⁵¹Cr-release assay was performed.

Stimulation and labeling of the target cells

On day 5 of the LDA culture PBMC of the initial stimulator were thawed and resuspended in culture medium supplemented with 10 µg/ml PHA-M (Boehringer Mannheim, Mannheim, Germany). Cells were grown in a 24-well plates (Greiner, Frickenhausen, Germany) at a concentration of 1 x 10⁶ cells/well. After 48 h incubation at 37°C in a humidified atmosphere containing 5% CO₂, cells were washed and resuspended in medium supplemented with IL-2 (50 U/ml) at a concentration of 0.5 x 10⁴ cells/ml in 24-well plates.

Target cells to be used in the ⁵¹Cr-release assay were then incubated at 37°C in a humidified atmosphere containing 5% CO₂. After 2 days the cells were centrifuged (5 min at 500 g), the supernatant was removed and 100 µCi ⁵¹Cr was added to the pellet. After 1 h incubation at 37°C the cells were washed five times in phosphate-buffered saline, resuspended in culture medium and then ready for use in the ⁵¹Cr-release assay.

Target cells to be used in the ‘JAM’ test were incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 2 days of which the last 16 h were the presence of 10 µCi [³H]TdR. Cells were washed once, resuspended in culture medium and then ready for use in the ‘JAM’ test.

Chromium-release assay

⁵¹Cr-labeled target cells (1 x 10⁴ in 50 µl) were added to each well of the LDA. After 4 h of incubation at 37°C in a humidified atmosphere containing 5% CO₂, 100 µl of the
supernatant was transferred to tubes and counted in a gamma counter (Wallac, Turku, Finland). A well was scored positive if the 51Cr-release exceeded the mean plus three times the standard deviation of the wells containing irradiated stimulator cells alone.

`JAM` test

[3H]Tdr-labeled target cells (1 x 10^4 in 50 μl) were added to each well of the LDA culture. After 4 h of incubation at 37°C in a humidified atmosphere containing 5% CO₂, the wells were harvested using a Micromate 196 96-well harvesting device (Canberra) and counted in the Packard Matrix 96 Direct Beta counter (Canberra). A well was scored positive if the [3H]Tdr retention was less then the mean minus three times the standard deviation of the wells containing irradiated stimulator cells alone.

Combined [3H]Tdr-based HTLp/CTLp frequency assay

A limiting dilution was set up as described. On day 3 the plates were centrifuged (1 min 200 g) and 50 μl of the supernatants was transferred to new plates and frozen at -30°C until use in the HTLp assay. Fresh medium containing IL-2 was added to the wells of the LDA culture to obtain a final concentration of 15 U/ml and this was repeated on day 6. On day 10, cells were resuspended, 150 μl of the suspension was transferred to new plates and a `JAM`-test or 51Cr-release assay was performed as described above. For determination of HTLp frequencies, the supernatants were thawed and 1 x 10^5 CTL cells in 25 μl medium were added to the wells. After 24 h incubation at 37°C in a humidified atmosphere containing 5% CO₂, 0.5 μCi [3H]Tdr was added to each well and the wells were harvested the following day.

Calculation of HTLp and CTLp frequencies

Frequency estimations were calculated using the computer program as developed by Strijbosch et al.37 Frequencies were estimated using the jackknife version of the maximum likelihood analysis. Data were excluded when the goodness of fit was higher than 12.5 (corresponding to P values >0.05). Statistical analysis of the data was performed using the Sign test.

Results

Determination of HTLp and CTLp frequencies from a single limiting dilution assay

Several effector/stimulator combinations were tested in the combined assay and compared to the outcomes of the separately performed `standard` assays. As shown in Table 1, in eight out of nine combinations the HTLp frequencies were slightly lower when determined from supernatants as compared to those determined when CTL-L2 cells were added directly to the irradiated LDA cultures. Although this slight decrease in sensitivity is significant according to the Sign test, the 95% confidence intervals overlap in six out of nine combinations (Table 1). Notably, in one HLA-identical sibling combination this slight decrease in sensitivity gave rise to a frequency that was below threshold value. Removal of the supernatant on day 3 had no influence on the outcome of the CTLp assay in the combinations tested (data not shown).

Adaptation of the `JAM` test for use in a human setting

Simultaneously, we adapted the `JAM` test ([3H]Tdr retention assay) for use in a human setting to replace the more cumbersome 51Cr-release assay as a read-out for the CTLp assay. The `JAM` test, which is based on DNA fragmentation after CTL-induced cell death, was originally developed in a mouse model and turned out to be an easier and more sensitive assay as compared to the standard 51Cr-release assay.14

The LDA protocol we used for generation of cytotoxic T lymphocytes was similar to that used in the 51Cr-release-based CTLp assay as described by Roosnek et al.11 However, with respect to the target cells a prerequisite for the `JAM` test is that these cells are in log growth phase to obtain optimal [3H]Tdr incorporation. Therefore, we tested several stimulation protocols using different incubation times, cell numbers and PHA and IL-2 concentrations (data not shown). The protocol that appeared most optimal was the 5 day protocol as described in Materials and methods, which resulted in sufficient high levels of [3H]Tdr incorporation and low spontaneous cell death during the assay (<5%).

To address the question whether in our hands the `JAM` test was more sensitive or at least as sensitive as the 51Cr-release assay, we tested several effector/stimulator combinations (n = 9) exhibiting different degrees of HLA match, using both `JAM` test and 51Cr-release as a read out system. As shown in Table 2, the frequencies as found in the `JAM` test and 51Cr-release assay are not significantly different. Only one out of nine combinations gave a significantly higher frequency in the `JAM` test as compared to the 51Cr-release assay, i.e. there is no overlap in 95% confidence intervals. Overall, it appears that the sensitivities of the `JAM` test and 51Cr-release assay are comparable in our setting, while the `JAM` test is far more easy to apply.

Determination of HTLp and CTLp frequencies with the complete [3H]Tdr-based method

The final step was to combine the `JAM` test and HTLp assay in a single assay. To validate this experimental set-up several combinations exhibiting multiple HLA mismatches were tested. Moreover, as this assay will be applied in donor search for unrelated as well as HLA-identical sibling donors, several combinations were tested that would fit these categories, namely HLA-identical combinations and combinations exhibiting only few HLA mismatches.

The data show that the assay has a broad sensitivity and frequencies detected range from 1:395 to 1:500 000 in the HTLp assay and 1:4367 to >500 in the `JAM` test (Table 3). Although there tends to be an increase in both HTLp and CTLp frequencies with an increase in the number of HLA antigens mismatched
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Table 1  Comparison of HTLp frequencies determined either by adding the CTLL-2 cells directly to the irradiated wells or by adding the CTLL-2 cells to the supernatants (combined assay)

<table>
<thead>
<tr>
<th>No. of mismatches</th>
<th>HTLp (irradiated wells)</th>
<th>95% CI</th>
<th>HTLp (supernatants)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Completely/partially mismatched combinations</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 A/B and 2 DR mm*</td>
<td>1:791</td>
<td>547-1425</td>
<td>1:672</td>
<td>483-1109</td>
</tr>
<tr>
<td>3 A/B and 1 DR mm*</td>
<td>1:100</td>
<td>852-1589</td>
<td>1:1512</td>
<td>1196-2079</td>
</tr>
<tr>
<td>3 A/B and 1 DR mm*</td>
<td>1:3448</td>
<td>2710-4762</td>
<td>1:4975</td>
<td>3953-6666</td>
</tr>
<tr>
<td>2 A/B and 1 DR mm*</td>
<td>1:759</td>
<td>1388-2538</td>
<td>1:4830</td>
<td>3636-7194</td>
</tr>
<tr>
<td>2 B and 1 DR mm*</td>
<td>1:536</td>
<td>396-831</td>
<td>1:1105</td>
<td>844-1602</td>
</tr>
<tr>
<td>2 B and 1 DR mm*</td>
<td>1:37037</td>
<td>27778-55556</td>
<td>1:47619</td>
<td>34482-76923</td>
</tr>
<tr>
<td>1 B mm*</td>
<td>1:125000</td>
<td>17429-500000</td>
<td>1:500000</td>
<td>20000000-2x106</td>
</tr>
<tr>
<td>1 DR mm*</td>
<td>1:3376</td>
<td>2463-5319</td>
<td>1:5952</td>
<td>4504-8772</td>
</tr>
</tbody>
</table>

HLA-identical sibling combination

*Number of mismatches as detected by serology.

Table 2  Comparison of CTLp frequencies determined with either the 'JAM' test or Cr release as a read-out for CTLp frequencies in completely mismatched combinations (5-6 A, B, DR antigens mismatched), partially HLA-matched combinations (0-2 A, B, DR antigens mismatched), and genotypically HLA-identical sibling combinations

<table>
<thead>
<tr>
<th>No. of mismatches</th>
<th>CTLp Jam test</th>
<th>95% CI</th>
<th>CTLp Cr release</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Completely/partially mismatched combinations</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 A/B and 1 DR mm*</td>
<td>1:1631</td>
<td>1191-2593</td>
<td>1:6536</td>
<td>4854-10101</td>
</tr>
<tr>
<td>4 A/B and 2 DR mm*</td>
<td>1:1938</td>
<td>1376-3279</td>
<td>1:1118</td>
<td>714-2384</td>
</tr>
<tr>
<td>4 A/B and 2 DR mm*</td>
<td>1:2625</td>
<td>1894-4274</td>
<td>1:2710</td>
<td>2016-4132</td>
</tr>
<tr>
<td>1 A locus mm*</td>
<td>1:11904</td>
<td>8261-19230</td>
<td>1:9346</td>
<td>7246-13333</td>
</tr>
<tr>
<td>2 DR mm*</td>
<td>1:7299</td>
<td>5263-12048</td>
<td>1:7404</td>
<td>4504-21277</td>
</tr>
<tr>
<td>0 mm*</td>
<td>1:55556</td>
<td>400000-90909</td>
<td>1:90909</td>
<td>62500-166667</td>
</tr>
</tbody>
</table>

HLA-identical sibling combinations

*Number of mismatches as detected by serology.

Table 3  HTLp and CTLp frequencies in E/S combinations exhibiting different degrees of HLA matching determined by the combined [3H]TdT-based method

<table>
<thead>
<tr>
<th>No. of mismatches</th>
<th>HTLp frequency</th>
<th>95% CI</th>
<th>CTLp frequency</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Completely/partially mismatched combinations</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 A/B and 1 DR mm*</td>
<td>1:395</td>
<td>295-944</td>
<td>1:4717</td>
<td>3425-7634</td>
</tr>
<tr>
<td>3 A/B and 2 DR b*</td>
<td>1:1186</td>
<td>902-1733</td>
<td>1:4367</td>
<td>2653-12346</td>
</tr>
<tr>
<td>3 A/B and 2 DR b*</td>
<td>1:4255</td>
<td>3322-5952</td>
<td>1:16667</td>
<td>12821-24390</td>
</tr>
<tr>
<td>2 A/B mm*</td>
<td>1:8264</td>
<td>6289-11905</td>
<td>1:9901</td>
<td>7752-13699</td>
</tr>
<tr>
<td>1 A mm and DR subtype mm*</td>
<td>1:16666</td>
<td>12987-23255</td>
<td>1:28315</td>
<td>20408-38461</td>
</tr>
<tr>
<td>DR subtype mm*</td>
<td>1:3460</td>
<td>2646-5000</td>
<td>1:14286</td>
<td>10638-21739</td>
</tr>
<tr>
<td>DR subtype mm*</td>
<td>1:76923</td>
<td>52632-166667</td>
<td>1:200000</td>
<td>11111-33333</td>
</tr>
</tbody>
</table>

HLA-identical sibling combinations

*HTLp frequencies were determined on supernatants as described in Materials and methods. The 'JAM' test was used as a read-out for CTLp frequencies.

*In effector to stimulator direction as detected by serology.

"In effector to stimulator direction as detected by serology for HLA-A and B and by high resolution oligotyping for HLA-DRB and DQB.

*Genotypically HLA-identical sibling combination.
(completely mismatched vs partially/fully matched vs HLA-identical sibling combinations), a wide spread in frequencies can be seen within each group. The CTLp frequencies in HLA-identical sibling combinations were relatively low compared to those in less well matched combinations, but still detectable in two out of three combinations. As in a clinical setting the HTLp and CTLp assays are applied to facilitate donor selection procedures, both frequencies were determined in the case of two patients and their potential (unrelated) donors (Table 4). While all three potential donors for patient A were equally well matched for HLA using standard typing techniques, major differences in HTLp and CTLp frequencies were observed. Whether this was caused by unknown HLA mismatches and/or minor antigen mismatches is unclear at present. For patient B no completely HLA matched donor was available. Here a clear difference was observed in the CTLp frequencies as measured in the case of donor 1 as compared to donors 2 and 3, while HTLp frequencies differ between all three donors. Apparently, the detected or undetected HLA mismatches and/or minor antigen mismatches give rise to different types of in vitro alloresponses in these combinations.

### Discussion

Improvement of bone marrow donor selection procedures is a matter of concern since after allogeneic BMT a high number of patients still develop severe GVHD. In addition to high resolution HLA-typing techniques, functional in vitro histocompatibility assays, ie HTLp and CTLp frequency assays, are promising methods for prediction of alloreactivity and the occurrence of GVHD. Application of both assays separately is laborious and requires a high number of patient cells.

By combining the HTLp and CTLp assays in a single LDA and using the ‘JAM’ test as a read out for cytotoxicity, we developed an easy and cell-saving assay which enables us to quantify different subsets of T cells in a single ongoing alloreaction. As one might expect, the nature of the HLA and/or minor antigens mismatched will determine the initial route of the alloresponse (cytokine production/cytotoxicity), consequently the use of two different read-out systems will provide more detailed information on the in vitro histocompatibility. So far, it has been claimed that the CTLp assay mainly detects class I mismatches although class II restricted alloreactivity has been described. The HTLp assay has been claimed to be sensitive to both HLA class I and class II mismatches as well as minor antigen mismatches.

By performing a single LDA to obtain both frequencies more insight will emerge in the effect of (isolated) HLA mismatches on the initial alloresponse. Furthermore, the alloreactive profile of each separate well of the culture can be determined.

A major advantage of using the ‘JAM’ test as a read-out for CTLp frequencies is that it simplifies the performance of the CTLp assay. Handling of target cells and harvesting of the plates is relatively easy in this system as compared to others. Although the CTLp frequencies detected by 51Cr release and ‘JAM’ test were comparable for the combinations tested, it should be emphasized that the ‘JAM’ test is based on a different aspect of cell death as is the 51Cr-release assay, ie DNA fragmentation vs disruption of the cell membrane. Because the retention of [3H]TdR-labeled DNA during the assay is very stable, longer incubation times can be applied, ie 6 h instead of 4, the advantage being that the discriminative power (scoring of positive vs negative wells) has increased.

With respect to the HTLp assay two points have to be considered. First, as preliminary data already indicate, determination of HTLp frequencies, by adding the CTLL2 cells to the supernatants of the LDA cultures instead of adding the CTLL2 cells to the irradiated LDA cultures, gives rise to a slight decrease in sensitivity of the HTLp frequency assay. This confirms the findings of Schwerer et al and is probably due to continued IL-2 production/cytotoxicity, consequently the use of two different read-out systems will provide more detailed information on the in vitro histocompatibility. So far, it has been claimed that the CTLp assay mainly detects class I mismatches although class II restricted alloreactivity has been described. The HTLp assay has been claimed to be sensitive to both HLA class I and class II mismatches as well as minor antigen mismatches.

### Table 4

<table>
<thead>
<tr>
<th>HLA antigens mismatched with the patient</th>
<th>HTLp&lt;sup&gt;a&lt;/sup&gt;</th>
<th>95% CI</th>
<th>CTLp&lt;sup&gt;2&lt;/sup&gt;</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patient A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A3 A24 B18 B62 Cw<em>0303 Cw</em>0304 DRB1<em>1101 DRB1</em>1301 DRB3<em>0202 DQB1</em>0603/0607 DQB1*0301</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Donor 1</td>
<td>Cw<em>0303 Cw</em>0700</td>
<td>1:5076</td>
<td>3774–7692</td>
<td>1:32 258</td>
</tr>
<tr>
<td>Donor 2</td>
<td>Cw<em>0303 Cw</em>0700</td>
<td>1:58 824</td>
<td>41 667–111 111</td>
<td>1:200 000</td>
</tr>
<tr>
<td>Donor 3</td>
<td>Cw<em>0303 Cw</em>0700</td>
<td>1:500 000</td>
<td>250 000–/</td>
<td>1:333 333</td>
</tr>
<tr>
<td><strong>Patient B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A<em>0201 B</em>1800 B<em>4402 Cw</em>0501 Cw<em>0700 DRB1</em>0401 DRB1<em>0701 DRB4</em>0101 DQB1<em>0200 DQB1</em>0301</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Donor 1</td>
<td>B<em>4403 B</em>1800 Cw<em>0400 Cw</em>0700 DQB1<em>0200 DQB1</em>0303/0305</td>
<td>1:3546</td>
<td>2755–4975</td>
<td>1:10 526</td>
</tr>
<tr>
<td>Donor 2</td>
<td>Cw<em>0501 Cw</em>0501</td>
<td>1:43 478</td>
<td>13 699–25 641</td>
<td>1:90 909</td>
</tr>
<tr>
<td>Donor 3</td>
<td>DRB4*0103</td>
<td>1:17 857</td>
<td>1:100 000</td>
<td>66 667–200 000</td>
</tr>
</tbody>
</table>

<sup>a</sup>As detected by PCR-SSP for HLA-A, B and, Cw and high resolution oligotyping for HLA-DRB and DQB. Mismatched antigens are shown in italic.  
<sup>2</sup>In GVH direction.
secretion after irradiation of the cultures. Whether this phenomenon has any bearing on the functional relevance of the test is currently under investigation.

A second point of consideration, is the production of IL-2 by irradiated alloreactive stimulator cells in response to the effecter cells. This so-called 'back stimulation' is caused by alloreactive T cells in the stimulator PBMC fraction which although being irradiated, continue to produce IL-2. In our hands, 30 and 50 Gy irradiation doses were not always sufficient to abrogate IL-2 production in every effecter-stimulator combination. This confirms the findings by Reisaeter et al.\(^{21}\) who stated that irradiation doses of up to 100 Gy are necessary to completely abrogate the 'unwanted' IL-2 production. This, however, leads to a reduced antigen presenting capacity of the stimulator cells, thereby affecting the outcome of both HTLp\(^{21,22}\) and CTLp assays (unpublished observation). Therefore, the depletion of the IL-2 producing cells in the stimulator PBMC fraction as described by Reisaeter et al.\(^{21}\) seems more favorable and is currently being evaluated in our experimental set-up.

The HTLp and CTLp assays provide additional information on in vitro histocompatibility that cannot be deduced directly from HLA typing of patient and donor. Looking at two extremes with respect to HLA-matching, ie HLA-identical siblings on one side and completely mismatched unrelated combinations on the other, the HTLp and CTLp frequencies were always relatively low or high, respectively (Table 3), which is in concert with previously published data.\(^{5,9}\) However, in the group that most closely resembles the clinical situation of unrelated patient/donor combinations, ie the unrelated combinations with only few or no HLA mismatches, a wide spread in frequencies can be observed (Table 3), ranging from frequencies similar to those found in HLA-identical siblings up to those found in completely mismatched combinations. Its potential relevance in donor selection was emphasized even more when the combined HTLp/CTLp assay was applied in two patients and their six potential donors (Table 4). Major differences can be seen in both HTLp and CTLp frequency when comparing the donors, while at least in patient A, no correlation can be drawn between the frequencies found and the HLA phenotype of the patient and the potential donors. It should however be taken into account, that in addition to minor antigen mismatches, still several presently undetected HLA mismatches may exist in these combinations that can induce an alloresponse which is observed in the combined HTLp/CTLp assay. Whether the differences in in vitro alloreactivity against a single patient also reflect the post-transplant in vivo reactivity remains uncertain, as only one patient will actually serve as bone marrow donor. Although the predictive value of the combined HTLp/CTLp assay for development of GVHD is yet to be determined, the current policy in our transplant center is that next to serological typing for HLA-A and B, and high resolution typing by molecular techniques for HLA-C, DRB and DQ\(\beta\), the outcomes of the combined HTLp/CTLp assay are taken into consideration for selecting the optimal donor. Furthermore, when no donor is available that completely fits the HLA matching criteria, the functional cellular assays may provide additional information to identify more acceptable mismatches. An extended study governing a complete subtyping of HLA class I and class II antigens, in combination with the outcome of the combined HTLp/CTLp assay and the outcome of transplantation is currently being performed to validate the value of the combined HTLp/CTLp assay and to gain more insight into the functional in vitro relevance of certain HLA mismatches, and its effect on the development of GVHD.

In conclusion, the combined HTLp/CTLp assay as described here is a practical and sensitive tool for the detection of in vitro histocompatibility and can be applied in both related and unrelated patient/donor combinations. Furthermore, this assay might prove to be helpful in the identification of mismatched HLA antigens that do not give rise to an alloreaction, the so-called permissible mismatches.

Acknowledgements

We are grateful to M van den Beucken for performing HLA-B*12 subtype analysis. We thank Prof Dr TJM De Witte for critically reading the manuscript. This work was financially supported by Bone Marrow Donorbank Nijmegen.

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