Evaluation of Alloreactivity in Responder-Stimulator Pairs by Determination of Gamma Interferon-Producing Cells and Cytotoxic-T-Lymphocyte Precursor Frequencies

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We used the enzyme-linked immunospot (ELISPOT) assay and the cytotoxic-T-lymphocyte precursor frequency assay to evaluate alloreactivity in responder-stimulator pairs. High frequencies of responder cells among cells from HLA-mismatched pairs and low frequencies among cells from pairs of siblings with identical HLA types were detected by both assays. The ELISPOT assay thus illustrated the helper and cytotoxic-T-cell response to allogeneic HLA antigens.

Histocompatibility before transplantation is determined by serological or molecular methods. These techniques define HLA antigens and alleles shared between the donor and the recipient but do not provide any functional information from which complications after allografting can be predicted. Perfect HLA matching, in fact, does not ensure the absence of rejection or graft-versus-host disease (1, 5), while certain HLA mismatches do not seem to preclude successful transplantation (2, 4). In vitro functional methods, namely, the helper precursor frequency and cytotoxic-T-lymphocyte precursor frequency (CTL-p-f) assays, have been used to measure the strengths of alloreactions promoted by T cells. Yet these assays are difficult to perform and require a large number of cells and radionuclides. New methods for determining T-cell functions include serological or molecular methods. These techniques define HLA matching, in fact, does not ensure the absence of complications after allografting can be predicted.

In preliminary experiments performed with cells from an HLA-MM pair, increasing irradiation doses (30, 45, and 60 Gy) inhibited cytokine secretion but reduced the stimulatory alloreactivity promoted by T cells. Then streptavidin-peroxidase was added and spots were revealed with a chromogenic substrate. All reagents were purchased from Mabtech (Stockholm, Sweden). Plates were examined with an automatic reader, and results were shown as the number of IFN-γ-producing cells (IFN-γ-pc)/10⁶ R cells. Experiments were performed with RPMI 1640 containing fetal calf serum (FCS) or human AB serum. In ELISPOT HLA-blocking experiments, S cells from two HLA-MM pairs were incubated with 40 μg/ml of mouse anti-human HLA class I and II mAb for 15 min at 4°C and then incubated with R cells.

**CTL-p-f assay.** Limiting numbers of R PBMC (5 × 10⁴ to 3.125 × 10⁵) were cocultured in 96-well, U-bottom plates with 5 × 10⁴ irradiated (30 Gy) S PBMC. The medium was RPMI 1640 with human AB serum. Twenty replicates for each R cell dilution were set up. As a negative control, irradiated S cells were incubated without R cells. On day 3, cultures were fed with interleukin-2. On day 7, OKT3 mAb-induced blasts obtained from the original S cells were incubated with 51Cr for 1 h and added to the cultures. Plates were centrifuged and incubated for 4 h. Then, 50 μl of supernatants was harvested and transferred onto solid-scintillator plates. 51Cr in supernatants was detected by a β-radiation counter. Wells were scored as positive when the count was higher than the mean plus 3 standard deviations of the negative control. All data were processed using software that gave the CTL-p-f. Results were shown as the number of cytotoxic-T-lymphocyte precursors/10⁶ R cells.

In preliminary experiments performed with cells from an HLA-MM pair, increasing irradiation doses (30, 45, and 60 Gy) inhibited cytokine secretion but reduced the stimulatory capability of S cells (data not shown). To reveal cytokines secreted mainly from R cells, 30-Gy-irradiated S cells were seeded at 2 × 10⁵, 1 × 10⁶, and 5 × 10⁵ in the same final volume (200 μl) with 2 × 10⁴ R cells in a limiting dilution. The most appropriate concentration of S cells to obtain a surrogate

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one-way reaction proved to be $5 \times 10^4$ cells/200 μl. The number of spots, in fact, titrated linearly ($r^2 = 0.99$) with the R cell concentration, suggesting a limited contribution from S cells in secretion. Spot values corresponding to the first R cell concentration ($2 \times 10^5$ R cells/200 μl) did not differ significantly (Grubb’s test; $P > 0.05$) among the three R/S cell ratios (962 ± 236, 985 ± 146, and 858 ± 104). This result suggests that the stimulatory capability was maintained.

An ELISPOT assay of three R-S pairs was then performed for 48 h with these parameter values. Results showed strong alloreactivity between the cells from an HLA-MM pair and few spots corresponding to the HLA-IS and MZ pairs (data not shown). The kinetics of cytokine secretion were determined, and cells from two HLA-IS pairs (pairs 6 and 8) were cultured for 48 h and HLA-IS and MZ cells were incubated for 144 h. In ELISPOT assays, HLA-MM R and S cells were incubated for 48 h and HLA-IS and MZ cells were incubated for 144 h. Results showed high frequencies of IFN-γ- pc among cells from HLA-MM pairs (mean, 371 IFN-γ- pc/10^6 R cells), and these frequencies decreased when anti-HLA mAbs were added to the S cells (Fig. 1). Among cells from HLA-IS (mean, 8 IFN-γ- pc/10^6 R cells) and MZ (mean, 3.5 IFN-γ- pc/10^6 R cells) pairs, low frequencies of IFN-γ- pc were found. High CTL-p-f among cells from HLA-MM pairs (mean, 61 cytotoxic-T-lymphocyte precursors/10^6 R cells) and low frequencies among cells from HLA-IS (mean, 6 cytotoxic-T-lymphocyte precursors/10^6 R cells) and MZ (mean, 2 cytotoxic-T-lymphocyte precursors/10^6 R cells) pairs were found. Results are summarized in Table 1.

The ELISPOT assay measures T-cell function and may be a useful aid in assessing the prognosis of a transplant. To illustrate the immune response mainly on the part of R cells, this being closer to the T-cell response after hematopoietic stem cell or solid organ allografting, S cells were irradiated with 30 Gy and the R/S cell ratio was increased. More precise methods employ purified non-T cells as S cells (3), but these cells are often difficult to obtain in sufficient numbers, especially from oncohematological patients. Our approach is a trade-off between the maintenance of stimulation capability and the conditions for a one-way reaction and the collection and holding of a larger quantity of cells for repeated determinations, such as immunological follow-up.

The determination of the kinetics of IFN-γ secretion disclosed a nonspecific production of cytokine probably due to factors in the FCS. Human AB serum did not elicit this nonspecific increase of IFN-γ- pc and would seem preferable in prolonged cultures before ELISPOT analysis. High frequencies of IFN-γ- pc among cells from HLA-MM pairs and lower frequencies among cells from HLA-IS and MZ pairs were found. The CTL-p-f assay showed a similar pattern of alloreactivity detection. The determined R cell frequencies, especially among cells from HLA-MM pairs, were lower in the CTL-p-f assay than in the ELISPOT assay. This result could be due to different sensitivity thresholds as well as the kinds of responses detected by the two techniques (direct IFN-γ secretion by R

![FIG. 1. IFN-γ- pc among cells from pairs 1 and 2 as assessed by using S cells treated with (+) anti-HLA (α-HLA) class I and II mAbs.](image)

### Table 1. IFN-γ- pc and CTL-p-f in 10 R-S pairs

| Pair no. | Pair type | Cell type | Results of HLA typing | Gender | No. of IFN-γ- pc/10^6 R cells ± SD | No. of CTL precursors/10^6 R cells |
|----------|-----------|-----------|-----------------------|--------|----------------------------------|----------------------------------|
| 1        | HLA-MM    | R         | A*2,3; B13,50(21); DRB1* 03,14 | F      | 172 ± 57                         | 83                               |
|          |           | S         | A29(19); B44(12),63(15); DRB1* 07,11 | F      | 670 ± 78                         | 71                               |
| 2        | HLA-MM    | R         | A*2,3; B44(12),49(21); DRB1* 02,11 | F      | 275 ± 73                         | 20                               |
|          |           | S         | A22,4(9); B8,51(5); DRB1* 03,07 | F      | 365 ± 61                         | 71                               |
| 3        | HLA-MM    | R         | A24(9),30(19); B35,49(21); DRB1* 15,01 | M      | 7 ± 7.6                          | 6                                |
|          |           | S         | A12,4(9); B61(40),63(15); DRB1* 13,14 | M      | 20 ± 18                          | 10                               |
| 4        | HLA-MM    | R         | A29(19),30(19); B53,39(16); DRB1* 11,13 | M      | 4 ± 2.9                          | 4                                |
|          |           | S         | A12,4(9); B61(40),63(15); DRB1* 13,14 | M      | 0                               | 3                                |
| 5        | HLA-IS    | R         | A*2,24,25; B*35,13; DRB1* 07,11 | F      | 3 ± 5.8                          | 2                                |
|          |           | S         | A*2,24,25; B*35,13; DRB1* 07,11 | M      | 2 ± 20                           | 2                                |
| 6        | HLA-IS    | R         | A12,4(9); B61(40),63(15); DRB1* 13,14 | M      | 4 ± 2.9                          | 4                                |
|          |           | S         | A12,4(9); B61(40),63(15); DRB1* 13,14 | M      | 0                               | 3                                |
| 7        | MZ        | R         | A*2,02,30; B*13,18; DRB1* 07,11 | F      | 3 ± 5.8                          | 2                                |
|          |           | S         | A*2,02,30; B*13,18; DRB1* 07,11 | F      | 2 ± 20                           | 2                                |
| 8        | MZ        | R         | A*2,02,30; B*18; DRB1* 01,03 | F      | 3 ± 5.8                          | 2                                |
|          |           | S         | A*2,02,30; B*18; DRB1* 01,03 | F      | 2 ± 20                           | 2                                |

* Asterisks indicate HLA alleles. F, female; M, male; CTL, cytotoxic T lymphocyte.
cells as detected by the ELISPOT assay versus the cytotoxic response after the activation and expansion of cytotoxic-T-lymphocyte precursors present at the beginning of the culture). ELISPOT analysis may also reveal different T-cell populations (helper and cytotoxic) secreting IFN-γ. The incubation of S cells with anti-HLA class I or II mAb decreased the number of IFN-γ-pc. Hence, the spots detected may represent both CD8 and CD4 IFN-γ-secreting T lymphocytes recognizing allogeneic HLA molecules. In conclusion, the ELISPOT assay may be a useful method for detecting alloreactivity elicited by HLA mismatches in R-S pairs. IFN-γ ELISPOT analysis is simpler than the CTL-p-f assay and may reveal both the cytotoxic and the T-helper response. It can thus be proposed for the detection of alloreactivity before and after allografting as a means of predicting immunological complications.

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