PART IV

TRANSPLANTATION IMMUNOLOGY & TECHNIQUES

Chairmen: W Drukker
           M Mebel
Defect in Cytotoxic Effector Cells in Long Term Renal Transplant Recipients

F THOMAS, J THOMAS, J DOANE, H M LEE
Transplant Immunology Laboratory, Medical College of Virginia, Richmond, Virginia, USA

Summary

In this study, a variety of cell-mediated immunity studies were performed in successful long term renal allograft recipients (all HLA non-identical). These studies showed that all recipients were responsive to donor in MLC and over 70% had MLC blocking factors. A marked and specific defect in recipient ability to generate anti-donor cytotoxic effector T cells in vitro was seen in all recipients at 10 years. This specific defect apparently increases in incidence in the transplant population with increasing time post-transplantation. This mechanism has not been well studied to date and may represent a previously unappreciated immune regulatory process perpetuating long term allograft survival in HLA non-identical transplants.

Introduction

Fourteen years have now elapsed since the first systematic human renal transplants between non-identical donors. Results from our institution (Thomas et al, 1975) and Starzl’s results (Starzl et al, 1974) have indicated that an actual 10 year survival of about 50% can be anticipated in a well-focused renal transplant program. The large majority (90% or more) of these 10 year related donor survivors have well functioning allografts despite HLA non-identity between donor and recipient in most cases. The extraordinary long term durability of these non-identical transplants logically raises the immunobiological question — why are these non-identical grafts not rejected? The rationale for this study is the supposition that recently developed tests of cell-mediated immunity (CMI) might provide some putative answers to this question.
MATERIALS AND METHODS

Patients studied were all long term (2–14 year) post-transplant recipients with living related haploidentical donors, 87% of which were parent to child combinations. All had relatively stable kidney function although four patients had clinical symptoms of chronic rejection. The CMI findings in patients with chronic rejection (especially LDA activity) are the subject of another communication (Thomas et al., 1976a) and will not be discussed here. Twenty-three recipients were studied. All patients were withdrawn from immunosuppressive drugs for 24 hours prior to blood drawing. Studies of mixed lymphocyte culture (MLC) were performed by the micro technique of Hartzmann et al. (1971). Direct lymphocyte mediated cytotoxicity assays were performed on freshly drawn $^{51}$chromium labelled target cells of the donor and unrelated third and fourth parties. A four hour incubation period was used according to the method of Garovoy et al., (1973).

Studies of cell mediated lymphyolysis (in vitro generation of cytotoxic effector cells, CML) were performed by the technique of Bonnard et al., (1974) as previously described (Thomas et al., 1976b). The MLC results are expressed here as the stimulation index (SI) according to the formula $cpm_{AXm}/cpm_{AAm}$, where A = responder cells and Xm = mitomycin treated stimulator cells. Studies of serum blocking activity in MLC and CML were performed by comparing the counts per minute of tritiated thymidine in MLC or the % specific cytolysis in CML between tests run in normal pooled AB serum and tests run in serial dilutions of recipient’s serum. Blocking was considered to be present if a statistically significant reduction in test results were obtained in recipient’s serum when compared to normal pooled plasma. Studies of T cell levels (E rosettes) and T cell reactivity (phytohaemagglutinin induced blastogenesis) were also performed.

RESULTS

All patients studied had T cell levels and reactivity within normal limits (E rosette range of 53–78%, PHA range of 96,000–159,000 cpm). The lymphocytes of all 23 long term (LT) renal transplant recipients (A) with related haploidentical donors (B) responded with significant incorporation of $^3$H-thymidine in one-way MLC assays to the specific donor and to unrelated individuals. The individual MLC stimulation index (SI) of ABm cultures ranged from 3.9 to 29.0 with a group mean of 10.8 ± 6.4 (standard deviation). Since 87% of the recipients had parent donors, we compared the SI in ABm cultures to the SI obtained in normal adult offspring to parent MLC assays in our laboratory. The mean SI of both groups was almost identical, 10.8 ± 6.4 (SI) and 10.4 ± 6.6 (SD), respectively. When the recipients were divided into very long survivors (8–12 years) and 2–5 year survivors, the mean SI of ABm cultures
was lower (8.3 ± 7.2) in the 8–12 year group compared to the 2–5 group (12.6 ± 7.1), but the differences were not significant (p > 0.10). Finally, the MLC responses of both groups to unrelated third and fourth party stimulating cells were within the range of the mean SI 31.54 ± 20.7 (SD) obtained between the normal unrelated control individuals included in these experiments.

Studies of blocking activity of recipient serum on one-way MLC responses indicated that the majority (73%) of recipients exhibited significant MLC blocking at serum dilutions of 1/5 – 1/500. In three recipients, blocking was still detectable at 1/5000 dilutions. In 70% of 8–12 year recipients and 58% of 2–5 year recipients, respectively, the serum blocking effect was specific for donor cells. Non-specific inhibition of MLC and PHA responses was detected in the serum of only two recipients both at 10 years post-transplant.

The serum of five recipients exhibited no detectable MLC blocking activity at dilutions of 1/5 – 1/500. In contrast, MLC responses were potentiated in their serum, reaching a peak response at 1/50 – 1/500 dilutions. The potentiation of responsiveness was specific for recipient cells, not affecting donor or third party cells as responders. PHA responsiveness was also potentiated by recipient serum.

None of the long term recipients demonstrated significant positive (>5% cytolysis) LMC activity to donor cells in the presence of normal serum. In an effort to determine whether the recipient lymphocytes were capable of generating cytotoxic effector cells to the donor, we performed CML assays in 17 of these recipients. In the six day sensitisation phase of the CML assay, cultures were initiated with the following combinations in each experiment: ABm, ACm, CBm, and CDm (positive control). A = recipient, B = donor, C and D are unrelated third and fourth parties. The third party C cells were mismatched with the recipient (A) for 1–3 HLA antigens, and did not share the mismatched donor (B) antigens. In all 17 recipients tested normal levels of specific cytolysis were obtained after six hours of incubation against C targets in the ACm effector cultures compared to the control CML responses between mismatched normal unrelated CDm and DCm combinations. In the 8–12 year group none of the recipients (n=9) developed cytotoxic T cell effectors against the donor. The mean per cent lysis of ABm effectors against B target cells in this group was 3.9% ± 1.7 (SE). This is sharply contrasted to the mean CML response of 37.1% ± 6.2 (SE) lysis against C targets in ACm cultures. In the 2–5 year group (n=8) 50% of the recipients responded in CML to donor cells with specific cytolysis ranging from 31% – 42%, and 50% of the recipients failed to generate anti-donor cytotoxic effector cells giving a group mean cytolysis of 19.7% ± 6.4 (SE) in ABm effectors. The control CML responses of normal unrelated third party lymphocytes to donor stimulating lymphocytes (CBm effectors) demonstrated that donor cells in all cases were capable of stimulating in vitro generation of cytotoxic effectors comparable to normal unrelated CDm controls.
The deficient CML responsiveness against HLA haploidentical donor cells seen in 76% of these LT recipients and most strikingly in the 8–12 year group is not simply explained by IS drug therapy, since normal effector cell responses were obtained against third party cells. Nor is it readily explained by the relatively low MLC responsiveness to donor cells, since a comparison group of normal adult offspring with similar specific MLC stimulation index values responded in CML to their haploidentical parent cells with a mean cytolysis of 34% ± 5.2 (SE). Moreover, no correlation was seen between degree of MLC responsiveness (SI) to donor and ability to generate effector cells in CML assays (per cent lysis) within the 17 haploidentical combinations tested.

In the few LT recipients who were CML responsive to the donor in the presence of normal serum, their serum was tested for blocking activity at the afferent and efferent levels of the CML assay. ABm effector cultures initiated in the presence of 5% (v/v) recipient serum were inhibited up to 50% of the control response in normal serum. Recipient serum also inhibited the cytotoxic activity (% lysis) of ABm effectors which had been sensitised in NPS. These inhibitory effects of recipient serum in CML were specific for donor cells.

**DISCUSSION**

These studies began as an attempt to explain the excellent long term durability of non-HLA identical related donor transplants. The results indicate that (1) all recipients are responsive in MLC to the donor, indicating a lack of tolerance in the proliferating helper cell population, (2) most have specific blocking activity in their serum which is capable of inhibiting MLC responsiveness, and (3) most have a marked and specific defect in ability to generate cytotoxic effector T cells against donor cells. This lack of MLC effectors after in vitro sensitisation to MLC stimulating, HLA mismatched donor cells has not been described, in transplant recipients to our knowledge, and may represent an important immune modulation mechanism related to long term allograft survival.

Direct lymphocyte-mediated cytotoxicity (LMC) was early described by Rosenau and Moon (1966). This mechanism of cytotoxicity has been elegantly studied in lower animals (Brunner and Cerottini, 1974, Hayry et al, 1972, Wagner et al, 1973). However, there is less information on the operational parameters of T lymphocyte-mediated cytotoxicity in human transplantation. We have previously studied both levels and reactivity of circulating T cells in the early post-transplant period and suggested that the T cell has a primary role in early acute allograft reactivity (Thomas et al, 1976c). Stiller et al (1976) have recently reported an association with anti-donor LMC activity and early acute rejection of human renal transplants. Strom and co-workers (1975) have demonstrated that the infiltrating cells in acutely rejecting human allografts execute primarily a T cell cytotoxicity. These studies all suggest that the cytotoxic T cell subpopulation may be an important effector of human allograft
rejection, and, conversely, a specific deficiency in recipient capability to generate cytotoxic effector cells to donor alloantigens may be important in long term graft survival.

Bach et al. (1976) have studied the subpopulations of lymphocytes responding in MLC and CML and suggested that the blastogenic response of T cells (as measured by MLC) and the cytotoxic effector cell activity (as measured in CML) are functions of separate, but co-operating subpopulations of T cells. Although an LD locus disparity is considered necessary in most cases for in vitro generation of detectable levels of CML, the cytotoxic activity is usually directed at the SD, HLA-A and B locus antigens.

In this study, the donor-recipient LD disparity as measured by MLC stimulation index showed no definite relationship to long term allograft survival. Patients with excellent 8–12 year graft survival had MLC stimulation indices similar to those with failed transplants and to recipients at shorter post-transplant periods. In contrast, the very long term successful recipients all had a striking deficiency in ability to generate anti-donor cytotoxic effector cells in culture. These observations support the concept that T cell subpopulations in the human may be functionally disparate in a manner similar to that seen in lower animals, and that the T cell subpopulation responsible for generating cytotoxic effector cells against specific donor cells may be ‘operationally tolerant’ in many long surviving transplant recipients.

We did not find MLC tolerance or non-reactivity as seen in 25% of long term successful patients reported by Bach and colleagues (1972). Our studies indicate that tolerance in the proliferating helper cell subpopulation is not critical for long term allograft survival among HLA non-identical donor-recipient combinations. On the other hand, it should be noted that the majority of recipient sera had high titres of MLC blocking activity, so that the full expression of MLC reactivity seen in vitro is probably not achieved in vivo.

The mechanism or mechanisms responsible for production of the marked and specific CML defect seen in long term successful transplants is of great interest, both conceptually and clinically. Our studies suggest that this CML defect occurs with a progressively greater frequency as the time post-transplant increases. In the 2–5 year interval, the defect was seen in only 50% of recipients. At the 10 year mean interval, 100% of successful transplant recipients had this defect. Only serial longitudinal studies of patients over these time periods can definitively answer the question of whether this defect in cytotoxic effector cell generation is, in fact, a progressive one. It is of interest that among the 24% CML responders found in this study, the serum of these recipients was able to specifically inhibit the ABm CML reaction both at the sensitisation and cytotoxicity phases of the assay. Thus all of the 17 patients studied at 2–12 years post-transplant can be considered deficient or compromised in CML reactivity to the specific donor either at the cellular level or by the blocking activity of their serum.
It has been of some surprise to clinicians that some long term recipients can undergo both major and minor lapses of immunosuppressive therapy with little or no adverse effect on the transplanted kidney (Seigler et al, 1972, Uehling et al, 1976). This suggests a rather high degree of operational tolerance. The data reported here indicates that all the long term patients studied had normal T cell levels and reactivity, but would appear to have donor-specific immune modulation mechanisms leading to partial or operational tolerance. Clearly, this tolerance is functional in most cases only with immunosuppressive drug cover. These studies have described two in vitro correlates of operational tolerance – a specific defect in recipient capability to generate cytotoxic effector T cells and serum blocking factors which have been previously described by others in the MLC system (Revillard et al, 1973) and in the microcytotoxicity assay (Quadricci et al, 1974).

The mechanism of this selective deficiency in cytotoxic T cell generation is thought most likely to be related to any of four factors: (1) in vitro generation of blocking antibody or anti-receptor antibody, (2) depletion of donor reactive clones, (3) suppressor T cell activity, and (4) poor immunogenicity of the mismatched antigens. We are currently studying the potential role of in vitro production of blocking antibody by selectively removing B cells from the CML responder lymphocytes. The in vitro production of antibody directed against donor alloantigens or against recipient receptor sites, would be expected to interfere with cytotoxic effector cell generation and activity. In preliminary experiments we have noted a statistically significant and specific blastogenic effect of some recipient sera on recipient (but not donor) cells. This effect is reminiscent of anti-idiotypic antibody stimulation of helper cell activity in lower animals (Hammerling et al, 1976).

Another hypothesis which we consider plausible as an explanation for this cytotoxic T cell effector defect is the presence and activity of suppressor T cells. Suppressor T cells have been described in lower animals (Gershon, 1974) and recently in man (Waldmann et al, 1974) and could conceivably suppress cytotoxic effector cell generation. Six of the CML negative recipients were tested for the ability of their lymphocytes to suppress the CML responses of third and fourth parties to the donor cells. In 4/6 there was evidence of suppressor cell activity. Thus, this may be a reasonable explanation for a majority of patients exhibiting the cytotoxic effector cell defect. Experience in our unit had indicated that elevated T cell levels are related to acute rejection in the early post-transplant period (Thomas et al, 1976) so that suppressor T cell activity is probably not operative to any great degree at this time. On the other hand, in the late post-transplant period, the period under study here, T cell suppressor activity might be operational.

It is also possible that the lack of CML responsiveness to HLA haploidentical related donors is a result of poor immunogenicity of the mismatched antigens. Eighty-two percent of the recipients we studied had a HLA-B locus mismatch with the donor, and 42% were mismatched for both an A and B locus antigen.
Other studies have demonstrated variability in CML responses to specific HLA antigens, noting, however, that HLA-B locus antigens in general are important targets in CML (Eijsvoogel et al., 1973) (Grunnet et al., 1974). It is possible, nevertheless, that the particular HLA antigen mismatches represented in the donor-recipient combinations we have studied may be poor target antigens for CML. Thus, a lack of CML activity may reflect a functional histocompatibility.

In summary, these studies have demonstrated that long term successful renal transplant recipients with related HLA non-identical donors have a specific defect in capability to generate cytotoxic T cells in vitro against donor cells, despite MLC responsiveness. This finding appears to be related to suppressor cell activity in some cases and may represent a previously unappreciated mechanism of long term human allograft acceptance.

Acknowledgments

Supported in part by NIH Grants #2PO1HE0820308, 1R01AI12586–01, and 1R01AI12822–01, ACS1N105, and a grant from the Richmond Heart Association.

References

Garavoy, M, Franco, V, Zschaack, D, Strom, T, Carpenter, C B, and Merrill, J, (1973) Lancet 1, 573
Hayry, P, Andersson, L, Nordling, S, and Virolainen, M, (1972) Transplantation Reviews, 12, 91
Quadricci, E, Tiemann, J, Marchioro, T and Striker, G (1974) Transplantation, 17, 361
Rosenau, W and Moon, H (1966) Journal of Immunology, 96, 80